SIXTH EDITION

Hayes' Principles and Methods of Toxicology

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HAYES' PRINCIPLES AND METHODS OF TOXICOLOGY

EDITED BY

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Contents

Preface

This is the sixth edition of *Hayes' Principles and Methods of Toxicology*. It has been revised and updated while maintaining the high standards necessary to serve as a reference to the concepts, methodologies, and assessments integral to toxicology. As was the case with the first five editions of the book, new chapters have been added that address the advances and developments in the field of toxicology. These chapters deal with the importance of dose–response, systems toxicology, food safety, the humane use and care of animals, and neurotoxicology. A number of new authors have been added and the glossary has been expanded. Every effort has been made to maintain this book as a tome useful to both graduate students beginning their educational journey as well as the more seasoned toxicologist.

In many ways, toxicology remains a paradox. The words of Paracelsus, the sixteenth-century German-Swiss physician and alchemist, continue to remind us that the line between light and dark, good and evil, poison and medicine is but a fine one that we as toxicologists have been given the awesome responsibility to divine, "Alle Ding' sind Gift, und nichts ohn' Gift; allein die Dosis macht, daß ein Ding kein Gift ist." The first chapter reminds us of the evolution of toxicology, as it has matured over the centuries from its genesis as a science focused on the discovery and use of poison as an expedient tool to commit murder to the advances that have resulted in the use of toxicants to benefit mankind as medicines.

Toxicology is much more than the science of poisons. Practitioners of toxicology need to understand the hazard and the underlying mechanisms of toxicity, as well as the principles of extrapolating experimentally derived hazard information to the assessment of risk under the conditions of exposure in the species of primary interest (humans). The vastness of the field of toxicology and the rapid accumulation of data preclude any individual from absorbing and retaining more than a fraction of the methods, techniques, and information being developed on a daily basis. However, an understanding of the principles underlying these methods is not only manageable but also essential for the practicing toxicologist, and it is to this end that this book was conceived and continues in this edition.

"Training is everything. The peach was once a bitter almond; cauliflower is nothing but cabbage with a college education." (*The Tragedy of Pudd'nhead Wilson and the Comedy of the Extraordinary Twins*—Mark Twain). And thus, we hope to provide in this edition, as before, an experience that continues to educate, elucidate, nurture the inquisitive, and foster the motivation to learn. We begin with four basic principles of toxicology—dose matters, people differ, everything transforms, and timing is crucial. The relevance of these principles is waiting to be discovered, challenged, and applied in the following chapters.

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Foreword to the Sixth Edition

How do you see *toxicology*—as molecular magic, a web of biochemical circuitry, a sequence of immunological or pharmacological actions and interactions, or perhaps as clinical harms and pathological findings from which ideas about causes and mechanisms might be deduced? To you is it a set of *effects* for which causes must be found? Is it a process of evaluation of one or many risks leading to a critical and doubtless criticized judgment balancing harm and cost against benefit? Or, put more simply, is *toxicology* one of those uncomfortable disciplines that extends from the precision of basic sciences to the uncertainties of personal and societal judgments required to be accurate and yet to contain the fog of economic, political, and even philosophical debates? Is it really a combination of all these areas in which the practitioner and the student may concentrate on one aspect when a balanced view requires awareness of all the other factors affecting what they study, how it is studied, and how their discoveries and findings will be used?

These questions may seem rhetorical, but the competent toxicologist striving to exert professional skills to their best ends must always be aware of the place of his or her work as it affects people, the environment, and society in general. In this respect, toxicology is unusual in its combination of several natural sciences with the softer and often more controversial disciplines of economics, the law, and political science. To me, *toxicology* is now sufficiently mature to be seen as an example of *systems biology* in which account must be taken of many processes and their proximate and distant interactions.

To do the best job that he or she can for the present and future generations and the environment in which we live, the toxicologist requires broad awareness and understanding of why something is being investigated and how to explore it. The methods employed should reveal or exclude effects with accuracy, consistency, and economy of effort and resources, and in ways that will support subsequent risk–benefit decisions because experience and theoretical knowledge have demonstrated the validity of the procedures and the results they give. The value of the results as indicators of more fundamental biological processes must never be ignored because toxicology both borrows from and supports other sciences.

One of the several particular strengths of this book, the sixth edition, lies in the breadth of its coverage of established and newer methods and tactics for the detection and investigation of toxic effects and for studying the causes of those actions at many levels from populations to individuals and down through tissues and cells to molecules. Toxicology is a derivative science, using the understanding and techniques of other sciences to find, analyze, and evaluate harmful actions. The continuous growth of toxicology has come from its usage of and gifts to its parents of medicine, pharmacology, biochemistry, pathology, immunology, genetics and embryology, and, more recently, development of molecular biology. All those aspects and related disciplines are described here from the viewpoint of the toxicological scientist and practitioner needing awareness of the newest as well as of classical ideas and techniques.

Comparing the contents of the present and previous editions of this book illustrates the continuing but jerky progress of toxicology, sometimes accelerating thanks to new discoveries and methods developed elsewhere, as in the introduction of genetics and immunotoxicity and appreciation of the endocrine disruptors and nanomaterials, sometimes adapting to the novel issues of, for example, genetic manipulation, advanced biotherapies, and the unanticipated exposures of modern urban life, and sometimes taking account of the less than certainties pronounced by some epidemiological and environmental surveyors.

Toxicology is and must remain a vigorous and as far as possible rigorous discipline. Its practitioners and other commentators, who ought to be more aware than they sometimes seem to be of its strengths and weaknesses before making toxicological pronouncements, will find much help in this book in phrasing questions that can be answered, in deciding how best to answer them, and in helping others to understand the realities and practicalities underlying the detection, assessment, and use of toxicological knowledge for protection and as a means to probe biological mechanisms better suited to study by other sciences.

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Foreword to the Fifth Edition

Toxicology is an evolving science with ongoing development of methods, concepts, and understanding. It has been only some five years since the fourth edition of this book was published, but a wealth of novel information has been reported in the scientific literature since that time. This is especially true regarding *toxicogenomics*, a term that was coined in 1999 to describe the marriage of toxicology and genomics. Since that time, gene expression analysis has been used as mechanistic toxicology screens, for more sensitive and earlier toxicity discovery, in drug discovery and in drug and chemical safety assessments. Proteomic technologies have also recently been much applied in toxicology, allowing for the examination of the entire complement of proteins in an organism, tissue, or cell type. Using large-scale, high-throughput methods, protein expression, posttranslational modifications, and protein interactions may be studied. Also coming to the fore is metabonomics, where high-resolution ^{1}H-NMR spectroscopy is used in conjunction with pattern recognition to provide a fingerprint of the small molecules contained in a given body fluid that may be applied to define the dynamic phenotype of a cell, organ, or organism. These promising technologies are described in this edition in a new chapter on toxicopanomics.

During the last few decades, a wealth of toxicological information has become available, making it impossible for any individual toxicologist to keep abreast of all new information; for example, the TOXLINE file contains over 3 million bibliographic citations; thus, the need for comprehensive and readily accessible information resources has become apparent. Many web-based, searchable databases are now available that make it possible to retrieve information related to specific toxicological questions. This edition now contains a new chapter on information resources for toxicologists.

Already in antiquity it was well known that nature was not always benevolent and that human poisoning was possible via animal venoms and plant extracts. Such toxins were in earlier times used for hunting, waging war, and assassinations; in modern times, accidental poisoning with natural toxins is rather prevalent in many parts of the world. This fifth edition includes a new chapter on plant and animal poisons.

Because of the considerable societal pressure with respect to reducing the use of animals in toxicology, nonanimal methods are being developed in an attempt to predict what happens when animals, including humans, are exposed to toxic levels of drugs and other chemicals. Such methods include a number of cellular and subcellular systems, as well as mathematical models based on correlating a compound's chemical or structural variation with measured toxicological responses. One new chapter in this edition covers the use of nonanimal methods in toxicology.

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Foreword to the Fourth Edition

Publishing a toxicology book on the entrance into the third millennium is an appropriate time to reflect on the progress that has been made in that discipline since its inception. Almost 500 years ago, Paracelsus published a treatise based on his intuitive observations; he merely argued that these observations should convince one that the dose makes the poison. Now science has advanced enough so that we can prove that Paracelsus was correct, because the law of mass action tells us that the degree of perturbation of a system is proportional to the chemical potential of a substance in that system. We still, however, must rely heavily on observational epidemiology to determine the details of exactly what dose effects what change in humans.

The fourth edition of this book is a magisterial, state-of-the-art compilation of the principles and methods that toxicologists must use to identify whether a causal relationship exists between specific doses of a chemical and an alleged adverse effect, observed primarily in humans. Proper integration of principles and methods of toxicology is extremely important since the primary purpose of toxicology is to predict human toxicity. Previous editions of this book have delineated in very useful detail the methods of toxicology and how these methods have been perfected steadily and rapidly in the last few decades. The necessarily heavy reliance on animal experimentation for determining causality in humans is obvious and certainly warranted.

This book was the first to chronicle the overall aspects of the use of animal experiments in toxicology. The exponential rate of growth of toxicology continued to be reflected in further editions of the book, which served as the authoritative and comprehensive source of methods used in this science. Proper and critical conduct of acceptable toxicological tests still continue to create the body of systemized knowledge essential to the science of toxicology.

The current edition continues this tradition but adds some very significant new chapters. These chapters are on epidemiology and exposure assessment, and a chapter on repeat dosing combines previous chapters that subdivided multiple dosing into arbitrary intervals. It is remarkable that we have returned, almost full circle, to an emphasis on direct exposure and effects in human populations after finally, firmly establishing the basic scientific foundations of toxicology. This thorough, complete compendium is a necessary addition to the library of everyone interested in this subject.

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Foreword to the Third Edition

Until 1982 when the first edition of this book was published, there was no specific source to which a student or an investigator could turn to for a comprehensive presentation of the methods used in modern toxicology. For anyone who was trying to teach the subject, the book filled a great void for both the teacher and the student. The book appeared at a time when technical achievements in the field related to toxicology were undergoing tremendous refinements. Techniques and the tools of experimental biology, pathology, mathematics, engineering, physics, and analytical/biological chemistry, which had been barely conceived 20 years earlier, were in common use. The rapid growth of toxicology at that same time created a need for scientists from all of these fields to apply their expertise to the science of toxicology. Toxicology borrowed freely from these related sciences so a developing, modern, scientifically acceptable body of procedures became identified as the methods of toxicology. Prior to the span of a single human life, the methods of toxicology consisted of some general, short-term tests for the determination of the overall aspects of this difficult area of toxicology. The exponential rate of growth of toxicology continues, and the third edition of the book continues to be an authoritative and comprehensive source of the methods that are currently used in this science.

If toxicology can be appropriately defined as the study of the harmful effects of chemicals on biologic systems, it must then embody a systemized knowledge of the effects of chemicals that are introduced into the simplest, as well as the most complex, of all biologic systems, and methods must be available to accomplish these experiments. The availability of methods to detect the harmful effects of chemicals allows for the creation of data, but those data become useful in toxicology only after they are suitably interpreted. An additional link toward understanding the subject of toxicology is the placing of results obtained from the available methods in their proper relation and perspective to the whole picture of the role that toxicology can play for the improvement of mankind. In order to accomplish this function, the toxicologist must not only develop an understanding of the methods used but also determine the significance or insignificance of their data in the complete picture of the toxicity of each compound. My graduate school mentor, Dr. Roger Hubbard, once told me that no scientifically valid experiment creates erroneous results, but inappropriate application of those results can create erroneous conclusions. An understanding of the principles together with the methods involved in the science of toxicology prepare the critical scientist for developing an insight with regard to the proper application of experimental results. Results that are properly obtained by acceptable methodology and that are suitably weighted for the conditions under which they were obtained certainly contribute to the development of proper conclusions. In this book, very highly qualified toxicologists present the procedures in detail that are currently used and accepted in the field of toxicology. Discussions of each procedure or category of procedures enable the conduct of acceptable toxicologic tests that create the body of systemized knowledge essential to the science of toxicology. Properly applied, that knowledge serves to protect mankind and the biologic realm in general from sudden, as well as delayed, insidious chemically induced harm.

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We express our warmest appreciation to the many knowledgeable individuals who have contributed knowingly and otherwise to the sixth edition of *Hayes' Principles and Methods of Toxicology*. The good contained within this tome is due in no small part to the various chapter authors; the bad resides solely with the editors. We most heartily thank each of the chapter contributors, who either revised their chapters or prepared new chapters, for keeping in mind that thoughtfully worded information is greatly appreciated, especially by the student but also by the more advanced reader. We thank Jill Jurgensen and Kathy Brailer for their skillful handling of the manuscript. Appreciation is also expressed to our publisher, Barbara Norwitz, and the staff at CRC Press.

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Dr. A. Wallace Hayes is a toxicologist with over 35 years of experience in industry and academics. He holds degrees from Auburn University (PhD and MS) and Emory University (AB). Dr. Hayes was an NSF predoctoral fellow at Auburn University, an NIH individual postdoctoral fellow at the Vanderbilt University School of Medicine, and a NATO Senior Scientist at the Central Veterinary Laboratory in Weybridge, England, and was a recipient of a NIH Research Career Development Award. Dr. Hayes has held tenured professorships at the University of Alabama, the University of Mississippi Medical Center, and Wake Forest University School of Medicine. He has served on committees and expert panels for the National Academy of Sciences, the National Institutes of Health, the Environmental Protection Agency, and the Department of Defense. He has also served on a number of Generally Recognized as Safe (GRAS) expert panels. Dr. Hayes has authored more than 225 peer-reviewed publications; is the editor of *Principles and Methods of Toxicology*, *Human and Experimental Toxicology*, and *Cutaneous and Ocular Toxicology*; and serves as coeditor of the Target Organ Toxicity Series. He is the editor in chief of *Food and Chemical Toxicology*. He has also served as the past secretary-general of International Union of Toxicology (IUTOX) (two terms); as past treasurer and board member of the American Board of Toxicology; as past president of the American College of Toxicology, the Toxicology Education Foundation, and the Academy of Toxicological Sciences; and as past member of the council of the Society of Toxicology. Dr. Hayes is a diplomate of the American Board of Toxicology, the Academy of Toxicological Sciences, the American Board of Forensic Medicine, and the American Board of Forensic Examiners. He is a fellow of the Academy of Toxicological Sciences, the Institute of Biology (UK), the American College of Forensic Examiners, and the American College of Nutrition. He is also a registered toxicologist in European Registered Toxicologist (ERT) and a certified nutrition specialist (food safety). Dr. Hayes was honored by the Society of Toxicology in 2006 with the Society's Merit Award, by the Mid-Atlantic Society of Toxicology with its Ambassador Award in 2012, by the American College of Toxicology in 2012 with its Distinguished Scientist Award, and by the International Dose-Response Society in 2013 with its Outstanding Leadership Award.

Dr. Claire Kruger is president of Spherix Consulting, Inc. With more than 25 years of consulting experience, her primary area of expertise is in foods, consumer products, and pharmaceuticals, where she provides scientific, regulatory, and strategic support to clients in both the United States and international regulatory arenas. She has conducted toxicity evaluations of foods and food contaminants, as well as health risk assessments and exposure assessments of drugs, cosmetics, and pesticides. Her clients include food, drug, and dietary supplement manufacturers, agricultural producers, biotechnology companies, trade associations, and law firms.

In her role as a consultant, she has been involved in the safety evaluation of a variety of consumer products, providing oversight of product compliance with current and emerging scientific and regulatory guidance. Dr. Kruger provides evaluations of the potential health risks related to employee and/or consumer exposure to consumer products. She has worked with clients to develop product stewardship programs to ensure appropriate guidance for sourcing of ingredients and for manufacturing, distributing, and using products.

Dr. Kruger has been involved in evaluating the safety of foods, novel foods, food additives, Generally Recognized as Safe (GRAS) substances, dietary supplements, food contact substances, and food contaminants. She has developed health risk assessment and regulatory compliance dossiers for ingredients used in dietary supplements, infant formulas, medical foods, novel foods, and functional foods.

In this role, Dr. Kruger has dealt with issues relating to all aspects of food production, from manufacturing process and ingredient specifications to preclinical safety evaluation and human clinical testing. She has prepared numerous GRAS determinations and notifications and has served as an expert for many GRAS determinations. Dr. Kruger has also assessed the safety of and prepared premarket notifications for a number of dietary supplements. In addition, she has developed novel approaches for evaluating the safety of bioactive components and has employed these approaches in completing GRAS determinations for several novel bioactive products and functional foods. Dr. Kruger has also assisted clients with the identification, screening, and documentation of data for candidate ingredients to assist companies in evaluating these products for potential label claims.

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Section I

Principles of Toxicology

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1 The Wissenschaften of Toxicology
*Harming and Helping through Time**

Richard W. Lane

CONTENTS

> Continuity with the past is a necessity, not a duty
>
> **Oliver Wendell Holmes, Jr.**

PREFACE

History is full of bad intentions, ignorance, and folly. Poisoning has been part of each of these for as long as humans have lived. Understanding poisons—to control them or avoid them—has therefore been an integral part of our past. As our understanding of poisons progressed, a unique field of knowledge grew into the science of toxicology. This chapter captures toxicology's *wissenschaften*, its emergence as a distinct field of study from its vague, sometimes dark, beginnings. Toxicology passed through a number of phases during its maturation into a recognized discipline. These include the use of poisons to kill, listing poisons and their effects (observation/phenomenology), guessing at possible antidotes, identifying occupational causes of disease, detection of poisons, experimentation to deduce the mechanisms of toxicity, development of rational therapeutic measures, and finally quantification of effects. As toxicology moved along this path, it evolved from harming people to helping them by protecting them from the adverse effects of chemical exposure at home, in the environment, and in the workplace.

The use, abuse, and misuse of chemical agents by individuals, groups, and societies over time form the basis of toxicology and how it developed as a discipline. This chapter will demonstrate how toxicology has developed as a science from the study of chemicals to induce harm (the art of poisoning) to a study of chemicals in order to prevent harm (the science of preventing poisoning) and benefit humankind. Toxicology has come forward to be a critical and respected member of the scientific and medical communities.

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The title and emphasis of this chapter are slightly revised to focus on the deliberate and systematic examination of poisons. *Wissenschaften* is generally translated as *science* or *the sciences*, but the word also refers to the growth and development of any field of intellectual inquiry, such as literature, religion, law, and the sciences. It is the latter meaning that is used here to emphasize the emergence of toxicology as its own discipline. This chapter looks at the evolution of toxicology, the events and people that influenced and shaped its course. There will be more weight on situations that show the understanding of poisons and how the science of toxicology developed.

The observant reader will note other changes in this chapter. The most important is the absence of Dr. Joseph Borzelleca as an author. His work on this chapter over the years was seminal, and everyone in the field owes him a debt of gratitude for it. From his lead, the examination of the history of toxicology flourishes. The amount of new information and insight now available has grown substantially. The Society of Toxicology has published articles and posters. Numerous books have been published. Articles in major journals abound. Information on the Internet is sometimes overwhelming. There is even a society for this endeavor! With all this new information, some parts of the earlier versions have been revised or removed. Events after around 1970 have been omitted unless continuity is important. The style and intent of previous versions have been maintained to the extent possible, but differences are inevitable.

INTRODUCTION

Poisonings—accidental, intentional, and unintentional—form the basis of toxicology. Poisonings have occurred throughout history and is intertwined with most of the important aspects of human life, such as eating, politics, working, religion, folklore, murder, suicide, and warfare. Poisoning evokes both dread and fascination. Toxicology, the study of poisons, takes something that is inherently emotive and forces the practitioner to rationally deal with agents that can harm. By rationally applying his/her knowledge, the toxicologist can positively influence individuals' and the public's health.

A toxicologist uses his/her knowledge for the *proper* use of chemicals and mixtures. In some cases, this involved how to kill or harm. In others, the toxicologist allows the appropriate use of chemicals by establishing safe limits of exposure so that harm does not occur. In both instances, toxicology provides the basis for obtaining the desired outcome.

Initially, toxicology involved listing poisons, noting their onset of action and the nature of the harm produced (painful or painless, terminating in death or not, etc.). Observations in humans following exposure to toxic plants, minerals, or animals and recommendations for treatment—sometimes based on limited evidence—are hallmarks of the early phases of toxicology. As medicine evolved and observations about disease became more astute, epidemiology became possible, and effects could be associated with a cause. As chemistry evolved, detection of poisons became possible and toxicology moved into forensics. As the basic sciences evolved, poisons could be studied at the cellular level. The famous French physiologist, Claude Bernard, used poisons more than 150 years ago to investigate basic physiological phenomena. This marked a major turning point in toxicology as poisons were now being used as experimental agents to dissect the basis of physiology and cell biology. These studies could elucidate the mechanism of action of poisons, which could lead to the development of appropriate treatment modalities (i.e., antidotes) and/or to identifying safe exposure limits. Today, the study of poisons includes knowledge of the physiological, biochemical, and morphological effects of a chemical and understanding the mechanism of action at the organ, cellular, and molecular level. In this way, toxicology reflects the development of society: a progression from simplicity to sophistication, from crude to cultured, from elemental to elegant, from superstitious to scientific, and from taking lives to saving lives.

Poisons also cause psychological harm. The threat of poisoning can be terrifying, and poisons have generated fear throughout time, right up to today. Because poisons are tasteless or can be disguised, people have become fearful of them in any amount. The mere instance of a chemical can inflame the public, sometimes even when no physical harm can be demonstrated. There are numerous periods in history when the fear of poisoning far surpassed any physical harm. At times like those, rumors and inaccurate reports become facts. The misinformation can lead to hysteria, which can lead to countermeasures that often results in secondary problems worse than what started the problem in the first place. Unfortunately, proper communication of correct interpretation of the data is difficult and is drowned out by the clamor for action.

HARMING AND HELPING THROUGH TIME

Initially, poisoning was accidental. As man learned about toxic agents through experience, he could avoid them, a proper use of knowledge. But poisoning also could be deliberate, an abuse of knowledge. Once the value of poisons was recognized, they became very appealing solutions to difficult problems, like how to overcome superior physical force. Initially, killing involved the use of physical agents (clubs, spears) that required strength and skill, favoring large humans. How could smaller individuals level the field? Poisons were an answer. Knowledge of the poison, along with cunning, was required for success.

Once a need for poisons had been established, an industry of suppliers and practitioners developed for implementation. As people became more aware of poisons, laws were developed to prevent their use. Subtler, more sophisticated (i.e., undetectable) poisons were needed. The do-it-yourself poisoners were replaced by professional poisoners (early, applied toxicologists), who offered advice, provided materials, and perhaps even performed the required services. New agents developed by practitioners could make the

poisoning fast or slow, painful or painless. As poisoning developed into an art, its practitioners became infamous. The popularity of poisoning grew until it reached epidemic proportions in some countries. The resulting fear was enhanced by the inability to detect poisons and to prove that poisoning had occurred. Identification of the perpetrator was extremely difficult since determination of the cause of death (proof of poisoning) required analytical techniques that had not yet been developed.

Prevention of poisoning was accomplished by using bioassays (e.g., official tasters of prepared food and drink), taking precautions (only eating food of known origin, not eating foods that contained lumps or were highly seasoned), and developing tolerance/adaptation through the repeated ingestion of small doses of toxins.

Forensic toxicology began when advances in analytical techniques were applied to the detection of poisons. The Marsh test, developed in 1836 by British chemist James Marsh, allowed arsenic, the most popular poison at that time, to be identified unambiguously. More tests followed and more poisons could be identified. This had the intended effect of reducing poisonings because the poison could be identified, the perpetrators tried in court, and appropriate action taken. Practitioners became ever more sophisticated in their attempts to avoid detection, but they were no match for the chemists who continued to develop more sensitive and specific analytical methods. Once chemists turned their skills from developing poisons to detecting them, the popularity of such agents declined rapidly and poisoning became less common. Although subtle and ingenious means of poisoning are available today, forensic methods have made undetected poisoning nearly impossible.

As the use of poisons to dispatch people declined, their use as tools to understand physiological and pathological processes increased. Their redeeming value emerged. There are uses for poisons beyond harming! Agents commonly referred to as *poisons* have contributed to the health and safety of humankind and to the advancement of biological sciences, including medicine, in many ways. Claude Bernard, probably the first mechanistic toxicologist, used curare to study the neuromuscular junction. He wrote in 1878:

> Poisons can be used as agents for the destruction of life or as means to cure disease; but in addition to these uses – there is a third which particularly interests the physiologist. For him the poison becomes an instrument which dissociates and analyses the most delicate phenomena of the living machine and by careful study of the mechanism of death in different poisonings, he can gain knowledge, indirectly, of the physiological mechanism of life. (i.e. poisons can be used to explain physiological events). (Translation of P.N. Mage, 1965)

Dozens and dozens of other *poisons*, too numerous to list here, have helped us understand fundamental biology.

Further to helping mankind, knowledge of the proper use of chemical has been applied in many basic ways. Chlorine gas was one time used as a weapon of war due to its ability to cause pulmonary irritation, acute damage in the upper and lower respiratory tract, and eventually slow death by asphyxiation. Today, it is used as a disinfectant to treat public drinking water to prevent illness by dramatically reducing the threat of waterborne diseases. Its proper use has saved millions of lives. Smoke, an irritant also used in chemical warfare, is also used to preserve food. The use of pesticides, poisonous by their very design, which help feed a growing population by controlling unwanted plants and animals, has resulted in increased food production and subsequently in better nutrition, health, and longer life expectancy. Again and again, humans can use toxic compounds in a useful fashion to control their environment to their benefit.

When it became known that nondeliberate exposure to chemicals could produce adverse health effects (e.g., in the workplace and environment), efforts were directed at the prevention of the effects by defining safe conditions of exposure to protect humans and other life forms from injury. Dose–response relationships were established as correlations between the level of the chemical in blood and/or in tissues and biological activity were made. This was followed by the identification and quantification of the risk of adversity following exposure (risk identification, assessment, and management). Quantifying a risk, assigning a number to it, tends to decrease the uncertainty of extrapolation, lessen anxiety, and provide a degree of comfort. Quantification of the responses to toxic agents and the relationship of structure to biological activity are the basis for a great deal of scientific activity.

Starting from the art of poisoning, supplying and using poisons, the toxicologist now studies their mechanisms of action, develops analytical methods to identify and quantify poisons in body fluids and tissues, develops rational antidotes, establishes safe limits of exposure from carefully designed and executed studies, and quantifies and predicts adverse effects. The toxicologist now plays a critical role in the advancement of humankind.

PREHISTORY

Poisonings—it is not unreasonable to assume that harmful plants, moldy grains, and venomous animals were accidentally encountered with dire consequences—predate recorded history and make toxicology arguably the oldest biological science. The earliest view of poisons, based on everyday life and needs, began when man had only a rudimentary view of nature. As with many aspects of everyday life, the interpretation of the effects was frequently mixed with religion and mysticism. Finding food was a matter of chance, and if you picked the wrong plant, you could be in trouble. Cause and effect were generally unknown. Early man may have thought himself surrounded by poisons. After some trial and error, the distinction between poisonous and nutritious plants became known. But even food that was wholesome at one point could be contaminated by mold and rendered injurious. Thus, there was still a great deal that was unknown and uncontrollable. Life must have seemed capricious. It is easy

to imagine how poisoning could be seen as *magic* or an act of the gods. Mysticism and superstition made up for the lack of knowledge.

To establish that poisoning occurred, it is essential to demonstrate that a particular substance caused the adverse effects. This was often very difficult because cause and effect were not always evident when there was no understanding of pathogens, chemicals, and poor nutrition. Early man had to study hard to determine exactly what had caused an illness based on underlying assumptions about the nature of man himself and the world at the time.

Once cause and effect were established, toxic substances could then be used intentionally as tools to catch prey and dispose of unwanted persons. The initial instruments for killing were physical weapons that required strength and skill for effectiveness. Later developments, such as the bow and arrow, required more skill and less physical strength, but something more was needed to feed growing families and control one's surroundings. Might poisons be what were needed to solve some problems? As early humans learned through experience that plants were beneficial and were poisonous, the poisonous ones were used as aids in hunting, such as arrow poisons like curare from the resinous extract obtained from several tropical American woody plants. Poisonous animals were also discovered and the people in South America used the secretions from amphibia to kill animals for food. The adverse effects of venomous insects and animals were probably also noted, but the practical utility of these venoms was limited.

Poisons proved to be very useful in killing animals, and it did not require a great a leap of reasoning to extrapolate from the effects seen in animals to humans. Although killing people was not sanctioned (*Thou shalt not kill*.), could humans be dispatched as readily as animals with the use of poisons? It is unknown when the first human intentionally used a substance to kill another human, but humans have an instinct to control their own destinies and to satisfy their lust for power, wealth, and pleasure. The age of poisoning, of practical toxicology, the seduction by toxicology, had begun. Poisons moved from being random problems to predictable tools. Seduced by toxicology, man would use poisons for his advancement throughout history.

OBSERVATION/RECORDING OF PHENOMENA

With the development of civilizations and writing, the known causes of toxicity could be recorded so that others could learn about them. Many early cultures had lists (catalogs) of poisons and their effects in humans, based on keen observation. Interest in plants that are harmful to health and as tools for vindication evolved, as did their beneficial use, predominantly herbs, for medicinal purposes. The cures for the problems of humankind (healing or killing) could be found in nature. With time, the lists began to include detailed descriptions of preparation, use, and effects of biologically active plant materials. Metals were used for therapies. There was some interest in the properties of animals, but most writings dealt mainly with avoiding venoms. Prevention and treatment of poisoning and envenomation emerged.

Egypt

Egyptian medicine was reputed to be the most advanced of the ancient world, and as expected, the first known list of poisons and antidotes appears in Egyptian writings. Egyptian medicine was based on the work of the gods and the presence of evil spirits in the sick person. Medicines such as herbs were mostly expected to lessen pain, while magic effected the cure. However, a portion of Egyptian medicine was based on experimentation and observation, including the effects of poisons. Menes, the first Pharaoh of unified Egypt and the founder of Memphis, the capital, was reported in Egyptian papyri to have had an interest in poisons. He cultivated and studied the effects of poisonous and medicinal plants somewhere between 3500 and 3000 BC. Unfortunately, there is no detailed written history of these activities.

The Ebers Papyrus (c. 1550 BC) is one of the oldest known writings pertaining to medicine. It contains 110 columns of hieratic (priestly) script (equivalent to about 110 pages). It reveals many customs, practices, and traditions of Egyptian doctors and describes over 800 recipes, many containing recognizable poisons such as hemlock, aconite, opium, and some of the heavy metals. The formulas also contain over 700 drugs (medicinal substances), specific indications, and dosages, together with appropriate spells and/or incantations. Forty-seven case histories are presented. Modes of administration include snuffs, inhalations, gargles, pills, troches, suppositories, enemas, fumigations, lotions, ointments, and plasters. Vehicles included beer, wine, milk, and honey. Drugs were identified on the basis of origin as plant (e.g., acacia, castor bean, wormwood, fennel, garlic), animal (e.g., honey, grease, milk, excrement), or mineral (e.g., alum, iron oxide, limestone, sodium bicarbonate, salt, sulfur). Insect and animal venoms were described.

The Egyptians had some correct general principles of toxicology, but the concept of cause and effect was missing that consequently led to poor treatments for disease. Herbs played a major role in Egyptian medicine as antidotes, as were some minerals.

The Egyptians used chemicals in the administration of justice. The penalty of the peach involved having the accused ingest the distillate from crushed pits of peaches that are high in amygdalin and forms hydrocyanic acid. If the accused died, it was a presumption of guilt. If the accused lived, it was a presumption of innocence. The practice of using chemicals in the administration of justice continued into other cultures (e.g., Greek, Roman) and persists to the present with injections of chemicals used in some state executions.

Thousands of years later, Cleopatra (c. 69–31 BC) poisoned her second brother after Caesar was murdered so she could jointly rule Egypt with her infant son. According to a recent theory by Christoph Schaefer, she likely committed suicide by hemlock, wolfsbane (aconitum), and opium, not by the bite of an asp.

China

Legend has it that the second of China's mythical emperors, Shen Nung, is the father of Chinese medicine and agriculture. He is credited with writing a 40-volume work entitled *Pen Ts'ao* or *Pun Tsao* (the Great Herbal, a Chinese *materia medica*) around 2735 BC. It contained lists of poisonous plants, plants with medicinal value (365), and drugs (265, 240 of which are of plant origin). The effects of plants and drugs and appropriate antidotes were described. Drugs and poisons were presented together, presaging the concept that the dose differentiates a poison from a remedy. Included among the drugs were iodine, aconite (also used as an arrow poison), opium, cannabis, rhubarb, alum, camphor, iron, sulfur, and mercury. Shen Nung was also reputed to have discovered a number of drugs and experimented upon himself.

Another emperor, Huang Ti (2650 BC), reportedly wrote *Huang Ti Nei Ching* (*The Yellow Emperor's Medicine Classic*), the oldest extant classic of traditional Chinese medicine. Although the *Huang Ti Nei Ching*'s authorship is attributed to the Yellow Emperor, it was more likely written by several authors over a long period of time, compiled roughly 2000 years ago. The book is divided into two sections, the second being *Lingshu* (*The Vital Axis*) and was written sometime in the second century BC with revisions taking place up to the Han Dynasty (206 BC–AD 25). This great work forms the theoretical basis of traditional Chinese medicine. As traditional Chinese medicine's history developed over the millennia, nearly all significant medical works benefited from the enlightenment of this unparalleled book. *The Yellow Emperor's Medicine Classic* demonstrates that even in ancient times, people accomplished scientific achievements that are applicable, relevant, and innovative in modern times. It still remains one of the most respected and studied texts on Chinese medicine.

Another medical text, found during an excavation of the Mawangdui tombs and dating back to 168 BC, is the *Wushier Bingfang* (*The Fifty-Two Prescriptions*). It detailed 52 ailments and 52 prescriptions, an early written reference of Chinese pharmacology.

The Chinese may have been the originators of chemical warfare. Chinese writings contain hundreds of recipes for the production of poisonous or irritating smokes for use in war, as well as providing accounts of their use. There are reports from the fourth century BC of the Chinese using bellows to pump smoke from mustard and other noxious vegetable matter into tunnels being dug by a besieging army in order to discourage the diggers. Cacodyl (tetramethyldiarsane, $As_2(CH_3)_4$), a colorless liquid possessing an intensely disagreeable garlic-like odor, in smoke is also mentioned in early manuscripts.

India

The *Rig Veda*, a Sanskrit document written between 1500 and 1200 BC, is the earliest account of Hindu medicine. It contains many references to alchemy, science, and magic in the treatment of disease. Medicinal and poisonous plants and antidotes (e.g., for snake bites) are listed. The influence of gold as a therapeutic agent and for longevity is discussed. A later work, the *Ayurveda*, the Veda of long life, is a Sanskrit document written about 700 BC. It discusses medicine and all its branches in eight parts; drugs and poisons are also mentioned.

Sushruta (c. 380–450), a Hindu surgeon, authored a medical/surgical text called *Sushruta Samhita*. The section on drugs listed 760 indigenous medicinal plants, of which many were used externally as ointments, baths, sneezing powders, and inhalations. It also listed animal and mineral remedies. The fifth section, the *Kalpa Sthana*, was the section on toxicology, dealing with the nature of poisons and their management and advanced treatments for venomous snakebites.

Greece

The Greeks borrowed heavily from the medicine of Egypt, and they took it forward through their system of philosophy, which included what we now call science. Over the centuries, this philosophy formed the theoretical basis of their attempts at a causal foundation for explaining disease.

The Greeks had lists of poisons and antidotes that were consulted by citizens and the government. They had a great deal of knowledge about plant poisons and metals, especially arsenic, antimony, mercury, gold, copper, and lead. Other and more significant contributions to the advancement of toxicology include detailed descriptions of the effects of various agents in humans, antidotes, and principles for the management of poisonings (e.g., hot oil and vomiting).

The Greeks executed criminals with poisons, with Socrates (469–399 BC) being the most famous victim of state poisoning in history. Socrates' iconoclastic attitude did not sit well with everyone, and at age 70, he was charged with heresy and corruption of local youth. Convicted, he carried out the death sentence by drinking hemlock (the state poison), becoming one of history's earliest martyrs of conscience.

Suicide and murder by poisoning was not uncommon since poisons were readily available. The Greeks also used chemicals in warfare. Solon of Athens used hellebore roots (which contains two glucosides, helleborin that is narcotic and helleborcin that is a highly active cardiac poison, similar in its effects to digitalis, and purgative) to poison the water of the River Pleistos during the siege of Kirra around 590 BC.

Pythagoras of Samos (c. 570–480 BC) may be the first Greek to have an influence on toxicology. Although best known as the mathematician who developed the theory of numbers and considered to be the founder of arithmetic, he was also a physician and scientist who was especially interested in procreation and animal physiology. His contributions to toxicology include his studies of the effects of metals (e.g., tin, iron, mercury, silver, lead, gold, copper) in the body. Since he left few, if any, writings, all of his teachings have come through his pupils.

Hippocrates (460–377 BC), known as the *father of medicine*, was born on the island of Cos, the son of Heraclides,

a physician, and Phenarete. His contributions to the advancement of medicine are legendary, due in great measure to his belief that the causes of diseases were natural and not supernatural. Writings attributed to him rejected the superstition and magic of primitive *medicine* and laid the foundations of medicine as a branch of science. Like other Greek physicians, he believed that health was the result of an equilibrium or balance in the body among the humors (blood, black bile, yellow bile, and mucus) and that disequilibrium resulted in ill health.

He apparently was the first physician of record who believed that environmental factors should be considered as probable causes of disease. In his book *Airs, Waters and Places*, he argued that environmental factors (overall weather, local weather conditions, and drinking water) can influence health. "Every disease has its own nature and arises from external causes, from cold, from the sun, or from changing winds."

Hippocrates identified about 400 drugs, mostly of plant origin, that included narcotics (e.g., poppy, henbane, mandragora [mandrake]), purgatives, and sudorifics (inducing perspiration; diaphoretics). He also advocated the use of emetics and enemas as part of the cleansing process. His contributions to toxicology include the use of sound observation, logical reasoning, and basic approaches to the management of intoxication (decrease absorption; if ingested, induce vomiting) and the use of proper antidotes.

Hippocrates recognized lead toxicity among miners and metallurgists, although no concern was demonstrated for their protection.

Diocles of Carystus (375–300 BC), also known as *the younger Hippocrates*, was one of the most prominent medical authorities in antiquity. A pupil of Aristotle, he wrote one of the earliest *materia medica* (*materials of medicine*, a reference that lists the curative indications and therapeutic actions of medicines), the *Rhizotomikon*. It is considered to be the first work on botany that included the names of the plants, their habitat, means of collection, and medical uses. His second book on plants described those used for food, and his third dealt with poisonous plants. His works indicate that serious attention to the pharmacology and toxicology of plants had begun. Along with Hippocrates, Diocles extended toxicology beyond merely listing poisons and antidotes. Rational methods for the study of the effects of poisons and the treatment of poisoning were proposed. Experimental studies to assess the biological effects of plants had begun.

> Nature does nothing without a purpose.
>
> **Aristotle**

Heraclides Ponticus of Tarentum (387–312 BC), a philosopher and student of Plato, was reported to have spent a great deal of time studying poisons and antidotes. Heraclides belonged to the *empiric* school, which rejected anatomy as useless and which relied entirely on the use of drugs. He may have been the first physician to indicate the value of opium in certain painful diseases.

Theophrastus (372–287 BC) studied in Athens under Plato and afterwards under Aristotle. He became the favorite pupil of Aristotle and one of his chief collaborators in the attempt to achieve a complete study of all the known fields of wisdom. Aristotle named Theophrastus his successor and bequeathed to him his library and manuscripts of his writings. Theophrastus was probably the most famous Greek botanist/herbalist. He wrote *De Causis Plantarum* (*About the Reasons of Vegetable Growth*) and *De Historia Plantarum* (*A History of Plants*) in 300 BC. These works may be considered the beginning of modern botany and served as an excellent text of medicinal and poisonous plants, as well as indications for the use of medicinal plants. They were an important influence on medieval science. His contributions to toxicology include a list of poisonous plants and the recognition of adulterated food.

An important figure in toxicology was Nicander of Colophon (185–135 BC), a Greek physician, poet, and grammarian who wrote, among other things, two didactic poems about poisons. They are the most ancient works devoted exclusively to poisons. The longest, *Theriaca*, is a hexameter poem (958 lines) on the nature of venomous animals and the wounds they inflict. The other, *Alexipharmaca*, consists of 630 hexameters on the properties of poisonous plants, including opium, henbane, poisonous fungi, colchicum, aconite, and conium (poison hemlock), and their antidotes. Although there were fanciful parts, much was accurate and reflected his powers of observation and his experiences. Nicander divided poisons into those that killed quickly and those that killed slowly. He recommended emetics in the treatment of poisoning. So important were these works that *theriac* has come to mean antidote against all poisons, a concept that survived into the eighteenth century.

An interesting person, although not a Greek *per se*, was Mithridates VI, or Mithridates Eupator, (132–63 BC), king of Pontus (now the northeastern part of Turkey). In 120 BC, while still a child, Mithridates became king. His mother, said to have assassinated her husband Mithridates V, ruled in her young son's stead. Afraid his mother would try to kill him, Mithridates went into hiding, at which time he started ingesting small doses of various poisons in order to develop protection. When Mithridates returned (c. 115–111 BC), he took command, had his mother imprisoned, and set about extending his dominion. Mithridates was obsessively possessed with a fear of poisons. As protection, he took poisons daily, beginning with very small doses and increasing the amounts ingested to develop a polyvalent tolerance. He drank the blood of ducks fed toxic chemicals and took mixtures of antidotes. It has been reported that he may have ingested all the known poisons and their antidotes every day of the year each day starting early in his life. Mithridatum, his universal antidote, was to be taken each morning before breakfast to effectively prevent poisoning. The term *mithridate*, meaning an antidote or preventive for poisoning containing many ingredients, immortalizes his contribution to toxicology.

Mithridates was a student of toxicology and one of the first to systematically study poisons in humans. He tested

the effects of poisons and their potential antidotes on slaves, criminals, and prisoners. He used his knowledge of poisons against his enemies when, in 67 BC, the Roman general Pompey led a large army against Mithridates. Mithridates slowly retreated over the course of a year, until he reached the southern shores of the Black Sea. Along the way, near the outskirts of the city of Trabzon, he left a large supply of locally produced honey in clay pots knowing that it would be found and eaten by the advancing Roman army. Three squadrons of Pompey's army found and ate the honey and then became violently ill. That honey, locally called *mad honey*, was left for the Romans because it was produced from the nectar of rhododendrons. Mithridates knew about the mad honey in that region because his adviser, the Greek physician Kateuas, had read about Xenophon's experiences in the same area in 401 BC when his whole army became sick after eating the local honey. The *mad honey* contained grayanotoxin. Although grayanotoxin poisoning is rarely fatal, the physical effects—vomiting, loss of coordination, muscular weakness, low blood pressure, and hallucinations—often last for 24 h or longer. Part of Pompey's army was rendered helpless to repel their attackers and many were killed. Pompey eventually prevailed over Mithridates and found the prescription for mithridatum. He sent it to Rome where efforts to improve upon it were made (described in the following).

It is said that when Mithridates saw that people supported his son over him, he attempted to take his own life, but failed because of the resistance he had built up to poison. He had to ask one of his mercenary soldiers to kill him with a sword.

Dioscorides (Pedanius Dioscorides) (AD 40–90), was born in Anazarbia in Cilicia (today's Turkey). He was a Greek physician, pharmacologist, and botanist who practiced in Rome at the time of Nero. He was a surgeon with the army of the emperor so had the opportunity to travel extensively, seeking medicinal substances (plants and minerals) from all over the Roman and Greek worlds. Dioscorides is famous for writing a text on botany and pharmacology free from superstition, *De Materia Medica* (*On Medical Matters*), that was a precursor to all modern pharmacopoeias. This five-volume set (*On Plant Materials*, *On All Manner of Animal*, *On All Manner of Oils*, *On Materials Derived From Trees*, and *On Wines and Minerals and Other Similar Substances*) became the leading text in pharmacology for 16 centuries. It covered 4740 medical uses of the materials and included descriptions of about 600 plants and 1000 simple drugs. Also discussed are the dietetic and therapeutic value of animal products (e.g., milk, honey) and mineral drugs (e.g., mercury, arsenic, lead acetate, calcium hydrate, copper oxide). He also described a surgical anesthetic made from opium and mandragora (mandrake). He was the first to recognize the toxicity of mercury. His contributions to toxicology include classifying poisons into three major classes (animal, plant, mineral), identifying antidotes, and recommending decreasing absorption to control intoxication (e.g., by inducing vomiting or purgation; cf. Hippocrates of Cos).

Galen of Pergamum (AD 129–c. 216) may be second only to Hippocrates of Cos in his importance to the development of medicine. If the work of Hippocrates represents the foundation of Greek medicine, then the work of Galen, who lived six centuries later, is the apex of that tradition. He knew all of the medical knowledge of his day, gathered it together, and wrote about it voluminously and well. Greek medicine was transmitted to the Renaissance scholars essentially in the form of Galenism. It was Galen who first introduced the notion of experimentation to medicine. Galen argued that although apothecaries knew drugs, only the physician understood both the drug and the patient and, further, that drugs are tools only for physicians (hence, few experimental nonphysician pharmacologists in Greece). He introduced rationality into drug therapy. He recommended mixtures of drugs for treating disease, which is the basis for the term *galenicals* (medicinal preparations or remedies composed mainly of herbal or vegetable matter). He further developed the theriaca, the universal antidote, to include 100 substances, which was to be administered in honey and wine. Galen warned against the adulteration of herbs and spices.

Galenic physiology continued Hippocratic concepts and was a powerful influence in medicine for 1400 years. Galen and his work *On the Natural Faculties* remained the authority on medicine until Vesalius in the sixteenth century, even though many of Galen's views about human anatomy were incorrect since he had performed his dissections on pigs, Barbary apes, and dogs. His writings were a blessing to the ancient world, but they became a curse when, for more than a millennium, they were held to be an unassailable authority and paralyzed the progress of medicine, something Galen would have greatly deplored.

Paul of Aegina (AD 625–690) was a celebrated Greek physician during the Byzantine period and was probably the *last Greek compiler.* He was the quintessential student of the best medical authorities, Hippocrates and Galen, and authored a seven-volume medical encyclopedia, *Epitome*. Book 5 deals with toxicology, specifically bites and wounds of venomous animals. He also displayed a peculiar genius in the field of surgery, and the sixth book, *A Treatise on Surgery*, influenced European and Arabic surgery into the Middle Ages. *Paul of Aegina's Medical Handbook* or *pragmateia* was transmitted and transformed through Syriac and Arabic translations, to become one of the cornerstones of the Islamic medical tradition. Paul's influence on the development of medical theory in the Islamic world and beyond makes him an important contributor to Greek and Arabic medicine.

Rome

The Romans had an intense interest in poisons. Records back to the fourth century BC indicate that poisoning was common as a means of suicide and murder. Cicero's court speeches confirm the high incidence of murder by poison in the first century BC. Poisoning during the first century AD reached a peak during the reign of the Julio-Claudian emperors. The emperors poisoned members of their families and others who displeased them. Horace tells of the professional

poisoner Canidia, who with Martina and Locusta became an infamous trio of women poisoners. Locusta in particular gained infamy as a poisoner in Rome. Convicted of multiple crimes under Claudius, she was sentenced to death. But the sentence had not been carried out when Claudius died. The new Emperor, Nero, made use of Locusta to eliminate many of his rivals, including his half brother, Britannicus. Once Britannicus was dead, Nero suspended Locusta's death sentence and made her his advisor on poisons. Nero organized a school of poisoning where she could tutor others and conduct experiments to determine how to poison and defend the Emperor against poison. Locusta became one of the first to systematically investigate the use of poisons with state sponsorship.

Reports of poisoning continued during the reign of subsequent emperors during the first century AD, and poisoning almost became a status symbol with the moral decay of Rome. Mass poisonings were recorded. Suicide by poisoning was not uncommon, but Pliny the Elder defended euthanasia by poison in the elderly when so desired. During the second century AD when tensions and fear of the previous two centuries gave way to peace and prosperity, very few deaths by poisoning were recorded.

Our understanding of poisons available during Roman times is derived from the writings of Dioscorides, Scribonius Largus, Nicander, Pliny the Elder, and Galen (note the influence of the Greeks). Poisoners preferred plant poisons rather than animal or mineral poisons. Favorites included belladonna, aconite (wolfsbane, monkshood), hemlock, hellebore, colchicum, yew extract, and opium. The specific poisons used in poisoning incidents are rarely mentioned, but it is known that hemlock in honey was the poison favored by Canidia and that Seneca drank hemlock. Ovid called aconite *mother-in-law's poison*.

The first effort at improving the mithridatum was by Damocrates, one of Nero's body physicians, and is known as *mithridatum Damocratis*. Andromachus the Elder (c. AD 60) was another archiater (the chief physician of some cities and first body physician of princes), in this case, the royal physician to Nero. He too was ordered by Nero to improve on the existing antidote. Andromachus removed some ingredients from the mithridatum and added others: squill, opium, and, the most important, vipers' flesh. It was administered in honey to Nero. This became known as *theriaca Andromachi* or Venice treacle. (The name *Theriaca* or *Tiriaca* could have come from the work of Nicander of Colophon. It is also reportedly derived from the snake called tyrus, the flesh of which was added to the mixture by Andromachus.) The *Theriaca* contained 70 substances and was used until the eighteenth century.

Mercurialism as an occupational disease was recognized by the Romans. Mining in the Spanish cinnabar mines of Almadén, 225 km southwest of Madrid, was regarded as being akin to a death sentence due to the shortened life expectancy of the miners, who were slaves or convicts. We now know that this shortened life was due to the exposure of the miners to mercury. Cinnabar, mercuric sulfide, is the principal ore of mercury and was used as a red pigment. Later, it was a source of mercury metal, which was used for centuries as the best way to extract gold and silver from their ores. When Spanish prospectors discovered rich cinnabar deposits in central California, they named the site after the mines of Almadén, Spain. The ready availability of New Almaden mercury was a crucial ingredient in the California gold rush.

Chemical warfare was used by and against the Romans. The Romans catapulted bees and hornets at their enemies. Hannibal of Carthage hurled pots of snakes on the decks of Roman ships in a sea battle during the Punic Wars, 184 BC. When the pots broke, the Romans were forced to fight both the snakes and Hannibal's forces. The Romans were not above poisoning wells when it suited them.

The first law against poisoning, *lex Cornelia de sicariis et veneficis* (concerning assassins and sorcerers), was passed in the time of Sulla (82 BC). The law not only provided for cases of poisoning but contained provisions against those who made, sold, bought, possessed, or gave poison for the purpose of poisoning.

Aurelius Cornelius Celsus (30 BC–AD 50) was the author of the first systematic Roman treatise on medicine. It is the most important historical source of knowledge of Alexandrian and Roman medicine. Little is known of Celsus. It appears that he was not esteemed as a scientist in his time, and there is dispute as to whether he was even a physician. His fame rests entirely upon his *De Medicina*, in eight books. *De Medicina* was among the first medical books to be printed early in the Renaissance (in Florence, 1478), and more than 50 editions appeared. It became very influential largely because of its splendid Latin style. It was required reading in most medical schools into the 1800s. The surgical section, which even Joseph Lister studied in the nineteenth century, is perhaps the best part of the treatise. His four classical signs of inflammation—calor, dolor, rubor, and tumor (heat, pain, redness, and swelling)—are still used today. Book 5, *Toxicology and Rabies* (note the difficulty in separating poisons from pathogens), includes the works of Nicander and Dioscorides and covered poisons and antidotes. He cited others who believed that poisons and animal venoms depressed a vital factor resulting in the loss of innate heat. His contributions to toxicology include his list of poisons and antidotes and the management of poisoning. Consistent with Hippocratic teaching, Celsus advocated eliminating the poison as quickly as possible (acrid materials applied to wounds, cupping severe wounds, suction with palms of the hand, and the use of hypertonic salt solutions). In addition, he recommended the use of appropriate antidotes including the antidote of Mithridates: 37 ingredients in honey.

Gaius Plinius Secundus, better known as Pliny the Elder (AD 23–79), was a famous Roman naturalist, historian, military tactician, philosopher, and one of the most learned men of his time, writing 160 books. His most famous and one surviving work, *Historia Naturalis* (*Natural History*),

was published in AD 77. *Historia Naturalis* consists of 37 books that covered all that the Romans knew about the natural world in the fields of astronomy, geography, zoology, botany, mineralogy, medicine, metallurgy, and agriculture. Despite its flaws, *Historia Naturalis* remains a key resource on Roman life. Pliny's contributions to toxicology include lists of poisons and their biological effects and his questioning of the value of nonspecific antidotes like mithridatics. He was also interested in adulteration of foods and developed methods for the detection of adulteration (e.g., chalk in flour, herbs, and spices).

Galen (see in the previous discussion) became the chief physician of Rome in AD 164 and is credited with systematizing Roman medical practice. Through Galen, the totality of Greek medicine became part of the Roman world, and it was the Roman medicine that was passed down to posterity as *western* medicine.

After the collapse of the western Roman Empire in the fifth century, Europe lost touch with much of its medical heritage. The center of Europe's view became the Church, which exerted profound influence on medicine. The Church viewed care for the soul as far more important than care of the body, so much so that medical treatments and even physical cleanliness were little valued. In time, illness became seen as a condition caused by supernatural forces and cures could only be effected by holy men. This pre-Hippocratic belief that disease was punishment by God and treatable only by prayer and penance meant that licensed medicine as an occupation vanished. It would be centuries before its return to Europe.

Middle East

At roughly the same time that Europe was moving away from medicine, a new civilization was rising to the east. Pre-Islamic medicine in the Arab region had been negligible due to the unsettled, nomadic life. As Islam spread and conditions changed, the Arabs attempted to collect all knowledge that was available. Greek medicine was one of the first sciences studied by Islamic scholars. Translators rendered the entire body of Greek medical texts into Arabic by the end of the ninth century. These translations established the foundations of Arab medicine. Based on the Greek teachings, Arab physicians came to look upon medicine as the science that helps recognize the dispositions of the human body, with the goal of preserving health and, if health was lost, assisting in recovering it. The Arabs also learned from the Indo-Persian practices further east. They built on these traditions and made significant contributions to all the health professions.

The Arabs excelled in chemistry and are credited with inventing distillation, sublimation, and crystallization. Jabir ibn Hayyan (Latinized to *Gerber*, c. 705–769) may be the father of Arab alchemy. He was an expert in chemical procedures and was the first to discover mercury. He produced arsenic trioxide (arsenious oxide, As_4O_6) from realgar (arsenic sulfide, As_4S_4), a naturally occurring, red-colored ore found in lead and iron mining, and thus made available to mankind one of the most widely administered poisons for homicide. He wrote one of the first pharmacological treatises in Arabic.

The Arab pharmacopoeia of the time was extensive and gave descriptions of the geographical origin, physical properties, and methods of application of everything found useful in curing disease. Arab pharmacists introduced a large number of new drugs to clinical practice, including senna, camphor, sandalwood, musk, myrrh, cassia, tamarind, nutmeg, cloves, benzoin, saffron, laudanum, naphtha, and mercury. They were familiar with the anesthetic effects of cannabis and henbane, both when taken as liquids and when inhaled.

The practice of pharmacy was extended by Arab physicians and eventually became a separate profession run by highly skilled specialists who were licensed. Arab pharmacies are considered to be the forerunners of modern pharmacies. To keep patients happy, make the physician's job easier, and promote more effective healing, Arab pharmacists are credited with developing or perfecting syrups and juleps (words from the Arabic, to drink, and Persian, rose water, respectively), tinctures, confections, pomades, plasters, and ointments as means of administering drugs. They were the first to wrap medicines (pellets) in silver foil.

Abu Ali Husain ibn Abdullah ibn Sina (Latinized to *Avicenna*, 980–1037), the *prince of physicians*, was born in Bokhara, Persia (today, Bukhara, Uzbekistan). He was a child prodigy whose fame as a physician was so great that by the age of 18 he was appointed physician to the prince and became physician-in-chief to the hospital in Baghdad. He was a logical thinker and an astute observer. Some have referred to him as a second Aristotle. Avicenna wanted to develop a system of medicine, to make medicine *a quasi-mathematical discipline*. This would remove uncertainty from medical decisions (cf. Hippocrates, Galen). By the age of 21, he had written a 20-volume encyclopedia. His most significant medical works were *Book of Healing*, a medical and philosophical encyclopedia, and *The Canon of Medicine*, a codification of all existing medical knowledge. *The Canon* included descriptions of some 760 medicinal plants and drugs that could be derived from them. *The Canon* rapidly became the standard medical reference of the Islamic world.

Avicenna laid out the basic rules of drug trials that are still followed today. He discussed oral and parenteral poisons and bites and stings and their treatment, and classified and discussed poisons as plant, animal, or mineral.

His contributions to toxicology include mechanisms of action of poisons including neurotoxicity and metabolic effects. He also recommended the bezoar (from the Arabic *bazahr*, from the Persian *pad-zahr counterpoison*, from *pad protecting*, *guardian*, *master* + *zahr poison*) stone as an antidote for venoms and preventive of disease. Originally, it meant *antidote*, but later, it referred specifically to a solid mass found in the stomachs and intestines of

ruminants, which was held to have antidotal qualities. His work was the authoritative text on poisons and antidotes for 500 years.

Jewish medical erudition was of the background of Greek, Roman, and Arabic works. Rabbi Moses ben Maimon (Moses Maimonides, 1135–1204) was a famous Jewish philosopher and physician, court physician to Saladin, and rabbi of Cairo. His compendium on poisons, *Poisons and Antidotes/Upon Poisoning and Its Treatment*, was translated into Latin by Armend and Blasii in 1305, into German in 1813 by Steinschneider, and into French in 1865. He taught that the simplest method to poison someone was to add a single or compound poison to a highly spiced and/or chopped dish or in a victim's glass of wine, under the reasoning that the strong flavors and uneven texture would mask the bitter taste or consistency. He described poisonous insects and animals and noted that the most dangerous bite was that of a fasting human. His treatment of poisons included ligature of the bite, sucking out the poison by means of cupping glasses or with oiled lips (another extension of Hippocratic teaching to decrease absorption), and the use of external (e.g., salt, onions, asafetida) and internal remedies (e.g., emetics). His books on health were very advanced and resemble modern medical texts. He believed in the importance of preventive medicine and stressed the importance of hygiene. He wrote a four-volume treatise upon hygiene and diet (*Sepher Rephuoth*).

The medical works of Hippocrates and Galen were returned to the West by way of the Middle East and North Africa, recovered through translation of Arab medical references in Sicily, southern France, and Spain. Avicenna's *The Canon of Medicine* had a great influence on Europe during the Middle Ages and was a standard European medical text for centuries. Its *materia medica* was the pharmacopoeia of Europe. So great was the reputation of Arab physicians that Chaucer names four in *The Canterbury Tales* and Dante, in *The Inferno*, and placed Avicenna next to two other great physicians from ancient times, Hippocrates and Galen.

Middle Ages (c. 500–1450) and the Renaissance (c. 1450–1600)

As there was little medical work in Europe during the Middle Ages, information related to drugs and poisons is meager. European works on poisons were largely based on the remnants of classical works available and on the works of the Arabs.

Academic texts on poisoning were often written by monks since monasteries were the main seats of learning in a largely illiterate population. One example is *The Book of Venoms*, written by Magister Santes de Ardoynis in 1424. This was a reasonably comprehensive account of the poisons known at the time (e.g., arsenic, aconite, hellebore, laurel, opium, mandrake, cantharides), their effects, and treatment.

Pietro d'Abano (c. 1250–1316) was a teacher of science and medicine at the University of Padua and one of the most renowned teachers and skillful physicians of his time. His famous work *Conciliator Differentiarium* attempted to reconcile Arabic medicine and Greek natural philosophy. In another book, *De remediis venenorum* (*De venenis eorumque remediis*), he classified poisons as mineral, vegetable, and animal. Unfortunately many innocuous substances were often in the lists of ingredients thought to be poisonous, these were side-by-side with many truly deadly plants and minerals. He correctly noted that poisons can be absorbed from air and through the skin (*poisoning by touch*). The book was very popular and went through 14 editions. The power of the Church was great at that time, and he was tried twice by the Inquisition on charges of heresy and practicing magic. Acquitted at the first trial, he was found guilty at the second, after his death.

During the Renaissance, a shift in the notion of illness occurred. Instead of humoral theories dating back to the Greeks that had physicians regarding each patient's disease as unique, changed to a view where disease was no longer a unique experience but a process essentially similar in all patients. Medicine began to look a bit more like how we see it today. The period of observation (recording phenomena) and categorizing and listing poisons started to give way to the period of challenge and active investigation—experimental science. God was still used to describe conditions and cures, but the grip of the Church was weakening, and interpretation and experimentation were becoming more important.

Ulrich Ellenbog's *Treatise on Industrial Hygiene*, written in 1473, is the first known work on industrial hygiene and toxicology. The book dealt with occupational diseases and injuries among gold miners. Ellenbog also wrote about the toxicity of carbon monoxide, mercury, lead, and nitric acid.

George Bauer (Latinized to Georgius Agricola, 1494–1555) was born in Saxony during the early years of the Renaissance. In 1522, he began to study medicine, first at Leipzig and then at Bologna and Padua—Italian universities at the center for science, medicine, and philosophy. He took his degree in 1526 and became a practicing physician in the ore-producing region of Bohemia. He initially hoped to discover new medical drugs from mine ores. Agricola, who is considered the father of mineralogy, observed firsthand the ill effects of the mining operations on miners. The publication of his book (1556), *De Re Metallica*, included a section on industrial hygiene as he described how the miners suffered from diseases as a consequence of their work. He attributed the diseases to the dusts, stagnant air, and gases in the mines. The book included suggestions for mine ventilation and worker protection (masks), discussed mining accidents, and described diseases associated with mining occupations such as silicosis. At the same time, he also blamed the miners for their condition because of their carelessness. He died one year before the publication of his great work.

While most of this seems sensible today, Agricola's readiness to discard received authority, even that of classical

authors, is impressive. He was among the first to found a natural science based on observation and field experience, as opposed to dogma or conjecture. His works on mining were by no means the only ones available at the time, but his were not steeped in the ideas of alchemists, of whom Agricola had a low opinion. Herbert Hoover, a mining engineer who became a U.S. president, translated *De Re Metallica* into English in 1912. Hoover regarded Agricola as the originator of the experimental approach to science, *the first to found any of the natural sciences upon research and observation, as opposed to previous fruitless speculation*.

> The universities do not teach all things.
>
> **Paracelsus**

Philippus Theophrastus Aureolus Bombastus von Hohenheim (1493–1541) was born outside the village of Einsiedeln (near Zurich), Switzerland, the son of Wilhelm Bombast von Hohenheim, a German physician/alchemist. Following the death of his mother when he was very young, Paracelsus moved to Villach in southern Austria, where his father taught chemistry, practiced medicine, and became interested in the health problems of the local miners, eventually becoming an expert in occupational medicine. Paracelsus attended the universities of Basel, Tubingen, Wittenberg, Leipzig, Heidelberg, Cologne, and Vienna, from which he received a baccalaureate in medicine in 1510, at the age of 17. He received his doctorate from the University of Ferrara in 1516. It was the custom of that time to Latinize one's name after receiving a degree and Philippus von Hohenheim chose the name Paracelsus (*para*, above, Celsus), since he considered himself greater than Celsus. He traveled throughout Europe, England, Scotland, Egypt, the Holy Land, and Constantinople, attempting to learn the most effective means of medical treatment and the latest findings in alchemy. He returned to Villach in 1524 and became town physician and lecturer in medicine at the University of Basel in 1527.

He was a keen student of human behavior who believed that practicing physicians needed to use common sense, gain experience, travel, and practice humility. As his fame spread, his lectures became very popular and students thronged to them.

Paracelsus' enduring legacy is at times puzzling, though. He was of low personal hygiene, a drunk, and seemed not to have paid his bills. He never stayed in one place too long, possibly because of enraged patients and threats of lawsuits. Cope (1957) described Paracelsus as arrogant and conceited

> almost to the point of insanity… extremely effective in [his] criticisms of the then accepted doctrines… reveled in the wildest speculations and taught [his] mad conjectures as unassailable truths… bitter and unscrupulous controversialist… mystic… his writings… so confused and obscure as to be often quite unintelligible… braggart, scorner of authority… that Paracelsus scarcely ever lectured except when he was half drunk, or attended a patient until he was wholly drunk.

Paracelsus defended himself in his *Seven Arguments, Answering to Several of the Detractions of His Envious Critics*, written in 1537.

He was part medic, part mystic. This is understandable when considering that he was a product of his times, when magic and science existed together. It was a turbulent age, with the Reformation and all that it brought. In addition, the scientific revolution was starting. At the same time, people were also reconnecting with the classical teachings from Egypt to Greece and Rome. Thus, Paracelsus was an astrologer and alchemist and mystic as well as a physician. The paradoxes that surround Paracelsus can be explained from these perspectives.

An iconoclast, Paracelsus believed all physicians who preceded him were incompetent, liars, or fakers. His disdain for established authorities, for everything that had been said by his predecessors, reached its climax on June 24, 1527, when he publicly burned the books of Avicenna and Galen in front of the university. By doing this, he discarded the old ways, in order to demonstrate that reconnection to classical medicine was not a move forward. At the same time, he attacked the medical principles of his time and trusted only his own observations, ideas, and works.

Paracelsus was a deeply religious man. He was intensely concerned with the eternity, or soul, in man and felt that a doctor was neither *pillmaker* nor businessman, but a legate of God, the supreme physician. Medicine was therefore a divine mission, and the doctor must raise his eyes from *excrements and salvepots to the stars*. The perfect physician, he felt, was a philosopher, an astrologer, an alchemist, and, above all, a virtuous man. The character of such a doctor, Paracelsus proclaimed, was far more effective than mere mechanical skill (Bettman, 1979).

His approach to medicine and the body was chemical. He taught that it was more important to learn about the chemical composition of the body than about the muscles (i.e., more chemistry/biochemistry and less emphasis on anatomy and physiology). It was the role of the alchemist to find these chemicals and convert them to effective remedies, and Paracelsus, the physician/alchemist, began to do so with simple materials, the metals. He tried to bring chemistry into therapeutics and is credited with the introduction of mineral baths, laudanum, mercury, lead, arsenic, copper sulfate, and iron into the practice of medicine.

Paracelsus' positive contributions to medicine and toxicology outweigh their incongruity with his mystical approach. He is considered by some to be the father (founder) of chemistry and/or medicinal chemistry and the reformer of *materia medica*. He forever destroyed the doctrine of the four humors as the basis for disease, and believed that diseases were specific/discrete conditions and are cured by specific/discrete treatments. He taught that observation and experience are essential for success in medicine. Although others made observations in humans (often after deliberately administering a poison), Paracelsus encouraged the use of animals. He also developed and promulgated certain basic principles of the action of chemicals

(e.g., dose–response) that still form the scientific underpinnings of modern experimental toxicology.

In the *Third Defense*, he wrote, "What is there that is not poison? All things are poison and nothing (is) without poison. Solely, the dose determines that a thing is not a poison." (Deichmann et al., 1986). This concept has been expanded to include no-effect level, threshold, extrapolation, and dose–response relationship. His other contributions include target organ toxicity, animal experimentation to study the effects of chemicals, and the use of inorganic salts in medicine. He was thoroughly seduced by the complexity of chemical–biological interactions and spent his lifetime trying to solve the mysteries of these interactions.

His principal works include *Chirurgia magna* (1536), *De gradibus* (1568), and *A Treatise on Diseases of Miners* (1567).

Paracelsus died at age 49, some say in a brawl at the White Horse Tavern in Salzburg on December 24, 1541. Despite his early death, Paracelsus permanently changed the course of medicine and toxicology.

Attempts to explain the action of toxins attracted the attention of other giants in the biomedical sciences of that time, such as Ambroise Paré (1510–1590). Paré practiced surgery in France and is considered a founding father of modern surgical practice, *the greatest surgeon of the sixteenth century*, and one of the most famous anatomists of all time. Paré is remembered mainly for innovations in treating war wounds and for the treatment of skin ulcers, but he also investigated carbon monoxide poisoning and published a report in 1575. He proved that bezoar stones could not cure all poisons. He may have been the first person to state that each chemical has a specific toxic response. Another French physician, Jacques Grevin (1538–1570), the *father of modern biotoxicology*, published his classical work, *Deux Livres des Venins*, in 1568 and further developed the concept of chemical–biological interactions.

The beginning of the Renaissance also began a period of a great number of notorious poisonings. Poisons and their effects were being studied by alchemists mainly to create the most potent concoction. Life was not valued as it is today and poisons became a leading weapon, due to their relative inconspicuousness, to remove rivals or partners. Perhaps the most notorious of the poisoners were Cesare (1476–1507) and Lucrezia (1480–1519) Borgia, the illegitimate children of Rodrigo Lenzuoli Borgia (1431–1503) who become Pope Alexander VI in 1492. They dispatched several of their rivals with a secret poison, *La Cantarella*. The exact composition of *La Cantarella* is not known, but it may have included copper, arsenic, and phosphorous, which reflected the trend in alchemy at that time to make the most potent mixtures from known toxic substances. The poor quality of historical records makes it difficult to know who actually committed the crimes, but Cesare is the primary suspect. The death of Pope Alexander VI was likely due to poisoning, but it is not known whether he drank poisoned wine intended for a cardinal, or the cardinal had him poisoned, or there was a mix-up in the kitchen!

In Venice, during the sixteenth century, a body of alchemists known as the *Council of Ten* met regularly to arrange poisonings for the state. The Council's written records have been preserved, showing they planned, voted, and carried out the demise of any chosen person for a sum of money. Victims were named, prices agreed upon, and contracts with poisoners recorded. Payment was made after the deed was accomplished. The Council seems to have had a number of poisonous ingredients available: corrosive sublimate (mercuric chloride), white arsenic (arsenic trioxide), arsenic trisulfide, and arsenic trichloride. In 1543, John of Ragusa, a Franciscan brother and mercenary poisoner, confronted the Council, declaring that with his collection of poisons, he could remove any person from society. He also added, "The farther the journey, the more eminent the man, the more it is necessary to reward the toil and hardship undertaken, and the heavier must be the payment." Perhaps, he may be considered the first consulting toxicologist. His estimate was carefully considered by the Doge and Council of Venice.

Poisoning had become such an art and so rampant that schools for poisoners were established in Venice and Rome. A publication on the art of poisoning appeared in 1589. Written by Giovanni Battista Porta (1535–1615), *Neopoliani Magioe Naturalis* describes various methods of poisoning, particularly drugging wine, as this was perhaps the most popular method at that time. Described as *a very strong poison*, Porta gives a formula for *Veninum Lupinum*. This was a concoction of aconite, yew, caustic lime, arsenic, bitter almonds, and powdered glass. Mixed with honey, it was made into pills the size of walnuts.

Even Leonardo da Vinci (1452–1519) experimented with poisons, trying to make them more potent by passage through animals and did some musing about their use for chemical warfare in the form of throwing powdered chalk and arsenic trisulfide on enemy ships.

Royalty used poisons on their rivals and on the poor, just to study the effects on humans. Catherine de Medici (1519–1589) of Florence and later queen of France tested and carefully studied the effects of various toxic concoctions on the poor and the sick, noting onset of action, potency, site of action, and signs and symptoms.

Marie-Madeleine-Marguerite d'Aubray, Marquise de Brinvilliers (1630–1676), was a French poisoner who worked with her lover Jean-Batiste de Godin de St. Croix. Brinvilliers poisoned her father, two brothers, and a sister in 1670 for their inheritance. She attempted to poison her children's tutor, Briancourt, with whom she had shared romantic relations, but his quick wits saved him. His intelligence also saved the lives of Brinvilliers' sister-in-law and sister, cloistered in a convent, whom she also tried to poison. Brinvilliers even went so far as to poison her own daughter, merely because she thought her stupid! She regretted it immediately afterward however and made her drink a great quantity of milk as an antidote. She appears to have used Tofana poison (see in the following), whose recipe she seems to have learned from her lover, who had learned it from Exili, an Italian chemist and poisoner whose real name was probably Nicolo Egidi or Eggidio, who had been his cellmate in the Bastille. Sainte-Croix betrayed her upon his death, with incriminating

documents found among his belongings. After several years on the run in England and the Netherlands, Madame de Brinvilliers was tried and convicted on all charges of poisoning. She was forced to do public penance, was put to torture, both ordinary and extraordinary, and was beheaded.

The pinnacle of this period in France occurred with Catherine Deshayes (c. 1638–1680), popularly known as *La Voisin*. La Voisin started her career by practicing fortune-telling to support her family. She practiced medicine, especially midwifery, and performed abortions. She sold aphrodisiacs to those who wished for people to fall in love with them and poison to those who wished for someone to die. La Voisin was interested in science and alchemy and financed several private projects and enterprises, some by con artists. She was part of a network of fortune-tellers in Paris who dealt with the distribution of poison. La Voisin was convicted of witchcraft and burned in public in Paris on February 22, 1680.

Throughout time, women have taken a particular interest in poison for criminal purposes, and the extent of this can be seen in an account from Rome in 1659. A society of women was formed in secret, meeting regularly at the house of a reputed witch, Hieronyma Spara. Usually married, the members of this society were issued the poison they required with instructions for its use. Spara was eventually arrested by the Papal police and tortured on the rack. She refused to confess. Nevertheless, she was hanged along with a dozen other women suspected of having been her aides.

Perhaps the most notorious poisoner of seventeenth century Italy was a woman from Palermo named Giulia Tofana (executed in Rome, 1659). She invented a poisonous mixture, *Aqua Tofana*; although some records suggest her mother invented it and passed the recipe to her. The solution was mostly arsenic and lead and possibly belladonna; it was a colorless, tasteless liquid and easily mixed with water or wine to be served during meals. It was sold in vials that bore the representation of a saint, usually Saint Nicholas of Bari. She managed to sell it under the pretense that it helped a woman's complexion. This was not a complete misrepresentation since the active ingredient was arsenic that was used to treat various skin disorders. However, the real purpose of her vials was made known to those with whom Tofana had a *rapport*. She made a good business for over 50 years selling a large production of Aqua Tofana—she employed her daughter and several other lady helpers—to would-be widows. It has been estimated that Tofana aided the murder of over 600 people between 1633 and 1651, usually husbands, making her one of the *greatest* homicidal poisoners of all time.

By the end of the Renaissance, despite the poisonings, science was flourishing; it was the beginning of the period of enlightenment. Competition among ideas allowed old thinking to be discarded. New ideas provided a path forward to allow for the development of theories that could be tested (cause and effect) and the practical application of scientific information.

Mathematics also flourished. While generally not regarded as a key part of toxicology, risk assessment has its roots in mathematics. The work on numbers by Greeks like Diophantus was lost over the centuries, but even so, he lacked zero that meant mathematics could not progress very far. Europe labored under Roman numerals for centuries. The Arabs, however, used a revolutionary numbering system. These numbers, especially zero derived from their contact with Hindus, allowed mathematics to be its own field of study. Mathematics in the form of algebra was practiced in Arab lands in the 800s. The Arabic numbering system and algebra were introduced to Europe by the Moors in Spain and the Saracens in Sicily around the year 1000. The new numbers were in general use by the thirteenth century.

The use of numbers and mathematics from the Arabs was important for two reasons. The obvious one is better quantification and accuracy. Everything in science must be quantified and the new numbers allowed that to happen. Additionally, the new numbers and mathematics allowed probability theory to be developed. Without it, the risk assessments we perform today would not be possible.

In 1654, Blaise Pascal was asked to solve a problem by a gambler: how to divide the stakes of an unfinished game when one player is ahead. It had stumped mathematicians for 200 years and Pascal turned to Pierre de Fermat for help. The outcome of their collaboration led to the theory of probability. Their solution meant that people could for the first time make decisions based on mathematics. Before this, when people made decisions, it was based on superstition, mysticism, or tradition. Probability theory allowed risk to be understood in a rational way.

SYSTEMATIC TOXICOLOGY

Felice Fontana (1720–1805), an abbot, physician, physiologist, naturalist, and professor of philosophy at Pisa and director of the Natural History Museum at Florence, investigated the physiological action of poisons, particularly of snakes. He is the first modern scientist to study venoms (*Ricerche fisiche sopra il veleno della vipera*, 1767). After a series of impressive and ingenious experiments, Fontana believed the action of the bite of the viper to be an alteration in the irritability of the fibers, which he maintained was mediated by the blood: in other words, the poison directly alters the blood, coagulating it, and this in turn alters all parts of the organism—especially the nerve fibers—that the blood would normally nourish. Through this work, he advanced the concept of target organ toxicity and secondary toxicity, that is, the symptoms of poisoning may not be the result of poisons acting on a particular organ but may occur as a result of effects on other organs/tissues. Fontana extended his toxicological experiments to other substances, especially to the laurel berry and curare. Although he did not hold a chair in chemistry, Fontana was perhaps the greatest Italian chemist of the end of the eighteenth century.

Richard Mead (1673–1754) was a British physician (medical degree from Padua) who worked at St. Thomas' Hospital and was physician to many of the leading figures of the day including King George I, George II, Isaac Newton, and Robert Walpole (the first British prime minister).

He attempted to explain the action of poisons (venoms) in his book *A Mechanical Account of Poisons* (1702). The book was well received and established Mead's reputation, although it has been said that the rules of treatment laid down are sounder than the arguments. Its publication excited so much attention that an abstract of it was printed in the *Philosophical Transactions* for 1703. Mead dissected vipers and gave an exact account of the mechanism that provides for the erection of the fang when the snake opens its mouth. He described snake poisoning and noted that the venom is only effective parenterally. He swallowed the poison and confirmed Galen's experiment on fowls, that puncture is necessary to produce the effect. Mead also considered other poisonous animals, plants including opium, and toxic natural gases. *A Mechanical Account of Poisons* was later republished with many additions in 1743.

Ellenbog, Agricola, and Paracelsus drew attention to the plight of miners, but little consideration was focused on the effects of nondeliberate exposure to chemicals in other work environments. It was the brilliant Italian physician Bernardino Ramazzini (1633–1714) who effectively and convincingly brought the entire workplace situation to the attention of the world, especially to the field of medicine. He was the first to describe in a comprehensive, systematic, and detailed fashion industrial health problems in his *De Morbis Artificium Diatriba* (*Diseases of Workers*). This is the first comprehensive work on occupational diseases and was published in 1700, although Ramazzini lectured on this topic as early as in 1690. It is considered a milestone in the history of occupational medicine. *De Morbis Artificium Diatriba* outlines the health hazards of irritating chemicals, dust, metals, and abrasive agents. The book describes the hazards of 52 occupations, such as tanners, miners, potters, masons, farmers, nurses, and soldiers. He noted the high incidence of breast cancer among nuns, which he attributed to their celibate life (which is now known to be due to the unabated presence of estrogen). In discussing the etiology, treatment, and prevention of these diseases, Ramazzini often cites Hippocrates, Celsus, and Galen and, after summarizing their observations, relates his own experience with the various diseases. By recognizing the social significance of occupational diseases, he earned the title, the *father of industrial hygiene*.

The observations of Ramazzini concerning the relationship between workplace exposure and disease were extended by the classical studies of Sir Percival Pott (1714–1788), British physician and surgeon to St. Bartholomew's Hospital (from 1749 to 1787), who achieved fame in two areas: surgery and occupational medicine/toxicology. His contributions to toxicology include describing in 1775 the relationship between squamous cell carcinoma of the scrotum (*sooty warts*) in London chimney sweeps, whose job it was to clean the residue from the chimneys and fireplaces, and the soot from burning coal. French counterparts were less prone to developing skin cancers because they bathed more frequently after working. This was the first identification of occupational chemical carcinogenesis and represents the beginning of the study of occupational cancer. He also noted the increased sensitivity of children to some chemicals. The sweeps were usually children who worked from age 8 to adulthood, although *apprenticeships* could start at age 4. Pott noted that this was an occupational disease and postulated that the cancer was caused by an ingredient in the residue from the burning coal. Pott's pioneering work resulted in *The Chimney Sweepers Act* of 1788, the year of his death.

With the Industrial Revolution, which occurred between 1760 and 1830, workers no longer owned the means of production. The demand for goods had grown to a point whereby the only means of meeting it was through mass production. This production was achieved through the invention of machines, such as James Hargreaves' spinning jenny, which could do the work of several individual workers. With the machines came the textile mills and factories, which in turn generated a proportionate increase in the exposure to chemicals needed for processing textiles such as acids, alkalis, soaps, and mordants (substances that fix a dye in and on textiles and leather by combining with the dye to form a stable, insoluble compound). As more workers were used to increase production, exposures to chemicals and dusts increased. The factory owners realized the benefits of increased production, but the risks were borne by the workers—not an acceptable or sustainable risk/benefit relationship. Charles Turner Thackrah (1795–1833) developed an interest in the diseases he came to see among the poorer classes of people living in the city of Leeds. His observations led him to develop some of the basic principles of occupational hygiene to improve the health of his patients. He advocated the elimination of lead as a glaze in the pottery industry and ventilation and respiratory protection to protect knife grinders and suggested a change in the work practices of tailors and in the design of their work stations to eliminate their cramped postures that he felt contributed to their high prevalence of tuberculosis. He published a book in 1831 entitled *The Effects of the Principal Arts, Trades and Professions, and of Civic States and Habits of Living, on Health and Longevity, with Suggestions for the Removal of many of the Agents which produce Disease and Shorten the Duration of Life*. Although Ramazzini recognized the relationship between a worker's occupation and health, Thackrah's work occurred in the heart of the Industrial Revolution, England. Thackrah was the first physician in the English-speaking world to establish the practice of industrial medicine. His writing led to a raised public awareness of the abominable working conditions of the new working class such that public outcry and the efforts of reformers led to the passing of the *Factory Act* in 1833 and the *Mines Act* in 1842.

Forensic toxicology, the application of analytical techniques to the detection of poisons, had its beginning with Joseph Jacob Plenck (c. 1735–1807), who noted in his text, *Elementa Medicinae et Chirurgiae Forensis*, that the only proof of poisoning is the identification of the poison in the organs of the body. This remains a basic principle of forensic toxicology. Unfortunately, it was not accepted by the medical or scientific communities until the work of Orfila (see in the following). Plenck also wrote a treatise, *Icones Plantarum*

Medicinalium secundum systema Lynnaei cum enumeratione virium et usus medici, *chirurgici et diaetetici*, dealing with the therapeutic use of about 800 plants. It centered on 111 plants with diuretic properties that still appear in many pharmacopoeias. Plenck is a forerunner of modern dermatology and also worked in ophthalmology.

Fredrich Accum (1769–1838) is largely forgotten these days but contributed to important changes in society by raising awareness of food safety. The application of analytical chemistry to matters of food and drug safety formally began with Accum, although earlier attempts were made by Theophrastus, Cato, Pliny the Elder, Dioscorides, and Galen. Born in Buckeburg, Germany, Accum moved to London in 1797 as a pharmacist. In Accum's time, it was common to add all sorts of materials to food to make it cheaper to produce and yet still pass as a quality product. He was the first to use analytical chemistry to detect adulterants in food and published *A Treatise on Adulterations of Food and Culinary Poisons* in 1820, a very successful book that was acclaimed worldwide. Accum and fellow campaigners fought against food fraud and paved the way for the 1875 *Sale of Food and Drugs Act* in Britain. He also published *An Attempt to Discover the Genuineness and Purity of Drugs and Medicinal Preparations.* Accum also had an entrepreneurial bent and was successful in his business of selling laboratory chemicals and equipment. He equipped the first chemistry laboratories of both Harvard and Yale. He left England and returned to Germany because of unsubstantiated charges of embezzlement directed against him related to his position as a librarian.

ANALYTICAL AND MECHANISTIC TOXICOLOGY

> In physical science the first essential step in the direction of learning any subject is to find principles of numerical reckoning and practicable methods for measuring some quality connected with it. I often say that when you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meager and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely in your thoughts advanced to the state of *Science*, whatever the matter may be.
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> **—Sir William Thomson, Lord Kelvin (1883)**

Advances in chemistry, physiology, pathology, and clinical medicine in the eighteenth and nineteenth centuries resulted in significant advances in toxicology. The time had come for analytical techniques to be formally incorporated into toxicology. Up until this time, it had been difficult to establish poisons as the cause of death since they could not be identified in tissues, the only scientifically valid proof.

Analytical (forensic) toxicology had its formal origins in the outstanding work of Mathieu Joseph Bonaventure Orfila (1787–1853). He was born on the island of Minorca, Spain, and educated in Valencia and Barcelona. He studied chemistry and medicine in Paris, receiving his medical degree from the University of Paris in 1811. Upon graduation, he became a private lecturer on chemistry. In 1813, when he was only 26, he published his monumental two-volume work, *Traité de Toxicologie: Traité des poisons tires des regnes mineral, vegetal at animal ou toxicologie generale considerée sous les rapports de la physiologie, de la pathologie et de la médecine légale* (Crochard, Paris). This classic work, the first of its kind, effectively combined forensic and clinical toxicology with analytical chemistry. It is a vast mine of experimental observation on the symptoms of poisoning of all kinds, on the effects poisons have in the body, on their physiological action, and on the means of detecting them. It earns Orfila the title, *father of forensic toxicology.* It is the first book devoted entirely to toxicology and established toxicology as an experimental science separate from pharmacology. He summed up everything known about poisons at the time and classified poisons into six categories: corrosives, astringents, acrids, narcotics, narcotico-acids, and septica and putrefacants. He presented the chemical, physical, physiological, and toxic properties of each chemical, methods of treatment, and chemical tests for their identification. An English translation of his work first appeared in 1816, and American editions were published in 1817 and 1826. In 1816, Orfila published *Eléments de chimie médecale* and in 1818, *Secours à donner aux personnes empoisonnées ou asphyxiées.* He provided a rational basis for some antidotes. He demonstrated the toxicity of strychnine in numerous experiments on dogs. At that time, strychnine was widely used in prescriptions and in tonics and was considered by practitioners of medicine to be a safe drug (Magendie, discussed in the following, later established the mechanism of action of strychnine). He later published *Leçons de médecine lègale* (1821), *Trait des exhumations juridiques* (1830), and *Recherches sur l'empoisonnement par l'acide arsenieux* (1841). Orfila's books were translated into many languages and this helped internationalize toxicology.

Becoming professor of medical jurisprudence in 1819, Orfila helped develop tests for the presence of blood and used a microscope to assess blood and semen stains. Four years later, he was professor of chemistry in the faculty of medicine at Paris. In 1830, he was nominated dean of that faculty, a high medical honor in France. Orfila was a capable physician and excellent analytical chemist. He assessed the various tests for poison detection and had found them to be highly unreliable. He was also an experimental toxicologist and administered known doses of poisons to animals, carefully observed the effects produced, examined organs for evidence of toxicity, and chemically analyzed tissues and body fluids to establish relationships between dose, response, and tissue levels. He was able to demonstrate conclusively and quantitatively that poisons are absorbed from the gastrointestinal tract and accumulate in tissues. He refined Rose's method for arsenic detection (in 1806, Valentine Rose showed how arsenic could be detected in human organs) to achieve greater testing accuracy. It was Orfila who showed with tests on animals that after ingestion, arsenic is distributed throughout the body. He consulted on many criminal cases due to his fame as an analyst and to his prominent university position. In one case, Madame Lafarge was accused of poisoning her

husband with arsenic and put on trial. Chemical tests conducted shortly after death were inconclusive. During the trial in 1840, Orfila had the body exhumed and found traces of arsenic in the man's organs (using the Marsh test, developed in 1836; see below). Madame Lafarge was found guilty and sentenced to the penitentiary for life. *L'affaire Lafarge* may have been the first trial in history in which convincing forensic evidence was successfully used in a court case.

Orfila's significant contributions to toxicology include the chemical detection of poisons in tissues and fluids, thereby permitting better diagnoses, furthering the concept of target organ toxicity by evaluating tissues grossly and histologically, relating symptoms to specific tissue injury, and extending the concept of dose–response. His investigations were also the forerunner of modern toxicokinetics and dynamics. His influence on modern toxicology is equal to that of Paracelsus and Bernard. His books were published in many languages and used in many countries. Few branches of science can be said to have been created and raised at once to a state of high advancement by the labors of a single man as Orfila did for toxicology.

Isidore Geoffroy Saint-Hilaire (1805–1861), a French zoologist, noted for his work studying anatomical abnormalities in humans and animals and coined the term *teratology* in 1832. His father had initiated studies on chicken eggs, but it was Isidore who first published an extensive work on teratology, *Histoire Générale et Particulière des Anomalies de l'Organisation chez l'Homme et les Animaux*, organizing all known human and animal malformations. Many of the principles governing abnormal development were described for the first time, and many hundreds of names for specific malformations are still in use.

James M. Marsh, an English chemist, developed a method for the detection of arsenic so sensitive that it can be used to detect minute amounts in foods or stomach contents (1836). Until the test was developed, arsenic, usually in the form of arsenic trioxide (As_2O_3), was a highly favored poison, for it is odorless, easily incorporated into food and drink, and untraceable in the body. Although there were tests developed to detect arsenic, they were insufficient for use in court.

In 1832, John Bodle was brought to trial for poisoning his grandfather by putting arsenic in his coffee. James Marsh, a chemist working at the Royal Arsenal, was asked by the prosecution to try to detect its presence. He performed the standard test by passing hydrogen sulfide through the suspect fluid. Marsh detected arsenic as a yellow precipitate, arsenic trisulfide (As_2S_3), but it did not keep well, and by the time it was presented in court, it had deteriorated. The jury was not convinced and Bodle was acquitted.

Angered and frustrated by this, especially when Bodle later confessed that he had killed his grandfather, Marsh decided to devise a better test. Marsh's test has a sample placed in a flask with arsenic-free zinc and sulfuric acid. Arsine gas forms and is led through a drying tube to a hard glass tube in which it is heated. Igniting the gas converts arsine to arsenic, which is deposited as a *mirror* just beyond the heated area on any cold surface held in the burning gas emanating from the jet. This test was sensitive enough, specific enough (antimony gives a similar result, but the deposit is insoluble in sodium hypochlorite, whereas arsenic will dissolve), and rigorous enough that it held up in court. The introduction of analytical methods for metallic poisons was such that it reduced their popularity, and practitioners turned to alkaloids isolated from plants.

The first half of the 1800s was an era of high-profile poisonings in Great Britain that left the public almost in a panic. The ready availability of poisons and the accessibility of scientific knowledge due to increased literacy and scientific education led the public to believe that poisoning was something new. The press fanned the panic by capturing and embellishing every detail. Additionally, poisoning seemed to pose a special problem to the new social order because of the rise of life insurance. People could now be murdered for money, not for being highly placed in society or a powerful ruler. One of the most famous examples of poisoning as a commercial transaction was the case of Dr. William Palmer, also known as the *prince of poisoners*. He was an English doctor convicted for the 1855 murder of his friend John Cook and executed by hanging the following year. He had poisoned Cook with strychnine. Before that, Palmer made large sums of money from the deaths of his wife and brother from collecting their life insurance. But the public's fascination with the case was of his suspected poisoning several other people including his brother, his mother-in-law, and four of his children who died of *convulsions* before their first birthdays.

This public outcry in the early 1800s stimulated the development of forensic toxicology in Great Britain. The result was the evolution of toxicology and medical and legal practices. Some of these changes are apparent today. For example, the registration of deaths by a physician, the banning of the sale of poisons by chemists (pharmacists), and rules of evidence in courts. A realistic appraisal of the situation, however, is that the hype was greater than the poisonings and there really was no poisoning epidemic in Britain at that time.

Alfred Swaine Taylor (1806–1880), British physician and the founder of British forensic medicine, is also the founder of modern medical jurisprudence, a natural continuation to the development of forensic toxicology. He received a diploma from the Apothecaries Society in 1828, his certificate to practice from the Royal College of Surgery in 1830, and presented the first course in medical jurisprudence in England in 1831 at Guy's Hospital. He was probably the most famous expert witness of his time and published his *Manual of Medical Jurisprudence* in 1842. It became very popular, and the 10th edition was published in 1879. His books on medical jurisprudence and on poisons became the standard works throughout the world. He codified the legal precedents, judicial rulings, and anatomical and chemical data that bore on the subject.

Sir Robert Christison (1797–1882), a noted Scottish physician with a medical degree from Edinburgh, studied toxicology under Orfila. He was appointed to a Chair of Medical Jurisprudence at the University of Edinburgh in 1822. In 1832, he transferred to a Chair of Materia Medica at the same

institution, which he held for the next 45 years. He published *A Treatise on Poisons* in 1829. He became a recognized authority on poisons, and in the course of his inquiries, he did not hesitate to experiment on himself. He took large doses of calabar bean (the seed of the woody vine *Physostigma venenosum* of western African and source of physostigmine; African natives used this as an ordeal: if the accused person eats the bean and vomits within half an hour, he is judged innocent, but if he succumbs, he is found guilty). Christison's attainments in medical jurisprudence and toxicology procured him the appointment of medical officer to the crown in Scotland, and from that time until 1866, he was called as a witness in many celebrated criminal cases. His works helped develop the basis for expert witnessing. He strove to provide a sound scientific basis for toxicology.

Christison was approached by the Scottish firm of W. and G. Young that wanted a harpoon that would kill a whale quickly to prevent it from diving under the ice. In 1831, the Youngs, harkening back to times before history, asked him to invent a harpoon that would utilize poison as the killing agent. Christison accepted the challenge and chose pure prussic acid (hydrogen cyanide) because of its extreme potency. Christison's harpoon contained two glass cylinders of the liquid, together containing almost 60 g. In 1833, the *Clarendon* was sent out with prussic acid harpoons. According to Christison, the harpoon gun was fired for the first and only time. The harpoon was buried deeply in the whale, which immediately *sounded*, or dived perpendicularly downwards. But in a very short time, the rope relaxed, and the whale rose to the surface quite dead. Apparently, the men were so appalled by the horrific effect of the harpoon that they declined to use any more of them. Christison was required by the Youngs to keep his invention secret, and he remained silent until 1860 (although the crewmen must have discussed it because a U.S. patent was granted for a prussic acid harpoon very similar to the Christison design in 1835). After the Youngs had died and their heirs were no longer involved in whaling, they released Christison from his promise of silence. In 1860, Christison explained his harpoon design in an article, *On the Capture of Whales by Means of Poison*, in the *New Edinburgh Philosophical Journal*.

Other forensic toxicologists of note include Henry Coley, a New York City resident who published *A Treatise on Medical Jurisprudence: Part I. Comprising the Consideration of Poisons and Asphyxia* in 1832. Included in this book were mineral acids, caustic alkalis, ammonia, nitrates, phosphorus, cyanide, metals, and alkaloids, as well as their chemistry, uses, signs and symptoms of poisoning, cause of death, postmortem findings, and treatments. Theodore George Wormley (1826–1897) published *Microchemistry of Poisons*, the first American toxicology textbook (1867), which became the standard on the subject worldwide. A.W. Blyth published *Poisons: Their Effects and Detection*, an excellent analytical toxicological text (1884). Rudolph A. Witthaus and Tracy C. Becker edited a four-volume text, *Medical Jurisprudence*, *Forensic Medicine and Toxicology*, which became the standard reference text in the field (1894–1896). Walter S. Haines and Frederick Peterson wrote *A Textbook of Legal Medicine and Toxicology* (1903). Alexander O. Gettler (1883–1968), who probably influenced the development of forensic toxicology in America more than anyone else, began working in the Office of the Chief Medical Examiner in New York City in 1918.

The advances in forensic toxicology paralleled advances made in analytical techniques. Toxicology was beginning to be recognized as a scientific discipline. However, true understanding of the basic mechanisms of action of chemicals and drugs lagged. Little had been done to answer the basic question, *How do poisons kill?* But that changed as mechanistic toxicology began with the classical studies of two of the most famous physiologists in medical history: François Magendie and his pupil Claude Bernard. Although important contributions had been made by others, they were not as systematic, fundamental, and far reaching as those of Magendie and Bernard.

François Magendie (1783–1855) contributed significantly to the advancement of physiology, medicine, and toxicology. His interest in the functioning of the nervous system led him to establish the mechanisms of action of emetine and strychnine, leading to the scientific introduction of these compounds into medical practice. He also experimented on the effects and uses of morphine, quinine, and other alkaloids, for which he is sometimes called the founder of experimental pharmacology. He was the first, or one of the first, to observe and describe anaphylactic shock.

Magendie's most famous pupil was Claude Bernard (1813–1878). Bernard was the son of a Burgundian vinegrower who studied pharmacy and enjoyed science, but wanted to be a playwright. His friends told him to study medicine and fortunately for humankind he accepted their advice. In 1834, Bernard enrolled in the Paris School of Medicine, and after a few years, he obtained a position at a lab at the Collège de France, where he worked under Magendie. He enthusiastically endorsed Magendie's philosophy that physiologists must discover the laws of *vital manifestations* or physiological functions and that observation and experimentation were the only methods of investigation. He received his degree in 1843. In 1854, he accepted the newly created chair of physiology at the Sorbonne. When Magendie died in 1855, Bernard took over his post at the Collège de France. He held the positions at the Sorbonne and the Collège de France concurrently until 1868.

In his work in experimental physiology, Bernard insisted that an experiment should be designed to either prove or disprove a guiding hypothesis. He also maintained that an experiment should produce the same results again and again, so long as the starting conditions are the same. These two points are integral parts of the modern scientific method.

His specific contributions to toxicology include furthering the concept of target organ toxicity, establishing approaches to defining the mechanism of action of drugs and other chemicals (i.e., curare, nicotine, carbon monoxide), demonstrating that the basic principles of pharmacology and toxicology are identical, and showing that drugs and other chemicals can modify the function and structure

of tissues. He believed that *the physiological analysis of organic systems can be done with the aid of toxic agents* (a new use for poisons!). His works were published in 18 volumes. One of his most famous, *An Introduction to the Study of Experimental Medicine,* was published in 1865 and translated into English in 1949. It is a clear and penetrating presentation of the basic principles of scientific research. Bernard introduces his idea of homeostasis in this book and he explains how and why it works and how humans, as well as animals, could not live without it. It is a classic in the field of experimental biology and a *must* reading for all students of biology and medicine.

It can be argued that toxicology as we know it today began with Bernard. Paracelsus took some important first steps, but much of his work is instructional only in hindsight. Orfila made the crucial introduction of analytical chemistry and jurisprudence. But Bernard completely changed the focus of toxicology from a science of poisoning to a biomedical science that could help to explain basic physiological processes. Substances that were used to induce harm were now being used to help mankind.

> One must break the bonds of philosophic and scientific systems as one would break the chains of scientific slavery. Systems tend to enslave the human spirit.
>
> **Claude Bernard (Introduction to *l'Etude de la Medecine Experimentale*, 1865).**

Magendie's and Bernard's work stimulated others to establish the mechanisms of action of toxic agents experimentally and to publish textbooks. For example, the Florentine physician and scientist Ranieri Bellini Pisano (1817–1878) promoted research in toxicology and pharmacology. He authored the first experimental toxicology text, entitled *Lezioni Sperimentali di Tossicologia*. He was also the founder of the Istituto Tossicologico Fiorentino.

The discipline of toxicology was also advanced by the outstanding research efforts of such noted northern European pharmacologists as Rudolf Buchheim (1820–1879), the founder of modern pharmacology; Oswald Schmiedeberg (1838–1921), another of the founders of modern pharmacology and toxicology and a student of Buchheim; and Rudolf Kobert (1854–1918), a student of Schmiedeberg, who published a number of textbooks in the 1890s (e.g., *Practical Toxicology for Physicians and Students*). A contemporary, Louis Lewin (1850–1929), the father of psychopharmacology, published on the toxicology of alcohols, chloroform, opiates, and plant-derived hallucinogens and also wrote a toxicology text (1929).

The mechanistic studies of Bernard were furthered by the brilliant German chemist, microbiologist, and immunologist Paul Ehrlich (1854–1915), who significantly advanced toxicology and pharmacology. His keen interest in chemistry and biological structure and function led him to propose the concept of a receptor as the sensitive site for chemical–biological interaction—that *chemical substances in organisms had specific points of attachment*—and once these were known, specific remedies could be developed. He subsequently identified several receptors. His most famous remedy was the use of arsenic in the management of syphilis (Compound 606, arsphenamine). His successful bout with tuberculosis stimulated his interest in immunity (as did his association with R. Koch), and he subsequently formulated the concepts of active and passive immunity. In addition to his originating the concept of receptors, his contributions to toxicology and pharmacology include underscoring the importance of mechanistic studies and structure–activity relationships. He shared the Nobel Prize in Physiology or Medicine with E. Metchnikoff in 1908.

The epidemiological study of chemical carcinogenesis that began with Pott in 1775 continued in 1822 when Dr. John Ayrton Paris (1785–1856) surmised that arsenic fumes might be the cause of the frequent occurrence of scrotal cancer in copper and tin smelter workers in Cromwell, England. In 1875, Richard von Volkmann (1830–1889) observed occupational skin tumors among workers in the tar and paraffin industry at Halle, Germany. In 1876, Joseph Bell (1837–1911) of Edinburgh suggested that shale oil was responsible for certain skin cancers in Scotland. Bladder cancer among aniline dye workers was first described by Dr. Ludwig Rehn (1849–1930) of Germany in 1895; this was the first recognition that chemicals could cause cancer away from their first point of exposure.

The experimental study of chemical carcinogenesis began in 1915 when Katsusaburo Yamagiwa and Koichi Ichikawa at Tokyo University produced malignant epithelial tumors by application of coal tar to the ears of rabbits. This was the first demonstration of chemical carcinogenesis in an animal model and was an experimental proof of Pott's hypothesis 140 years before. In 1922, R.D. Passey produced malignant growths by painting the skin of mice with coal tar ether extracts. In 1925, James Murphy and Ernest Sturm produced a high incidence of lung tumors in mice when coal tar was applied to the skin without local irritation. In 1932, Cook et al. published findings that pure hydrocarbons cause cancer in mouse skin. In 1935, Sasaki and Yoshida showed that *o*-aminoazotoluene caused liver tumors in rats. In 1938, W.C.H. Hueper, F.H. Wiley, and H.D. Wolfe first reported successful induction of bladder cancer in dogs by repeated injections of 2-naphthylamine.

As a true understanding of toxicity began to develop, there was still the practical aspects of toxicology to be handled. The need for laws to protect the public from unscrupulous purveyors of foods and drugs became apparent in the United States in the late 1800s. Harvey Washington Wiley (1844–1930), physician and chemist, served as Chief of the Bureau of Chemistry of the United States Department of Agriculture from 1883 to 1912. His main goal was to provide effective food and drug legislation to protect an unsuspecting public. Wiley issued a number of bulletins summarizing his studies of the effects of food chemicals in human subjects, tested in his *Poison Squad* from 1902 through 1907. His efforts culminated in the Pure Food and Drug Act (1906). After serving at the Department of Agriculture,

Wiley was Director of Foods Sanitation and Health for *Good Housekeeping* magazine from 1912 to 1930. He wrote many books on food composition, food adulteration, and nutrition.

In the 1910s and 1920s, the United States Radium Corporation produced *glow in the dark* watch dials painted with radioluminescent paint consisting of zinc sulfide and radium 226. The paint was applied with a small brush. Many of the young women employed in this work *pointed* the brushes by licking them between applications and ingested a small quantity of radium each time. Most people thought radium was a miracle elixir that could cure cancer and many other medical problems. A few knew it was harmful including the owners of the United States Radium Corporation and scientists who were familiar with the effects of radium because the scientific and medical literature contained ample information about its hazards. Radium accumulated in the bone marrow of the women, eventually producing bone cancer. This is one of the first instances of an occupational hazard from radioactivity. Alice Hamilton (see in the following) worked on the case to obtain just compensation for the workers. The Consumers League and the news media as represented by Walter Lippmann served in the process as well. Radium watches were manufactured into the 1950s, but with strict controls.

Despite the rise of toxicology as a science to help, there remained a certain element of mankind who wanted to use chemicals to harm. Advances in chemistry allowed people to use new agents for chemical warfare. These were generally gaseous compounds intended to indiscriminately debilitate or kill hundreds or thousands of people. It seems to be the worst scenario for toxicology: the knowledge gained by advances intended to help man were deviously twisted to harm. Early chemical warfare agents were crude destroyers of tissues, but later agents became quite elegant in their wickedness.

Chlorine was the first lethal chemical used in modern warfare. At 5 p.m. on April 22, 1915, German troops at Ypres discharged 180,000 kg of chlorine gas from 5,730 cylinders creating a gas cloud that blew with the wind. It either killed the French and Algerian troops in the opposing trenches or caused them to flee, opening a gap in the Allied line. On April 24, the Germans conducted a second chlorine gas attack at Ypres, this time against Canadian troops. On May 31, chlorine was employed on the eastern front, by the Germans approximately 50 km southeast of Warsaw. This attack employed 12,000 cylinders, releasing 264 tons of chlorine along a 12 km line. All totaled, there were nearly 200 chemical attacks during World War I using gas released from cylinders. The largest attack occurred in October 1915 when the Germans released 550 tons of chlorine from 25,000 cylinders at Rhiems. The effect of chlorine on the lungs has been described in many places. Chlorine is now considered obsolete as a chemical warfare agent.

Fritz Haber (1868–1934), perhaps Germany's greatest chemist, is known by most toxicologists for *Haber's law* used mainly in inhalation work (concentration × time = constant biological response). Yet he was a Nobel laureate for his discovery of a process for synthesizing ammonia from nitrogen and hydrogen. Inexpensive ammonia-based fertilizers led to an agricultural revolution through increased food production worldwide and saved millions from starvation. He developed a synthesis of nitric acid from ammonia that allowed the development of explosives and insecticides. He was also the chief of the German chemical warfare service during World War I and personally directed the first chlorine gas attack at Ypres, Belgium, in April 1915. In fact, Haber is often referred to as the father of chemical warfare. While not a toxicologist, he greatly influenced the course of the field through his work on insecticides and agents of chemical warfare. His legacy is as complex as toxicology itself.

As World War I continued without signs of end, many compounds were tested for utility as chemical warfare agents. In addition to chlorine, phosgene, diphosgene, chloropicrin, hydrogen cyanide, cyanogen chloride, and mustard were produced and used in large quantities. Mustard gas (bis(2-chloroethyl) sulfide) was first used in an artillery attack on July 12, 1917, by the Germans. This agent caused the most casualties of any agent used during World War I.

It is estimated that close to 1,300,000 casualties were produced by approximately 125,000 tons of chemical warfare agents used by the combatants, but the official figures likely underestimate the true number. Furthermore, it is unclear to what degree the official figures include individuals who were injured in gas attacks but who developed serious effects only after the war. However, considering there may have been 10,000,000 battle deaths from the war, it is arguable as to whether chemical warfare was more or less horrific than the other methods used.

In one sense, what started in World War I was simply a continuation of the use of smokes and irritants against one's enemies. In another, it changed the way chemicals and toxicology would be viewed. A line had been crossed and the use of toxicology to deliberately and indiscriminately inflict harm was part of governmental programs. The next 50 years produced some of the most lethal chemicals and combinations of chemicals imaginable.

A number of materials developed during World War I were not terribly lethal, though they were quite irritating. These are lacrimators that irritate the mucous membranes of the eyes and cause a stinging sensation and tears. They also irritate the upper respiratory tract, causing coughing, choking, and general debility. The effects are short lived and rarely disabling, and lacrimators are used today by law enforcement. Tear gas has gained widespread acceptance as a means of controlling civilian crowds and subduing barricaded criminals. The most widely used forms of tear gas have been *o*-chlorobenzylidene malononitrile and 2-chloroacetophenone. Proponents of their use claim that when used correctly, the effects of exposure are transient and of no long-term consequence. But exposure is difficult to control and indiscriminate and may not always be used correctly. Lethal injury has been documented. In 1969, 80 countries voted to include tear gas agents among chemical weapons banned under the Geneva Protocol.

The Nazis continued the work on chemical warfare that was started in World War I. They produced 12,000 tons of tabun (ethyl *N*,*N*-dimethylphosphoramidocyanidate; the first of the so-called *G-series* nerve agents, also known as GA) between 1942 and 1945. Thankfully, it was never used. The ultimate chemical genocide occurred during World War II when the Nazis used cyanide (in the form of Zyklon B—liquid hydrocyanic acid adsorbed into a highly porous material), carbon monoxide, and even engine exhaust to kill millions of people deemed to be *undesirable*.

Mechanistic studies led to a better understanding of the toxic action of many chemicals and to the development of specific antidotes. A classic example is the development of British anti-Lewisite (BAL) (dimercaprol) as an antidote for Lewisite ($CHCl{=}CHAsCl_2$), an arsenic-based gas that was synthesized too late to be used in World War I. Still fearful of its use in World War II, Rudolph Peters (1889–1982) headed the Oxford University laboratory that searched for antidotes to chemical warfare agents and developed BAL in 1940 based on the work of Carl Voegtlin (1879–1969), a developer of the *arsenic receptor* in chemotherapy and the first director of the National Cancer Institute (1938–1943).

Understanding the mechanism of poisoning by organophosphorus compounds, the basis for many insecticides and chemical warfare agents, also led to a rational antidote. Atropine, a drug that blocks muscarinic acetylcholine receptors, counteracts the vomiting and diarrhea, excessive salivation, bronchial secretions, sweating, and bronchospasm. It is administered intravenously, if possible, in high doses at frequent intervals until signs of intoxication diminish. Pralidoxime chloride (2-PAM), codiscovered in 1955 by Davies and Green and Wilson and Ginsburg, reactivates nerve agent–inhibited cholinesterase. Diazepam or another anticonvulsant may be administered in severe cases to control seizures and thereby prevent seizure-induced brain damage.

A biochemical mechanism for cyanide antagonism was described by Chen et al. (1933, 1934). They suggested using a combination of amyl nitrite, sodium nitrite, and sodium thiosulfate. Nitrite converts hemoglobin to methemoglobin, which in turn competes effectively for cyanide with the mitochondrial cytochrome oxidase complex. Cyanide is then removed from cyanomethemoglobin by intravenous sodium thiosulfate, which serves as a sulfur donor for rhodanese (thiosulfate sulfur transferase). Rhodanese accelerates cyanide detoxification by forming the nontoxic metabolite thiocyanate. This therapy represented the development of one of the first antidotes based on knowledge of toxicological mechanisms. This combination of antidotes has stood the test of time and still represents one of the most efficacious antidotal combinations for the treatment of cyanide intoxication.

The LD_{50} test was first introduced in 1927, when it was used for the standardization of not only important but also highly toxic and possibly fatal drugs such as insulin, digitalis extracts, and diphtheria toxins (Zbinden and Flury-Roversi, 1981). Because the potency of these drugs might vary among different sources or batches, it was essential to have precise measurements. Accurate chemical methods were not yet available, so lethality was used as an indicator of potency. Over time, however, it came to be forgotten that the method was designed for biological standardization of highly active pharmacological agents for which no chemical analytical method was available. Eventually, determination of the LD_{50} was used to assess the acute toxicity of chemicals other than drugs, and many regulatory authorities required an LD_{50} test for all chemicals: food additives, cosmetics, pesticides, and industrial chemicals. In some countries, new drugs required acute LD_{50} data in rats, mice, dogs, and occasionally also in monkeys by the oral, subcutaneous, and intravenous routes. Recently, this was seen as an inappropriate use of animals, and acute toxicity is determined much differently today due to this awareness.

Josef Warkany (1902–1992) of Cincinnati Children's Hospital Research Foundation was the first to demonstrate that exposures to environmental chemicals and dietary deficiencies and excesses can be responsible for the production of congenital malformations. Until that time, it was widely believed that birth defects were due to chance or *God's will*. Warkany was born and educated in Vienna, Austria. In 1932, he accepted a 1-year fellowship at Cincinnati's Children's Hospital Research Foundation and ended up staying for 60 years. In the late 1930s, Warkany and Rose Cohen Nelson attempted to produce endemic cretinism in rats. Though they failed, they obtained a syndrome of congenital skeletal malformations that was even more interesting. More than 3 years of painstaking research was needed to show that the skeletal malformations were not caused by a dietary iodine deficiency in the mother before birth, as in endemic cretinism, but instead were due to a riboflavin deficiency in the diet fed the pregnant animals. At that time, medical scientists believed that malformations were always genetic in origin, and most were reluctant to believe that the environment could have such a dramatic effect on fetal development. For his work in this area, Dr. Warkany is known as the *father of teratology*. His 1300-page textbook *Congenital Malformations* is a medical classic.

Although there are more notorious poisons, alcohol may be responsible for more deaths than any other. Professor Rolla Harger (1890–1983) at Indiana University developed the *drunkometer* in 1937 for testing drivers presumed to be under the influence. This was the first practical roadside breath-testing device intended for use by the police, which was important as prohibition had ended in the United States in 1933 and at the same time cars became more available and attained higher speeds. The *drunkometer* collected a motorist's breath sample into a balloon and then the sample was pumped through an acidified potassium permanganate solution. If there was alcohol in the sample, the solution changed color. The greater the color change, the more alcohol there was present in the breath. The *Breathalyzer*® was invented by Robert Borkenstein in 1954, which encouraged the development of police alcohol testing programs worldwide.

Occupational medicine and industrial toxicology were identified by Agricola and Paracelsus, systematized and advanced by the pioneering efforts of Ramazzini, and

further advanced by one of America's foremost physicians, Alice Hamilton (1869–1970). Hamilton researched occupational diseases, publicized the hazards of industrial chemicals to workers, and wrote several books on industrial toxicology. She was the foremost female occupational physician and industrial hygienist, the first woman faculty member of the Harvard Medical School, and the only woman to serve on the Health Committee of the League of Nations. She graphically described the history of industrial toxicology/occupational medicine in the United States in her autobiography, *Exploring the Dangerous Trades* (1943). Others who contributed significantly to industrial toxicology include Cecil Drinker (1887–1919) also of Harvard who believed that toxicological information was accumulating very rapidly, that mechanisms of toxicity were being elaborated, and exposure to chemicals was increasing due to advances in manufacturing. Ethel Browning (1891–1979) of Great Britain received her doctorate in medicine in 1927 and wrote the *Toxicity of Industrial Organic Solvents* in 1937. Interestingly, this was the first book on this subject and was written when Browning had no personal occupational medical experience. Her other publications included *Ionizing Radiations* (1959), *Toxicity of Industrial Metals* (1961), and, her greatest work, *Toxicity and Metabolism of Industrial Solvents* (1965).

The increased exposure of consumers to unknown chemicals started to raise concern. In 1933, more than a dozen women were blinded, and one woman died from using a permanent mascara called Lash Lure. It contained *p*-phenylenediamine, an untested chemical. *p*-Phenylenediamine caused blisters, abscesses, and ulcers on the face, eyelids, and eyes of Lash Lure users. It led to blindness for some and in one case, the ulcers were so severe that a woman developed a bacterial infection and died. Although the factual basis of the story was disputed by the industry and has never been confirmed, the so-called incident provided a *smoking gun* to those who saw a need for protective action.

Another crucial incident at that time (1937) was when a Tennessee drug company, S.E. Massengill Co., manufactured sulfanilamide dissolved in diethylene glycol to create elixir sulfanilamide. The food and drug laws in the United States did not require toxicological testing before sale. When 105 people died in 15 states, the trail led back to the elixir.

These events caused the U.S. Congress to pass legislation for a new Food, Drug, and Cosmetic Act in 1938. Lash Lure was the first product seized by the U.S. Food and Drug Administration (FDA) under its new authority. The Lash Lure tragedy showed that a better way to test for eye and skin irritation was needed.

In 1944, John M. Draize, an FDA scientist, standardized the scoring system of a preexisting test for ocular irritation. Frequently referred to as the *Draize test*, a liquid or solid substance is placed in one of a rabbits' eyes, and changes in the cornea, conjunctiva, and iris are observed and scored compared to the untreated eye. Despite differences between the rabbit eye and the human eye, the Draize test, when performed by trained personnel, has proven quite accurate in predicting human eye irritants, particularly slightly to moderately irritating substances, which are difficult to identify using other methods. The Draize test performs its primary function of assessing both the damage and potential for recovery after exposure to irritants very well. Draize et al. (1944) also standardized the scoring of skin reactions as a method to evaluate skin irritation or corrosion using rabbits.

By this time, the discipline of toxicology was now recognized by the scientific community and society as a distinct entity, separate from pharmacology and drawing upon the chemical, biological, and physical sciences. The stage was set for the application of toxicological principles and findings to protect the public—from consumers to workers—from the adverse effects of chemical exposure.

POST–WORLD WAR II

As society developed following World War II, it recognized the value of toxicology and its demands on the science grew. Much of this demand was a result of the enormous growth of the chemical industry. New synthetic chemicals were being produced, and new uses were discovered for older chemicals. The amounts of chemicals produced were greater than ever. The production, use, and disposal of chemicals were not always conducted in the best interest of man or the environment. Advances in analytical chemistry and the biomedical sciences also dramatically impacted toxicology. This led to new laws and regulations and a new type of activism. Given the importance of the issues, academic, industrial, and governmental research laboratories and private research foundations advanced the frontiers of toxicology by seeking the molecular basis for toxic action. Some of the issues raised outstripped the capacity to generate data and form coherent theories. Some of the questions being asked of toxicology helped move the field forward, while others were beyond the scope of any science.

Basic Science

Almost all the work in the field of xenobiotic biotransformation grew from the research of a Welshman, Richard Tecwyn Williams (1909–1979). His early work on the ring structure of glucuronic acid by isolating bornyl glucuronide from the urine of dogs fed borneol allowed him to crystallize the conjugate and use it as a source material for his elucidation of its pyranoid structure. This work in 1931 stimulated his interest in the biotransformation of foreign compounds and led to a series of papers on the fate of phenols, terpenes, and sulfonamides. Williams became convinced that the biotransformation of compounds was just as important as the metabolism of natural body constituents. During the late 1930s, Williams began writing a book on the detoxification of foreign compounds but, because of the war, it was not published until 1947. It was a slim volume summarizing much of the work that had been done to date. In the 1950s, studies on a broad range of compounds added considerably to the systemization of the biotransformation routes of xenobiotics, culminating in publication of an expanded

Detoxification Mechanisms in 1959 that provided a systematic approach to biotransformation based on organic chemistry classification. Williams also expanded his concepts of the principal biochemical reactions whereby drugs and other foreign compounds are biotransformed in two distinct phases: one of oxidation, reduction, and hydrolysis and the other of conjugation reactions.

With the aid of ^{18}O and mass spectroscopy, Howard S. Mason et al. in 1955 found proof that enzymes can use molecular oxygen to oxygenate their substrates. At the same time, Osamu Hayaishi at the National Institutes of Health made a similar discovery of oxidative transformations. This class of oxygenases had requirements for both an oxidant (molecular oxygen) and a reductant (reduced NADP) and hence was given the trivial name *mixed-function oxidases*. In 1958, Klingenberg and Garfinkel independently announced the discovery of a carbon monoxide–binding pigment (hemoprotein) with an absorbance maximum at 450 nm in the microsomal fraction of rodent liver. This pigment was characterized as a cytochrome with typical absorption bands by Omura and Sato in 1964 through the use of detergent solubilization of microsomes and interaction with isocyanide ligands. Rosenthal, Cooper, and Estabrook in 1965 studied the biotransformation of codeine, monomethyl-4-aminopyrine, and acetanilide and found them to be inhibited by carbon monoxide, and the inhibition could be reversed by yielding the same action spectrum, demonstrating that cytochrome P450 is the oxygen-activating enzyme in xenobiotic biotransformation as well as in steroid hydroxylation. From the isolation of membrane-bound P450 by Lu and Coon in 1969 to the first crystallization of a mammalian P450 in 1999 by Eric Johnson and coworkers, this area of research has established the important role of P450s in the disposition of drugs and other xenobiotics.

A key figure in the xenobiotic biotransformation was James R. Gillette (1928–2001), who worked with Bert LaDu, Jr. at the Laboratory of Chemical Pharmacology/Heart and Lung Institute at the NIH where B.B. Brodie was chief. Gillette's studies on cytochrome P450 were influential and he succeeded Brodie as chief of the Laboratory in 1972.

James A. and Elizabeth C. Miller (1915–2000; 1920–1987) made seminal discoveries related to the biotransformation of synthetic and naturally occurring chemicals to toxic and/or carcinogenic electrophilic metabolites and to the regulation of xenobiotic metabolism. Their work began in the late 1940s when they demonstrated that a foreign chemical could be biotransformed to intermediates that covalently bind to macromolecules. The administration of hepatocarcinogenic aminoazo dyes to rats resulted in the covalent binding of metabolites to protein in the liver. Little or no covalent binding occurred in nontarget tissues that did not exhibit tumorigenesis. The Millers found that factors influencing the in vivo binding of aminoazo dyes to protein also influenced hepatocarcinogenicity, which led them to suggest that covalent binding of metabolites to liver macromolecules was required for carcinogenicity. This line of thinking was extended to carcinogenic polycyclic aromatic hydrocarbons when they found that metabolites covalently bound to protein only in the skin.

James Miller demonstrated the oxidation of a foreign compound in a cell-free system by enzymes that were later identified as cytochrome P450. He demonstrated that liver microsomes reduced the azo linkage of 4-dimethylaminoazobenzene and that NADPH was required for catalytic activity. He also reported that flavin adenine dinucleotide was required for azo dye reductase activity, and these results provided a mechanistic explanation for the protective effect of riboflavin on the carcinogenicity of aminoazo dyes. These observations suggested that a dietary vitamin can inhibit the carcinogenicity of a chemical by influencing its biotransformation, an early example of cancer chemoprevention. In the 1950s, Miller continued to make fundamental discoveries on the properties of enzyme systems that biotransform foreign chemicals. He discovered that the *N*-demethylation of an aminoazo dye by liver homogenate was an oxidative process. This study provided the basis for subsequent investigations that demonstrated the NADPH-dependent oxidative metabolism of drugs and carcinogens by liver microsomes. In 1957, the Millers made the important observation that the demethylase system was inhibited by carbon monoxide. Although they did not pursue this line of research, these early observations and the other studies described earlier helped pave the way for the discovery of cytochrome P450.

The Millers discovered that foreign chemicals can induce the synthesis of liver enzymes that biotransform the compound administered and other foreign chemicals. Studies on microsomal enzyme induction provided a mechanistic understanding of the inhibitory effects of certain polycyclic aromatic hydrocarbons on aminoazo dye carcinogenesis. This work was followed by that of Allan Conney in 1956, who showed that the increase in metabolism could be antagonized by inhibitors of protein synthesis. The induction of these enzymes is important toxicologically because it leads to an accelerated biotransformation of drugs and environmental chemicals in vivo and so alters their action and toxicity.

In the 1960s, the Millers elucidated the molecular events leading to the activation of 2-acetylaminofluorene, aminoazo dyes, aflatoxin B_1, safrole, estragole, and ethyl carbamate to chemically reactive products that react with macromolecules in cells. The later studies were the start of research on the biotransformation of naturally occurring carcinogens in our diet. Studies on the activation of several structurally diverse carcinogens led to the important unifying concept by the Millers who proposed in 1969 that most carcinogenic and mutagenic chemicals are not carcinogenic or mutagenic per se, but that these compounds must undergo biotransformation to reactive electrophilic intermediates that exert their toxic effects by covalently binding to critical sites on DNA, RNA, and protein. This discovery laid the foundation for subsequent research indicating a relationship between the mutagenicity of chemicals after activation and initiated a new era of carcinogenesis research that led to the

development of rapid mutagenicity tests for the screening of potential human carcinogens.

Other early studies in the field of carcinogenesis took a different approach. In the 1940s, J.C. Mottram (1880–1945), Isaac Berenblum (1903–2000), and Philippe Shubik (1921–2004) studied the development of tumors in mouse skin that gave rise to the two-stage, initiator–promoter model. Cancer models involving two or more stages helped researchers understand carcinogenesis at the level of the whole organ and served as a basis for the classical model of carcinogenesis, which now includes initiation, promotion, and progression. The initiation phase has traditionally been described as involving the induction of mutations and escape from DNA repair. Cell proliferation in the promotion phase plus additional genetic events and angiogenesis in the progression phase are needed for the process of carcinogenesis to result in cancer. Roswell Boutwell, Stuart Yuspa, Henry Hennings, Thomas Slaga, and others have contributed to this field.

Peter Armitage and Richard Doll (1912–2005) used the two-stage model for investigating the age distribution of human cancer incidences in 1954 and 1956 using a simple power function of age. Later, Armitage and Doll and Suresh H. Moolgavkar found better equations to fit cancer incidence data. Richard Peto and Doll used epidemiologic data to estimate the fundamental causes of human cancer.

Ernst Wynder (1922–1999), born in Germany and raised in New Jersey when his family fled to escape Nazi persecution, attended medical school at Washington University, St. Louis. During a summer internship at New York University, his curiosity was piqued during the autopsy of a two-pack-a-day smoker who had died from lung cancer. Wynder began collecting case histories of lung cancer victims, first in New York City and then in St. Louis. His research brought him to thoracic surgeon Evarts Graham, who, despite initial skepticism about Wynder's premise, granted access to his extensive case records and agreed to sponsor the medical student. In 1950, the *Journal of the American Medical Association* published Wynder and Graham's *Tobacco Smoking as a Possible Etiologic Factor in Bronchiogenic Carcinoma: A Study of 684 Proven Cases*. Wynder and Graham's retrospective study was not the first to link smoking and cancer (in 1950, Doll also demonstrated that smoking causes cancer), but its sophisticated design, impressive population size, and unambiguous findings demanded action. During the next decade, hundreds of reports were published linking cancer and smoking, including large prospective studies and animal investigations. The preponderance of evidence led to the publication of the Surgeon General's Report on Smoking and Health in January 1964, the first official recognition in the United States that cigarette smoking causes cancer and other serious diseases. This seminal report prompted a series of public health actions and changed societal attitudes toward the health hazards of smoking.

Another husband–wife team in the area of carcinogenesis was John and Elizabeth Weisburger, who both started at the National Cancer Institute in 1949. John studied the mode of action of chemical carcinogens in general and arylamines in particular. Some of these chemicals were important occupational carcinogens, and more recently, it has been discovered that they occur in cooked meats, as the cooking process generates heterocyclic amines. These products undergo a two-step biochemical activation to DNA-reactive chemicals, *N*-oxidation usually in the liver, followed by *N*,*O*-acylation in target organs such as the liver, the intestinal tract, the mammary gland, and the pancreas. Between 1961 and 1972, as director of the Bioassay Segment of the Carcinogenesis Contract Program Management Group of the National Cancer Institute, John Weisburger was also involved with testing methods for environmental and industrial compounds including the role of dose levels and the species and strains of animals to be used in these tests. He introduced the F344 rat and the B6C3F1 mouse as the standard animal models. With the discovery that many carcinogens are mutagens, he organized national programs to develop rapid in vitro bioassay systems to test for carcinogenicity. When he left NCI to become director of research at the American Health Foundation in 1972, the NCI Bioassay Program was transferred to the NIEHS in North Carolina, where it continues today as the National Toxicology Program.

Elizabeth Weisburger identified biochemical pathways of malignant growth and mechanisms of carcinogenesis and synthesized reference metabolites and analogs of a research carcinogen. In 1951, she was appointed to the NCI Laboratory of Biochemistry. Ten years later, along with John, she formed a research group to test for carcinogenic activity in environmental and industrial compounds. Elizabeth stayed at NCI and became head of the Laboratory of Carcinogen Metabolism. She was appointed assistant director for chemical carcinogenesis in the NCI Division of Cancer Etiology in 1981, where she remained until her retirement in 1988.

During the late 1960s and early 1970s, many questions were being raised about rodent carcinogenicity testing. A breakthrough in thinking occurred in 1975 when Dr. Bruce Ames and his colleagues at the University of California in Berkeley developed an easy, exquisitely sensitive biological method for measuring the mutagenic potency of chemical substances. This bacterial mutagenesis system is commonly known as the *Ames test*. The great interest in the Ames test was based on the proposition that any substance that is mutagenic to bacteria may be carcinogenic because the DNA bases are the same in all cells. The test is based on inducing growth in genetically altered strains of the bacterium *Salmonella typhimurium* that are unable to synthesize the amino acid histidine from the ingredients in its culture medium. When a test material is applied to the bacteria in the presence and absence of mammalian microsomal enzyme systems, some undergo a back mutation (*revert*) such that the bacteria can grow like the original *wild* (unaltered) strains without histidine and can be seen as visible colonies. By simply counting the colonies after a standard time under standard growing conditions, the mutagenic

potential of the parent compound and its metabolites can be estimated.

The test initially gave the impression that it correlated very highly with rodent carcinogenicity. This correlation diminished as more data became available. For example, many substances that caused cancer in laboratory animals did not elicit a positive response in the Ames test and vice versa. The Ames test alone does not demonstrate cancer risk, but the mutagenic potency does correlate with the carcinogenic potency for certain types of chemicals. Further, the ease and low cost of the test make it valuable for screening substances in the environment and new substances in the laboratory. It also sets a new paradigm by distinguishing between genotoxic carcinogens and nongenotoxic carcinogens.

James G. Wilson (1915–1987) introduced a slicing technique (freehand slicing) that standardized the examination of fetuses from teratology studies, especially the soft tissues. Fetuses used for the Wilson's soft tissue sectioning technique are first fixed in Bouin's solution, which is a mixture of saturated picric acid, formaldehyde, and glacial acetic acid. This fixes the tissues, hardens the soft tissues, and softens the bones in order to preserve the specimens and make it possible to slice them cleanly into thin sections with a razor blade. All the internal organs can be examined and any abnormalities or developmental variations documented. The sections can be saved for further examination, if desired. This technique has its limitations (e.g., the original coloration of the tissues is lost), but its simplicity made it very popular and it has been accepted internationally. In 1973, Wilson outlined the four major manifestations of abnormal development: growth alterations, functional deficits, structural malformations, and death.

The growth in toxicology continued to attract more students and practitioners, and this resulted in more research. As with other recognized, independent scientific disciplines, toxicologists realized a need for a learned society to provide a forum for the exchange of the burst of new scientific information. The Society of Toxicology was founded in 1961. This was the first international society for and by toxicologists. Since its founding and as a result of the tremendous growth of toxicology, almost every developed country has its own version—a testimony to toxicology's importance and growth. The need for appropriate journals in which to publish, and thus disseminate, the results of investigations was acute and noted by the Society. The journal, *Toxicology and Applied Pharmacology*, was founded by Fred Coulston; this was followed by *Fundamental and Applied Toxicology*, which is now *Toxicological Sciences*. These have been the official journals of the Society. The Society has published a wonderful, informative book covering its first 50 years (SOT, 2011). The excellent biographies in that book are easily available and have permitted me to remove some of the people mentioned in earlier editions of this chapter.

Other important journals included *Food and Chemical Toxicology* (originally *Food and Cosmetic Toxicology*, founded by Leon Golberg) and *Regulatory Toxicology and Pharmacology*. Board certification began in the early 1980s to assure a minimal level of competency among people calling themselves toxicologists.

Regulatory

Arnold J. Lehman (1900–1979) earned his doctorate from the University of Washington in Seattle in 1930 and his medical degree from Stanford University in 1936. He taught at a number of universities before joining the U.S. FDA as director of the Division of Pharmacology in 1946. He and his staff published *Procedures for the Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics* in 1955, the first attempt by the agency to provide guidelines for toxicological studies. He and his colleagues, most notably O. Garth Fitzhugh (1901–1994), laid the foundations of an acceptable daily intake (ADI) of a material in 1952. They developed the concept of safety factors (a number applied to the highest dose that did not elicit an adverse effect in a properly designed and performed toxicological study, the no-observed-adverse-effect level [NOAEL]) for use in extrapolating animal data to man.

Government toxicologists have made significant contributions especially in the area of safety evaluation, including the quantification of risk. Regulatory agencies demand adequate data of high quality to serve as the basis for establishing safe exposure levels. To assist toxicologists concerned with the safety of food and color additives, the U.S. FDA issued a series of guidelines in 1982, often referred to as *Redbook*, to standardize the necessary toxicological studies to establish the safety of materials added to foods. These guidelines are designed to encourage sound science and the conservation of resources while providing adequate data for determining safe exposure limits for consumers. The extent of testing was and is often determined by the nature of the chemical, its chemical and physical properties, and the extent of exposure. The Organization for Economic Cooperation and Development (OECD) issued guidelines generally consistent with those in the *Redbook* that are now accepted internationally.

Following the disclosure that some contract laboratories had falsified data used to support the safety of regulated materials, FDA and EPA instituted good laboratory practices (GLPs) in 1978 to assure the proper conduct of all toxicology studies to be submitted for regulatory review. The *Redbooks* and GLPs moved sound science into regulatory toxicological research.

At the international level, the World Health Organization (WHO), through the efforts of Frank Lu, Gaston Vettorazzi, and John Herrman, applied sound toxicological thinking to establishing safe exposure conditions for food chemicals and pesticides. They more thoroughly developed the concept of an ADI based on sound toxicological data and the proper use of appropriate safety factors. This concept, recognized and effectively used worldwide, has resulted in few significant problems with food chemicals including pesticides evaluated in this manner. The evaluations are conducted through the

auspices of the International Programme on Chemical Safety and implemented by the Joint FAO/WHO Expert Committee on Food Additives (first meeting in 1956) and the Joint Meeting on Pesticide Residues (first meeting in 1961).

Problems and Responses

Despite the successes of toxicology in understanding the mechanisms of toxicity and being incorporated into regulations, a number of incidents occurred that influenced society and caused toxicology to take on new responsibilities.

After issues arose about the improper use of food colors and other food additives in the 1950s, the U.S. Congress passed significant revisions to the Food, Drug, and Cosmetic Act requiring the approval by the FDA of all new food and color additives based on safety before they could be added to foods. A noteworthy part of the law was the *Delaney clause* that stipulated that no additive could be deemed safe (i.e., given an FDA approval) if it were found to induce cancer in man or experimental animals. Scientists agreed that the FDA had sufficient authority in the general safety clause to ban carcinogens but proponents justified the clause on the basis that some cancer experts were not able to determine a safe level for any carcinogen.

While well intentioned, the Delaney clause has created problems for the food and chemical industries and for regulatory officials since enacted. The trouble began on November 6, 1959, when FDA announced that residues of the pesticide amitrole, a rodent carcinogen, had been found in cranberries and recommended that the public stop buying cranberries. Tainted supplies were withdrawn and new inspection procedures put in place. The scare passed quickly but a year's sales were wiped out since people eat little cranberry sauce except at Thanksgiving and Christmas. Notwithstanding publicity critical of the FDA, this action had beneficial results, particularly in convincing farmers that pesticides must be used with care. The use of amitrole was phased out during the 1960s. Nonetheless, the cranberry scare of Thanksgiving 1959 left an indelible residue of suspicion and worry about chemicals in the public's mind.

A month after the cranberry scare, federal officials learned that diethylstilbestrol (DES) a nonsteroidal synthetic estrogen now known to have delayed carcinogenic effects in humans had been shown to cause cancer in laboratory animals. At that time, DES was widely used as an additive in chicken feed, and DES residues were measurable in chickens sold in grocery stores. Officials banned DES from chickens and the DES story faded from the newspapers. DES was still permitted as a feed additive for beef and sheep because residues had not been measured in those animals due to analytical difficulties. Some criticized the FDA claiming that it had stopped sampling to avoid getting squeezed between the meat industry and the Delaney clause. The politics of toxicology were becoming apparent.

The environment presented other problems. One of the worst post–World War II examples of pollution was in Minamata, Japan. This small factory town was dominated by the Nippon Chisso Corporation. From 1932 to 1968, Chisso dumped an estimated 27 tons of mercury compounds into Minamata Bay. The town consists of mostly farmers and fisherman, so as Chisso Corporation dumped this mercury into the bay, thousands of people whose normal diet included fish from the bay unexpectedly were ingesting mercury. In May 1956, four patients suffering from an unknown disease were brought to the city hospital. They all had in common severe convulsions, intermittent loss of consciousness, repeated lapses into crazed mental states, and then finally permanent coma. Death was usually preceded by a very high fever. It was discovered that the same type of patients had been seen in the fishing villages surrounding Minamata City and that 17 people died after showing the same signs and symptoms. The illness became known as the *Minamata disease*, and eventually, it was determined to be caused by methyl mercury. The same syndrome was discovered again at Niigata City, Japan, in 1965. The probable cause of the disease in Niigata was methyl mercury from effluent from the Showa Denko Company's Kase factory, located on the upper reaches of the Agano River. The second Minamata disease was recognized at an earlier stage so fewer cases were reported. Both incidents were attributed to the production of acetaldehyde using mercury as catalyst. Methyl mercury had been produced by plankton and accumulated in fish and shellfish. Those who ate the contaminated seafood developed methyl mercury poisoning. The long-term effects were sensory disturbances and constriction of the visual field, incoordination and walking difficulties, dysarthria, hearing problems, and tremors. These cases of organic mercury poisoning were the first known to occur through the food chain transfer of an environmental pollutant.

In the pharmaceutical area, in the early 1960s, thalidomide was an approved drug used by some pregnant women in Europe and Canada as a sedative/hypnotic to treat morning sickness. Thalidomide was not approved in the United States, despite intense pressure from the manufacturer, because Dr. Frances Kelsey of the FDA insisted there was insufficient proof of the drug's safety in humans. Women who took the drug in early pregnancy delivered children with a widely varying but recognizable pattern of limb deformities. The most well known was absence of most of the arm with the hands extending from the shoulders, phocomelia. Another frequent arm malformation called radial aplasia was absence of the thumb and the adjoining bone in the lower arm. Similar limb malformations occurred in the lower extremities. The affected babies almost always had both sides affected and often had both the arms and the legs malformed. In addition, the drug caused malformations of the eyes and ears, heart, genitals, kidneys, digestive tract (including the lips and mouth), and nervous system. The first published suggestion of teratogenicity in man was W.G. McBride's letter in *The Lancet* in 1961. Thalidomide was soon recognized as a powerful human teratogen and banned worldwide. Taking even a single dose of thalidomide during early pregnancy could cause major birth defects. It is estimated that more than 10,000 children around the world were born with major malformations.

As a result of the thalidomide tragedy, teratology studies became a requirement for new drugs and recommended for certain other chemicals.

Rachel Carson's (1907–1964) *Silent Spring*, published in 1962, provided the first look at the widespread ecological degradation. It touched off an environmental awareness that still exists. The book focused on chemicals used in agriculture that sometimes led to high levels in the environment. Carson argued that those chemicals were more dangerous than radiation and that for the first time in history, humans were exposed to chemicals that remained in their bodies from birth to death. Well written and presented with thorough documentation, the book alerted a large audience to the environmental and human dangers of the indiscriminate use of chemicals. *Silent Spring* became a bestseller with international impact, spurred revolutionary changes in the laws affecting our air, land, and water and remains a landmark work.

Following the publication of *Silent Spring*, rising concern about the environment swept across universities. The intensity of the discontent compared with that over the U.S. war in Vietnam. A national day of observance of environmental problems was held in the spring of 1970, Earth Day. This was a nationwide grassroots demonstration on behalf of the environment. The American people finally had a forum to express their concern about what was happening to the environment, and they did so with exuberance. Earth Day is now an annual observance. As a result of Carson's book and the ensuing activism, and in recognition of the problems in the land, air, and water, President Richard Nixon established the United States Environmental Protection Agency, which was launched on December 2, 1970. It has developed new testing methods intended to protect man and the environment.

New occupational issues began to emerge during the 1960s. In the United States and elsewhere, asbestos, thought to be an inert material, was widely used for its heat-resistant characteristics in a wide range of building materials (roofing shingles, ceiling and floor tiles, paper products, and cement products), friction products (automobile clutch, brake, and transmission parts), heat-resistant fabrics, packaging, gaskets, and coatings. Its usage peaked during World War II and into the 1970s. During the late 1960s, evidence emerged indicating that some asbestos fibers were a health risk. Breathing high levels of asbestos fibers for a long time could result in asbestosis, a disease that can eventually lead to disability and death. Breathing asbestos also increased the risk of lung cancer, mesothelioma, and possibly cancers in other parts of the body. Starting in the 1980s, the concern about asbestos resulted in the spawning of a new industry, asbestos removal and abatement.

In the late 1960s, there were implications of a cover-up by the chemical industry about the adverse effects of vinyl chloride, a major commodity. Early research conducted by producers and users of vinyl chloride focused on its toxicological properties. Later studies investigated chronic toxicity and carcinogenicity in which carcinogenic responses were observed in rodent inhalation studies. This occurred at almost the same time that case reports were published on a finding of a rare cancer, hepatic angiosarcomas, in workers exposed to high levels of vinyl chloride. More stringent occupational exposure limits were instituted, and further research on vinyl chloride was initiated, including epidemiological studies of workers, animal carcinogenicity bioassays, and mechanistic investigations. The studies firmly established an association between prolonged exposure to high levels of vinyl chloride and angiosarcomas of the liver. More detailed investigation showed that workers who inhaled high levels of vinyl chloride for several years had altered liver function, nerve damage, poor blood flow in the hands, and unusual immune reactions. Animal studies showed that long-term exposure to vinyl chloride might adversely affect male reproductive organs. Vinyl chloride is now classified as a known human carcinogen.

The carcinogenicity findings revealed marked differences in potency between humans and rodents. Research on the metabolic kinetics and molecular dosimetry of vinyl chloride and its biotransformation products provided a basis for reconciling the species differences in potency and provided a mechanistic basis for the very specific carcinogenic response, hepatic angiosarcomas. The research conducted on vinyl chloride may be viewed as a success story for mechanistic-based findings and their importance in establishing appropriate health protective standards. Seminal work by Perry Gehring (1936–2003) and Phil Watanabe of Dow Chemical and others brought a new level of understanding and importance of the role of biotransformation and pharmacokinetics in toxicity. More stringent exposure standards have been effective in protecting workers. Moreover, the research approach used with vinyl chloride has served as a template for evaluating the toxicity and carcinogenicity of other chemicals.

As a result of workplace problems involving asbestos, vinyl chloride, and other chemicals, the U.S. Congress passed the Occupational Safety and Health Act of 1970, a new effort to protect workers from harm. The Act established for the first time a nationwide, federal program to protect almost the entire work force from job-related injury, illness, and death. The Occupational Safety and Health Administration (OSHA) was established within the Labor Department to administer the Act, effective April 28, 1971. Building on the Bureau of Labor Standards, the new agency took on the difficult task of creating a program that would meet the legislative intent of the Act.

Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin and its congeners) first achieved notoriety in the 1970s when it was discovered as a contaminant in some batches of Agent Orange, an herbicide used to defoliate trees in large areas of Vietnam. In addition to Agent Orange, dioxin is unintentionally produced by the manufacture of certain industrial chemicals, the chlorine bleaching process of pulp, the burning of certain wastes, and forest fires. Dioxin is highly lipophilic,

resists environmental degradation, and, in some species of animals, is very acutely toxic. It also causes cancers in animals and humans. It continues to generate concern because of its widespread distribution as an environmental contaminant (although levels have been declining for decades), its persistence within the food chain, and its toxicity. For these reasons, it has driven many aspects of food, environmental, occupational, and, interestingly, forensic toxicology, even though interpretation of its effects at low levels has been controversial. It is interesting to note that humans appear to be among the least sensitive species studied.

Most of our information about the effects of dioxin in humans comes from occupational accidents. Workers exposed to dioxin after a March 8, 1949, explosion at a Monsanto plant in Nitro, West Virginia, developed skin lesions (chloracne), eye irritations, headaches, dizziness, and breathing problems in the immediate aftermath of the incident. On July 10, 1976, an explosion at an Icmesa factory in Seveso, Italy, released 1.3 kg of dioxin into the air. The residents of the area were not evacuated immediately after the accident. Studies later confirmed that the residents exhibited the highest levels of dioxin ever found in human serum and that the soil in the area was heavily contaminated. Epidemiological studies of the residents of Seveso for over a quarter of a century indicate increases in certain cancers. Because of exposure to Agent Orange, the Veterans Administration set up the Agent Orange Registry, a health examination program for Vietnam veterans who were concerned about the possible long-term medical effects of exposure to Agent Orange. The National Academy of Sciences, in its 1994 report on Agent Orange, concluded that individual dioxin levels in Vietnam veterans are usually not meaningful because of background exposures to dioxin, poorly understood variations among individuals in dioxin metabolism, relatively large measurement errors, and exposure to herbicides that did not contain dioxin. Thus, the Veterans Administration will treat a number of diseases presumed to have resulted from exposure to herbicides like Agent Orange: chloracne or other acneform disease occurring within one year of exposure to Agent Orange, Hodgkin's disease, multiple myeloma, non-Hodgkin's lymphoma, acute and short-term peripheral neuropathy, porphyria cutanea tarda, prostate cancer, respiratory cancers, and some soft tissue sarcomas.

Environmental contamination by dioxin has been extremely controversial. Dioxin was implicated in vague illnesses in Love Canal, New York. Love Canal, near Niagara Falls, was a development built on and around a chemical waste site. Some epidemiologists claimed high rates of cancers and birth defects in the town, and the residents were evacuated in 1980. Some houses were torn down and the rest boarded up. Careful studies subsequently showed diseases in Love Canal were exactly what would be expected in a community of that size. Some parts of the area are now repopulated. A few years later the entire town of Times Beach, Missouri, was purchased by the federal government and bulldozed because of dioxin in the soil of unpaved roads. The buyout caused the only biological effect ever identified at Times Beach: huge populations of wild turkey and deer in the area of the former town. In 1996, the EPA announced the purchase of 158 homes and 200 apartments in the Escambia section of Pensacola, Florida, and relocated residents because dioxin-like chemicals are present in the soil of a former wood treatment plant. The EPA, worried about the chemicals possibly contaminating groundwater, dug up the soil and covered it with plastic. The residents demanded that their homes be purchased and that they be relocated, but refused examinations by U.S. Public Health Service doctors.

Even though there is little hard evidence of adverse effects in humans from dioxin in the environment, toxicology has been challenged by the public to deal with it. The controversy over dioxin involves the extrapolation of exposure to high levels (such as animal studies and occupational or accidental exposure) to the low levels generally found in the environment. For most people, the major exposure to dioxin is from food, mainly dairy and meat. This knowledge has led to increased surveillance of the food supply with resulting decreases in levels. Levels in the environment have been lowered 10-fold through new manufacturing methods and controlled incineration. Nonetheless, the concern remains.

Safety and Risk Assessment

Predicting what will happen in the future is the hallmark of any science. Given the same starting conditions, what happened before should happen again. Reproducibility may occur in the laboratory, but the public does not care about laboratories. The questions they pose are about different species, different routes of exposures, different exposure levels, and different exposure periods. How, then, can toxicologists explain to the public what may or may not happen under conditions that have not been tested and without theories that are as solid as in other sciences?

The emergence of modern safety assessment can be traced to the early 1950s with the concept of the NOAEL and ADI by Lehman and Fitzhugh and others. The basic premises have not changed in 60 years. For example, to determine the ADI, take the NOAEL (the highest dose tested that causes no adverse effects in a test species in a properly designed and executed study) and divide it by an appropriate safety/uncertainty factor (generally 100, to account for inter- and intraspecies differences). The resulting value, expressed as mg of chemical/kg body weight/day, is an amount that can be safely consumed for a lifetime by all segments of society. This has withstood the test of time and has effectively protected the public.

Risk assessment for carcinogens took a different route in the 1960s. It uses information from lifetime studies in male and female rats and mice administered a material at the maximum tolerated dose (MTD) and extrapolates the findings to millions of people exposed at levels many orders of magnitude lower. Unfortunately, standardizing risk assessments

has also resulted in inflexibility, applying numerous levels of conservatism, and frequently the inability to properly characterize likely risks because of the layers of default assumptions causing calculations to be unrealistic.

These efforts were often based upon theories that were not always well tested. For example, the most basic tenet of toxicology, the dose–response relationship with its origins dating back to Paracelsus, is apparently not as well understood and appreciated as toxicologists might think. What exactly is a dose, and is the nature of the response linear or curved? Is there a threshold for every effect? Further, is the response always in one direction, that is, as the dose is increased, is the severity of the response increased? Should hormesis be considered in risk assessment? How risk assessments are eventually conducted, by science or dogma, will greatly influence public policy. Much more research into these basic concepts of toxicology and risk assessment are needed.

Intentional Poisonings

One might think that intentional poisonings would disappear as analytical and forensic procedures improved, but they continued. Most were the standard poisons used for centuries (arsenic, cyanide), but some were more clever. In the end, only a few made any impact on the study of toxicology.

Some intentional poisonings were worse than ever. Chemical weapons more lethal than their predecessors, continued to be produced in a number of countries. In the United States, chemicals for lethal injection were being used as a means of execution. Three classes of drugs that are generally used in lethal injections are a general anesthetic to induce unconsciousness (e.g., sodium thiopental), a paralyzing agent to stop breathing (e.g., pancuronium bromide), and a cardiotoxic agent to stop the heart (e.g., potassium chloride).

Training

> Even while they teach, men [they] learn.
>
> **Seneca**

As toxicology became a recognized scientific discipline, many training programs began at prestigious universities. Although it was difficult to develop programs that could address all the many facets of toxicology including chemistry and biochemistry, physiology and pharmacology, pathology, statistics, and epidemiology, excellent ones were developed.

The need for a standard, modern textbook became evident. Although several texts were available, none appeared adequate. This issue was addressed by Louis J. Casarett (1927–1972) and John Doull. Casarett received his doctorate in 1958 from the University of Rochester, where he studied respiratory toxicodynamics and morphological changes following exposures to potentially toxic materials, especially polonium. In 1967, he moved to the University of Hawaii, where he developed a program in toxicology. His research involved drugs of abuse and pesticides. Doull received both his doctorate in pharmacology and his medical degree from the University of Chicago. He remained at Chicago for a number of years and then moved to the University of Kansas Medical Center, where he established one of the more outstanding programs in toxicology. Casarett and Doull published *Toxicology: The Basic Science of Poisons* in 1975. Since then, a number of other excellent texts address various aspects of the principles and practices of toxicology, including *Hayes' Principles and Methods of Toxicology*.

Many corporations developed centers of excellence in toxicology to study product and workplace safety and produced scientists of great renown. Some of these laboratories include DuPont's Haskell Labs (established in 1935), Dow Chemical (VK Rowe, Perry Gehring), and Union Carbide/Carnegie Mellon Bushy Run (Carroll Weil).

As reports about pollution were threatening to overshadow the benefits of chemicals to society, industry's challenge was to overcome lack of knowledge about the health effects of chemicals. So 11 major chemical companies in the United States created the Chemical Industry Institute of Toxicology (CIIT) in 1974 to address growing concerns about the effects of chemicals on environmental and human health. They hired Leon Golberg (1915–1987) as its first president. CIIT (now The Hamner Institutes for Health Sciences) moved the understanding of toxicology to new levels of expertise in the area of mechanism of action.

Contract toxicology laboratories also made a significant contribution to toxicology by providing unique opportunities for those interested in the pragmatic aspects of applied toxicology, namely, the conduct of appropriate tests to establish safe conditions of exposure. These studies must consider both the latest developments and advances in toxicology and the needs of regulators internationally. This is especially challenging in an era of increased international trade and harmonization. Laboratories, such as Hazleton Laboratories (now Covance) founded by Lloyd Hazleton, Food and Drug Research Laboratories founded by Ben Oser, Biodynamics (now Huntingdon Life Sciences, United States) founded by Tom Russell, and International Research and Development Laboratories (now MPI) founded by Frank Wazeter, produced trained toxicologists that populated a number of influential positions in academia, industry, and government.

Toxicology was also advanced within trade associations as they assisted both industry and regulatory authorities in establishing safe limits of exposure by using the best science possible. These associations include the Flavor and Extract Manufacturers Association (FEMA), Cosmetic Toiletry and Fragrance Association (CTFA) (now the Personal Care Products Council), and the International Association of Color Manufacturers (IACM). Toxicologists were also involved with organizations to promote the basic science, such as the International Life Sciences Institute

(founded by Alex Malaspina and John Kirschman) and applied science such as the Toxicology Forum (founded by Philippe Shubik with the support of industry, academia, and governments).

Through these efforts, toxicologists were trained and then kept up with developments in the field past their formal education.

> Toxicology is the ultimate Renaissance science.
>
> **Gillett (1987)**

Toxicology continues to grow. Its critical position in society and the uniqueness of the issues it faces continue to attract and even seduce some of the brightest minds. There is something for everyone: from the molecular to the macro, from the gene to the whole animal to the human. Toxicology's strengths derive from the integration of the chemical and biological sciences and supporting disciplines. Toxicology is also one of the few sciences in which academic, industrial, and regulatory scientists can and do effectively interact to protect the public. The importance of toxicology is recognized by governments worldwide. Toxicology has evolved from listing poisons to protecting the public, from simply identifying effects (qualitative toxicology) to identifying and quantifying human risks from exposure, and from observing phenomena to experimenting and determining mechanisms of action of toxic agents and rational management for intoxication. As Claude Bernard noted:

> Where then, you will ask is the difference between observers and experimenters? It is here: we give the name observer to the man [human] who applies methods of investigation, whether simple or complex, to the study of phenomena which he [she] does not vary and which he [she] therefore gathers as nature offers them. We give the name experimenter to the man [human] who applies methods of investigation, whether simple or complex, so as to make natural phenomena vary, or so as to alter them with some purpose or other, and to make them present themselves in circumstances or conditions in which nature does not show them. In this sense, observation is investigation of a natural phenomenon, and experiment is investigation of a phenomenon altered by the investigator.
>
> **Bernard (1865)**

Toxicology has come a long way! As science continues to advance, toxicology will continue to draw from these advances in its constant quest to protect the public from harm.

QUESTIONS

1.1 Analytical (forensic) toxicology had its formal origins in the outstanding work of what Spaniard?

1.2 The penalty of the peach was used by what ancient society?

1.3 What is the name of the early U.S. regulator noted for studying/testing the effects of food additives in his *Poison Squad*?

1.4 Name the two U.S. regulators who laid the foundation of an ADI of a material in 1952.

1.5 Who wrote *Silent Spring?*

FURTHER READINGS

This is not meant to be an exhaustive list of citations for all the details in the chapter. Rather, it is a list of some key books and articles the reader may wish to consult to gain a fuller appreciation of the history and meaning of toxicology.

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2 Use of Toxicology in the Regulatory Process

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CONTENTS

BACKGROUND

Regulatory toxicology is that area of toxicology directed at protecting public health by regulating exposure to potentially harmful materials. Historically, regulatory toxicology has developed in a manner that has reflected humankind's ability to relate exposure to certain agents to adverse health effects. Thus, because effects were observable and could be easily associated with exposure, early regulatory attention generally focused on preventing the acute effects of chemical agents. Food and drugs were the focus of early regulation

due, no doubt, to the relative ease in associating acute health effects with exposure to materials in the diet or medications. Hutt[1] notes that adulteration of the food supply was a serious problem in the ancient world, and he quotes Pliny the Elder, writing in the first century AD, as saying, "So many poisons are employed to force wine to suit our taste – and we are surprised that it is not wholesome!"

Occupational exposures were another early focus of regulation, due again to the fact that the relationship between exposure and effect was often observable. Early industrial hygiene efforts were typically intended to prevent overt or frank effects of materials in the workplace. Some of the first observations of effects from chronic human exposures to certain chemicals were made in occupational settings. Hutt[1] notes that, during the sixteenth century, Paracelsus wrote about diseases characteristic of miners. Certain chronic occupational hazards affected the exposed individual at the point of contact, which made the connection between agent and effect easy to discern. The first epidemiological study linking human cancer to a specific cause is attributed to Sir Percivall Pott, who in 1775 identified occupational exposure to soot as being responsible for scrotal cancers in young British chimney sweeps.[2]

The development of regulatory toxicology during the twentieth century up through the present has continued to shadow the ability to detect both chemicals and effects. That is, as it has become possible to detect chemicals at lower and lower levels as well as smaller biochemical and physiological changes, regulatory attention has turned to what appear to be *new* problems. For example, small increases in airway resistance following exposure to certain air pollutants are currently used as one basis for regulating these air pollutants; historically, no one would have been aware of these subtle effects. Similarly, guidelines for occupational exposures to benzene have decreased by two orders of magnitude—from 100 parts per million (ppm) in 1927 to the current Occupational Safety and Health Administration (OSHA) standard of 1 ppm. In contrast, ambient criteria (which are typically not mandated standards) for nonoccupational benzene exposures in some states can be much lower. For example, the ambient annual guideline for benzene is 0.04 parts per billion (ppb) in New York state, a concentration that is below many ambient background samples.[3]

Because of the dramatic increase in our ability to detect smaller effects and lower concentrations, programs to regulate chemicals in the environment have increased at an astronomical rate during the last 40 years. Factors contributing to the recent increase in regulatory activity include the following:

- The realization of the vast number of chemicals that humans have dispersed into the environment and to which humans have been exposed. As of 2007, more than 80,000 commercial chemicals in commerce had been identified and listed under the Toxic Substances Control Act (TSCA).[4] Advances in analytical chemistry have allowed ppb levels of chemicals to be detected in *pristine* areas, as well as in wildlife, food products, and human body tissues. This message was delivered initially by Rachel Carson in 1962 with the publication of Silent Spring, which described the impact on the environment, particularly birds, of use of pesticides. Today, the U.S. Centers for Disease Control and Prevention (CDC)[5] conducts biomonitoring studies (i.e., the National Report on Human Exposure to Environmental Chemicals)* of human serum and urine, demonstrating the presence of more than 200 multiple exogenous chemicals in biological media, including anthropogenic compounds (such as polychlorinated biphenyls [PCBs]) and chemicals that have both natural and anthropogenic sources (such as lead and cadmium).[5]
- The realization that historical chemical management practices might today be associated with low-level risks, even though such practices were consistent with the state of knowledge at the time. For example, during the 1970s, residents of Love Canal, a neighborhood of Niagara Falls in New York state, realized that they had unknowingly been exposed to chemicals that had migrated into their basements from a nearby site formerly used as a landfill. The Comprehensive Emergency Response, Compensation, and Liability Act (CERCLA), also known as Superfund, was enacted shortly after the Love Canal incident (see Table 2.1 and NYS DOH).[6]
- The establishment over the past 50 years of causal relationships between certain diseases and chronic chemical exposures, such as leukemia and benzene[7] or mesothelioma and asbestos.[8]
- The reduction in illness and mortality due to microbial diseases and the improved standard of living, which have focused increasing attention on other causes of ill health.

The rapid increase in the number and complexity of regulatory programs to address potential health effects from chemical exposures is also a result of the increased scientific uncertainty about toxicology and risk that has evolved with our increased understanding of these subjects (i.e., the more that is learned, the more clear how much more there is to learn becomes). As the complexities of toxicology have become better understood, more complex procedures for characterizing toxic responses have been developed, such as the use of probabilistic risk assessment methods, refined analyses of mode of action (MoA), or identification of key events on a causal pathway for toxicity.[9]

When the Delaney Clause (forbidding the addition to food of any substance found to induce cancer in animals or humans) was passed in 1958,[10] the public generally believed that the intent of the law—to provide a *zero-risk* food supply—was achievable. No one foresaw that, 20 years later, scientists would have identified more than 500 animal carcinogens,

* Often referred to as the *NHANES* study.

TABLE 2.1
Federal Laws Related to Exposures to Toxic Substances

Legislation	Agency	Area of Concern
Food, Drug, and Cosmetics Act (1906, 1938, amended 1958, 1960, 1962, 1968, 1976, 1996 (also known as the FQPA), 1997)	FDA	Food, drugs, cosmetics, food additives, color additives, new drugs, animal and food additives, and medical devices
FIFRA (1948, amended 1972, 1975, 1978, 1988, 1996)	EPA	Pesticides
Dangerous Cargo Act (1952)	DOT, USCG	Water shipment of toxic materials
Atomic Energy Act (1954)	NRC	Radioactive substances
Federal Hazardous Substances Act (1960, amended 1981)	CPSC	Toxic household products
Federal Meat Inspection Act (1967); Poultry Products Inspection Act (1968); Egg Products Inspection Act (1970)	USDA	Food, feed, color additives, and pesticide residues
National Environmental Policy Act (1970, amended 1975, 1985, 1989, 1996, 1997)	EPA	Ecosystems and natural resources
OSH Act (1970, amended 1974, 1978, 1982, 1983, 1984, 1986, 1987, 1990, 1992, 1995, 1996, 1997, 1998, 2002)	OSHA, NIOSH	Workplace toxic chemicals
Poison Prevention Packaging Act (1970, amended 1981)	CPSC	Packaging of hazardous household products
CAA (1970, amended 1974, 1977, 1990)	EPA	Air pollutants
Hazardous Materials Transportation Act (1972)	DOT	Transport of hazardous materials
CWA (formerly Federal Water Pollution Control Act; 1972, amended 1977, 1978, 1981, 1987)	EPA	Water pollutants
Marine Protection, Research, and Sanctuaries Act (1972)	EPA	Ocean dumping
Consumer Product Safety Act (1972, amended 1981)	CPSC	Hazardous consumer products
Lead-Based Paint Poison Prevention Act (1973, amended 1976)	CPSC, HEW, (HHS), HUD	Use of lead paint in federally assisted housing
Residential Lead-Based Paint Hazard Reduction Act (1992)	EPA	Use of lead paint in all housing
Safe Drinking Water Act (1974, amended 1977, 1986, 1996)	EPA	Drinking water, contaminants
Resource Conservation and Recovery Act (1976, amended 1984)	EPA	Solid waste, including hazardous wastes
TSCA (1976); Asbestos Information Act (1988)	EPA	Hazardous chemicals not covered by other laws, includes premarket review
Federal Mine Safety and Health Act (1977)	DOL, NIOSH	Toxic substances in coal and other mines
Comprehensive Environmental Response, Compensation, and Liability Act (1981); Superfund Amendments and Reauthorization Act (1986); Emergency Planning and Community Right-to-Know Act (1986)	EPA	Hazardous substances, pollutants and contaminants
Radon Gas and Indoor Air Quality Research Act (1986)	EPA	Indoor air
Oil Pollution Act (1990)	DOT	Oil pollution
Pollution Prevention Act (1990)	EPA	Toxics use reduction
Bioterrorism Act (2002)	FDA, CDC, USDA, EPA	Biological agents and toxins used in acts of war
Consumer Product Safety Improvement Act (2008)	CPSC	Children's product safety; CPSC reform

been able to detect chemical concentrations between two and five orders of magnitude lower than could be detected in the 1950s, and found that many naturally occurring chemicals in food could be considered animal carcinogens.[11] Enormous quantities of chemical toxicity data are now being made available via the Registration, Evaluation Authorisation and Restriction of Chemicals (REACH) program in Europe—as of 2008, information had been submitted on more than 100,000 chemicals,[12] creating enormous challenges for development of new methods and interpretation of findings.

CURRENT REGULATORY FRAMEWORK

While this chapter focuses on regulatory approaches in the United States, the authors recognize the importance of *globalization* of risk assessment and risk management procedures. Therefore, some examples of regulatory frameworks outside the United States—although by no means exhaustive—will be presented as well.

At the federal level in the United States, four agencies bear most of the direct responsibility for the regulation of toxic chemicals—the Consumer Product Safety Commission (CPSC), the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and OSHA. Table 2.1 describes the acts that empower these and several other federal agencies.

It is clear from Table 2.1 that there is a broad range of chemical exposures with which federal regulatory authorities are concerned. Chemicals may be regulated on the basis of environmental medium (e.g., air, water), activity (e.g., food manufacture, chemical transport, ocean dumping), and type of exposure environment (e.g., workplace, residential).

TABLE 2.2
Advantages and Disadvantages of Epidemiological Studies

Advantages	Disadvantages
Exposure conditions realistic	Costly and time consuming
Occurrence of interactive effects among individual chemicals	Post facto, not protective of public health[a]
Effects measured in humans	Difficulty in defining exposure, problems with confounding exposure
Full range of human susceptibility frequently expressed	Difficult to see less than twofold increase in risk except in very large populations
	Effects measured can be relatively crude (morbidity, mortality)

[a] Use of biomarkers in epidemiological studies, rather than disease endpoints, can allow such studies to be public health protective.

While the statutes in Table 2.2 represent approximately 100 years of federal legislative history, 20 of the 26 have been written (and some earlier statutes have been updated) since 1970, illustrating the relatively recent increase in public concern about chemical exposures.

The language of each statute provides the implementing agency with the basis for issuing regulations under the law. Some statutes instruct the agency to limit chemical release or exposure by requiring the use of certain control technologies. Some statutes require the agency to develop and implement risk-based standards, while others require balancing risks with the costs of regulating (or the benefits of not regulating) a certain chemical. The latter two types of statutes are the most likely to involve regulatory toxicology in their implementation.

Section 307 of the Clean Water Act (CWA) is an example of a statute that requires technology-based standards for pollution control. Under this portion of the CWA, industries discharging to surface water must use the best available control technology to limit their pollutant discharges; installation of the appropriate control technology is required from the discharger to obtain a National Pollutant Discharge Elimination System (NPDES) permit.

Other statutes specify the standard for safety that regulations and standards issued under the law are supposed to provide. A commonly cited example of a law that required health-based, or risk-based, standards for pollution control is Section 112 of the 1970 Clean Air Act (CAA), which required the EPA to set emission standards for hazardous air pollutants under the National Emissions Standards for Hazardous Air Pollutants (NESHAPS) program that would *protect public health* with an *ample margin of safety*. Implementation of this standard of safety for carcinogenic air pollutants proved to be so troublesome that, between 1970 and 1990, NESHAPS were set for only seven air pollutants. The difficulty in setting the risk-based standards was that the statute provided no indication of what an *ample* margin of safety was or how such a concept might be applied to carcinogens, given that the agency considered carcinogens to act by a no-threshold mechanism.* The 1990 amendments to the CAA replaced the health-based NESHAPS standards with specific technology-based standards for controlling hazardous air pollutants. The 1990 amendments state that after installation of the control technology, health-based standards must be set to further control emissions where unacceptable risks remain.

The Federal Food, Drug, and Cosmetic Act (FFDCA) is another example of a law requiring health-based standards for limiting the public's exposure to chemicals. Section 409 of the FFDCA requires the sponsor of a food additive to demonstrate to a reasonable certainty that no harm to consumers will result when the additive is put to its intended use. This statute contains the Delaney Clause (discussed earlier), a special provision that forbids the use of any food additive that has been found to induce cancer in humans or animals. Essentially, the Delaney Clause specifies that the acceptable risk from carcinogens as food additives is zero. This bright line has proven to be very difficult for the FDA and (until passage of the Food Quality Protection Act [FQPA] in 1996) EPA to implement, because the law does not allow the implementing agencies to specify de minimis, or acceptable, levels of risk.

The EPA regulates pesticides under both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the FFDCA. Under the FIFRA, the agency was required to balance the risk from a given pesticide with the benefit associated with its use. However, the EPA was also bound by the *zero-risk* Delaney Clause under the FFDCA with regard to pesticides that may concentrate in processed foods above the level allowed on the raw agricultural commodity. This dichotomous standard (known as the *Delaney Paradox*) forced the EPA to regulate to zero-risk pesticides that may concentrate on foods during processing while regulating using risk–benefit analysis for the same pesticides on raw agricultural commodities.[13] In 1992, a Circuit court ruled that the Delaney Clause does not allow the EPA to permit the use of carcinogenic pesticides under the FFDCA, even if their use is associated with negligible risk.[14] The Delaney Clause has also been difficult for the FDA to implement as more chemicals (including naturally occurring chemicals in foods) have been determined to be carcinogens. As such, the FDA has searched for ways to establish acceptable risk levels from food additives. In 1988, the U.S. Court of Appeals for the District of Columbia struck down an effort by the FDA to interpret the Delaney Clause as allowing the agency to set a *de minimis* risk level for two color additives for use in cosmetics and drugs.[15]

Other laws require the implementing agency to balance the risks and benefits of alternative regulatory choices. For example, Section 408 of the FFDCA, which, until the

* With respect to risks from no-threshold mechanisms, decisions must be made as to acceptable risk, in which case the *ample margins of safety* concept is difficult to apply, other than via an expression of permissible risk.

FQPA of 1996, required tolerances for pesticide residues on raw agricultural commodities to be set at levels necessary to protect the public health while considering the need for *an adequate, wholesome, and economical food supply*.[13] "Evaluation of Noncancer Effects" section of the TSCA requires the EPA to consider the potential benefits of using a chemical and the economic consequences of restricting its use when determining whether the manufacture, distribution, use, or disposal of a substance presents an unreasonable risk of injury to health or the environment.

Language in the Occupational Safety and Health Act (OSH Act) specifies that the agency (i.e., OSHA) must "adequately assure(s) to the extent feasible…that no employee will suffer material impairment of health or functional capacity."[16] This statutory language also requires balancing of risks and costs, but the dual requirements of *feasibility* and *that no employee will suffer* may, because of the absolute nature of the language, be impossible to reconcile in certain situations.[17]

While the narrative terms *unreasonable risk* or *ample margin of safety* have not been clearly or consistently defined across agencies or statutes, agencies have generally interpreted this language as requiring a qualitative—and, frequently, quantitative—estimate of the health risks associated with an exposure and the reduction in risks resulting from regulatory action. A major factor in the increased use of risk analysis by regulatory agencies was the 1980 Supreme Court decision in Industrial Union Department vs. American Petroleum Institute. In this case, the OSHA proposed lowering the occupational standard for benzene from 10 to 1 ppm on the bases that benzene was a carcinogen, any reduction in exposure would result in a reduction in risk, and 1 ppm was technologically feasible. The Supreme Court did not find for the union, stating, "Before he can promulgate any permanent health or safety standard, the Secretary [of Labor] is required to make a threshold finding that a place of employment is unsafe – in the sense that significant risks are present and can be eliminated or lessened by a change in practices."[18] The Court left the decision of what constitutes a *significant risk* to the OSHA. This landmark decision has had a major impact on agencies in addition to the OSHA, resulting in an increase in the development and use of tools to quantify risks from exposure to environmental chemicals.

The FQPA of 1996, which addresses risks from pesticides in food through the setting of tolerance limits, is primarily risk-based, with limitations on the extent to which the EPA can consider benefits. This is in contrast to the risk–benefit balancing requirements of the FFDCA noted earlier. Only in certain narrow circumstances under the FQPA can the EPA set pesticide tolerance levels that do not meet health-based criteria. Specifically, the circumstances comprise those situations where the use of a given pesticide prevents even greater risks from occurring to consumers (a risk–risk balancing) or where the lack of the pesticide would result in "a significant disruption in domestic production of an adequate, wholesome, and economical food supply." In addition, the FQPA eliminates certain aspects of the *Delaney Paradox* discussed earlier. Tolerance limits for pesticides in raw agricultural products and processed foods, for carcinogens and noncarcinogens (the Delaney Clause considered carcinogens only), are now to be based on health only. There are several other important provisions of the FQPA: the requirement that EPA specifically consider exposures and risks to infants and young children in setting pesticide tolerance limits, allowing an up to tenfold additional safety factor; the need to consider all pathways of exposure (e.g., drinking water, soil/dust ingestion) to a pesticide in setting tolerance limits for that pesticide in food; the need to consider the cumulative risk for multiple pesticides that act via a common mechanism of action when setting a tolerance limit for any single pesticide of the *common mechanism* class; and the establishment of a very ambitious comprehensive screening and testing program for pesticides that exert estrogenic and, possibly, other endocrine-related effects. The FQPA represents a landmark piece of legislation, not only in terms of the regulatory implications but also with respect to the advancement in scientific understanding required for its implementation.[19] For example, the EPA's ambitious Endocrine Disruptor Screening Program, which resulted from this statute, has posed significant technical and financial challenges in achieving its goals of evaluating potentially hundreds of chemicals for endocrine activity.[12]

Since the prior version of this chapter, the Consumer Products Safety Improvement Act (CPSIA) of 2008[20] represents a major statutory update in U.S. chemical regulation. This statute contains new testing and documentation requirements for children's products, with particular emphasis on lead and phthalates. It is unusual in its specificity (e.g., *defining* a child as up to 12 years of age and establishing numerical criteria for lead in children's products), and it allowed the U.S. Congress, in certain provisions, to supersede the type of technical role usually delegated to the implementing agency.

The combined effect of the use of risk assessment to help make regulatory decisions and the significant uncertainty that accompanies most quantitative estimates of toxicological risk has resulted in considerable debate about the practice of risk assessment. The EPA, FDA, and other agencies have been criticized by the Office of Management and Budget (OMB) and some representatives of the regulated community for being too conservative in their risk assessment procedures.[21] In contrast, environmental advocacy groups such as Greenpeace have claimed that "[i]n the real world, quantitative risk assessments are used almost exclusively to justify pollution."[22] Others have noted that "current risk estimates are by no means routinely exaggerated, either for the entire populations they apply to or for highly exposed or highly susceptible individuals within those populations."[23] Much of this difference in interpretation is perhaps due to the fact that risk estimates are frequently defined and presented inadequately. It is not uncommon for risk assessors to provide single value estimates of risk that may apply to some unknown percentage of the population. Because the variability in the exposure and dose–response characteristics of a population are so large, the risk estimates for a small, highly exposed or sensitive subpopulation may be very different from the estimates

of the most likely risks for the entire population. Although the risk assessment results are supposed to be qualified and uncertainty discussed, the risk number is often used without appropriate qualification. To address this problem, in 1994, the EPA prepared guidance to risk assessors on the need to provide fuller, more explicit descriptions of risk when providing such information not only to risk managers but also to the general public.[24] More recently, EPA developed guidance to enhance the approaches to and practices of risk assessment at the agency. These broadly applicable principles address a number of issues, such as the need for enhanced transparency in data selection and choices for risk assumptions, as well as clarifying the purpose of a particular analysis (e.g., screening vs. comprehensive assessment) and ensuring that the level of analysis is commensurate with the overall purpose.

In addition to the agencies discussed earlier, governmental and nongovernmental agencies can influence the regulatory process as well. The American Conference of Governmental Industrial Hygienists (ACGIH) sets exposure limits based solely on health protection for approximately 600 workplace chemicals. These exposure limits, known as threshold limit values (TLVs),[25] do not carry any regulatory weight, but it is not uncommon for workplaces to adhere to TLVs for chemicals that the OSHA does not regulate or that have an exposure limit that has not been revised since the inception of the OSHA in 1970. The TLVs have also been used by several state environmental agencies to derive acceptable ambient levels for toxic air pollutants.

Agencies in the Department of Health and Human Services (DHHS) that influence the regulatory process include the National Cancer Institute; the National Institute of Environmental Health Sciences, in particular the National Toxicology Program (NTP); the National Institute for Occupational Safety and Health (NIOSH) and the Center for Environmental Health (part of the CDC); and the Agency for Toxic Substance and Disease Registry (ATSDR).[26] These agencies affect the regulatory process in several ways, ranging from decisions on which chemicals to test in long-term cancer bioassays to defining principles for evaluating carcinogens and conducting site-specific (as with a hazardous waste site) and chemical-specific risk assessments. International organizations such as the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) also have a significant role in the use of information by regulatory agencies.

The primary focus of this chapter is on the use of regulatory toxicology at the federal level in the United States. However, state governments have also been active in regulating exposure to toxicants in the environment. For example, in 1986, voters in California overwhelmingly adopted Proposition 65, the Safe Drinking Water and Toxic Enforcement Act of 1986 (known commonly as Prop 65). This act contains two major provisions—one prohibiting the "discharge or release [of] a chemical known to the state to cause cancer or reproductive toxicity into water" and the other, a labeling requirement, mandating that no person expose another individual to any carcinogen or reproductive toxin without providing *clear and reasonable warning*. Exemptions for the discharge requirements are provided for carcinogens at discharge levels that will pose a lifetime cancer risk to a person drinking the water of less than 1×10^{-5}, or, for reproductive toxicants, discharges resulting in exposure levels less than 1000 times smaller than the *no observable effect level* (NOEL) for reproductive effects.

Some states in the United States have also developed their own risk assessment procedures and health-based standards, particularly in those situations where federal criteria are lacking, federal approaches and analysis have been viewed as outdated, or state and federal agencies have different interpretations of science. For example, several states have developed their own maximum contaminant levels (MCLs)* for chemicals in drinking water. In some cases, the state criteria can be more than an order of magnitude more restrictive than the federal limit (see, e.g., Cadmus).[28] For example, the federal drinking water limit for cis-1,2-dichloroethylene (cis-1,2-DCE) is 70 ppb (1 ppb = 1 μg/L), whereas the California standard is 6 ppb. Both values are based on noncancer liver toxicity in animals, with the differences mainly due to varying interpretations of toxicological findings.

Of particular interest with respect to non-U.S. agencies are risk assessment and risk management approaches undertaken by the European Commission (EC). The precautionary principle was first presented in Principle 15 from the Declaration of the 1992 Rio Conference on the Environment and Development, which states "in order to protect the environment, the precautionary approach shall be widely applied by States according to their capability. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation."[29]

The precautionary principle has engendered much debate. For example, under what conditions is action to mitigate risks appropriate? Under what conditions is development of a new technology considered so potentially *risky* that restrictions on development are warranted?[30] The REACH program in the European Union (EU) may be said to embody elements of the precautionary principle. This program requires companies that manufacture or import more than one ton of a particular chemical substance per year to register the chemical in a central database. Specifically, the regulation "is based on the principle that it is up to manufacturers, importers and down-stream users of substances to ensure that they manufacture, place on the market, import or use such substances that do not adversely affect human health or the environment."[31] Thus, overall, the emphasis of the precautionary principle is on demonstrating that a chemical *does not* present a significant risk and risk management action is unnecessary. This is a subtle, but important, difference from other approaches taken by the EC, which typically require demonstration that

* MCLs are health-based standards, as mandated under the Federal Safe Drinking Water Act.

a chemical *does* present a significant risk and, hence, risk management action may be necessary.

In addition to its influence on expansion of the precautionary principles, REACH has created important new challenges for the toxicology community. REACH has advanced to the stage where submissions on approximately 144,000 compounds have been received, dwarfing original expectations.[12] Toxicologists must now interpret toxicity findings from numerous submissions, creating potential concerns for the lack of sufficient technical support, the potential for false-positives, and the need to resolve differences in interpretation among submissions for the same compounds.

RISK ASSESSMENT PARADIGM

In response to a directive from the U.S. Congress, the FDA contracted with the National Research Council (NRC) of the National Academy of Sciences (NAS) to evaluate the risk assessment process in the federal government and make recommendations on how the process could be improved. As a result of this effort, the Committee on the Institutional Means for Assessment of Risks to Public Health published a book in 1983 titled *Risk Assessment in the Federal Government: Managing the Process*.[32] The book summarized past experiences and, although it did not propose new ways to evaluate risks from environmental chemicals, it has nevertheless had an important effect on the use of scientific information by regulatory agencies in its codification of the risk assessment process. The book has been particularly influential in two areas: (1) the separation of the risk assessment process from the risk management process and (2) the classification of the risk assessment process into four broad components—*hazard identification, dose–response assessment, exposure assessment,* and *risk characterization*.

More recently, the Committee on Risk Assessment of Hazardous Air Pollutants (established by the NRC under the direction of the EPA) and the Presidential/Congressional Commission on Risk Assessment and Risk Management (CRARM) reevaluated risk assessment and risk management approaches. The findings of the NRC committee were published in a 1994 book titled *Science and Judgment in Risk Assessment*,[33] and the findings of the CRARM were published in a 1997 report titled *Risk Assessment and Risk Management in Regulatory Decision-Making*.[34] While the separation of risk assessment and risk management and the four components making up the basic risk assessment paradigm remain key underlying principles, both committees recommended refinements in risk assessment and risk management approaches. For example, the NRC committee highlighted the importance of an iterative approach to risk assessment to reduce uncertainties, with each iteration incorporating fewer default assumptions and more specific information, balancing the use of *better science* with the constraints of the available resources.[33] The CRARM proposed a framework for risk management that encourages early and frequent involvement of all groups affected by the risk management problem and decision-making based on the context of broader, real-world goals of risk reduction and improved health status.[34]

Risk assessment is defined as the "systematic, scientific characterization of potential adverse effects of human or ecological exposures to hazardous agents or activities," and it involves assessment of the strength of the evidence as well as evaluation of the uncertainties associated with risk estimates.[34] In contrast, risk management is "the process of identifying, evaluating, selecting, and implementing actions to reduce risk to human health and ecosystems."[34] Risk managers choose actions that will mitigate risks, considering not only the information derived from risk assessment but also cultural, ethical, political, social, economic, and engineering information in the decision process.

The distinction between risk assessment and risk management has been an important characteristic in framing how risk analyses have been conducted and results applied.[32] There have been concerns that risk management issues on the risk assessment process, such as the economic significance of a product, can seriously undermine the credibility of the risk assessment. This concern is exemplified in the separation between the NIOSH and OSHA. The NIOSH, part of the DHHS, is responsible for recommending health-based standards for workplace exposures to OSHA, part of the Department of Labor. As the federal agency responsible for setting and implementing standards for workplace exposures, the OSHA is required to consider feasibility in the choice of exposure limits. It is not uncommon to find that permissible exposure limits (PELs) set by the OSHA are less strict than recommended exposure limits (RELs) set by the NIOSH.[35] In some cases, the differences may reflect the date when a particular value was set. However, other factors, such as differences in scientific interpretation or considerations of technical feasibility, may explain the differences. For example, the OSHA PEL for benzene is 1 ppm, whereas the NIOSH REL is 0.1 ppm.

The distinction between risk assessment and risk management is not nearly so clear in practice. This is because each component of a chemical risk assessment is associated with considerable uncertainty. In the face of this uncertainty, regulatory officials have generally resorted to erring on the side of caution by including health-protective assumptions. For example, the choice of a linear no-threshold model for carcinogens, which leads to a higher estimate of risk than other models, represents a risk management decision as much as a science policy decision. That is, the approach is conservative and provides the regulator with a greater level of confidence that the true risk to the human population is likely less than that expressed through the model. This approach has historically been justified as consistent with prudent public health policy when uncertainty is so great that it is difficult to provide a precise estimate of risk (i.e., in the face of uncertainty, it is easier to say the risk is less than x than to say the risk equals y). However, this practice can lead to inconsistent levels of protection for different chemicals and may direct resources away from the more significant risks.[36] For example, the potential cancer risks associated with chemical disinfectants

should be compared to the risks of waterborne microbial diseases when making decisions about treating public drinking water supplies; yet, such risk–risk trade-offs cannot be accurately weighed if health-protective assumptions have been used to different extents in the underlying risk assessments.[37] The practice of using health-protective assumptions in conducting risk assessments has been described by some as an inappropriate application of risk management to the risk assessment process.[21]

In 2009, an NRC panel provided recommendations for a more nuanced approach to the integration of risk assessment and risk management. The NRC panel expressed concerns that the risk assessment process at the EPA had, in several cases, become slow and inefficient; certain analyses, such as the risk assessment for trichloroethylene (TCE), could take years—if not decades. Similarly, concerns were expressed for the risk management process; disconnects were identified between the available scientific data and information needs of risk managers. To improve the process, the NRC panel recommended a framework, reproduced here in Figure 2.1, to enhance the utility of risk assessment in risk management. Importantly, the framework seeks to increase the level of upfront planning in the conduct of a risk assessment to ensure that the findings are more relevant. For example, consideration of exposure under existing conditions and under various control options could be incorporated into dose-response risk assessments.

Although risk assessments are commonplace at many federal and state agencies, there are no uniform guidelines that specify how regulatory officials should calculate chemical risks. There are also no uniform criteria that indicate how the findings of a risk assessment should influence regulatory decisions.[38] As a result, cancer potency estimates (i.e., the estimated upper bound on lifetime cancer risk associated with the lifetime daily dose of a chemical) developed by different regulatory agencies for the same chemical can vary substantially.[39] Differences in cancer potency estimates for chemicals can also vary among European agencies.[40] Furthermore, the level of risk sufficient to trigger regulatory action can vary considerably among agencies and even among different programs within a single agency.[41]

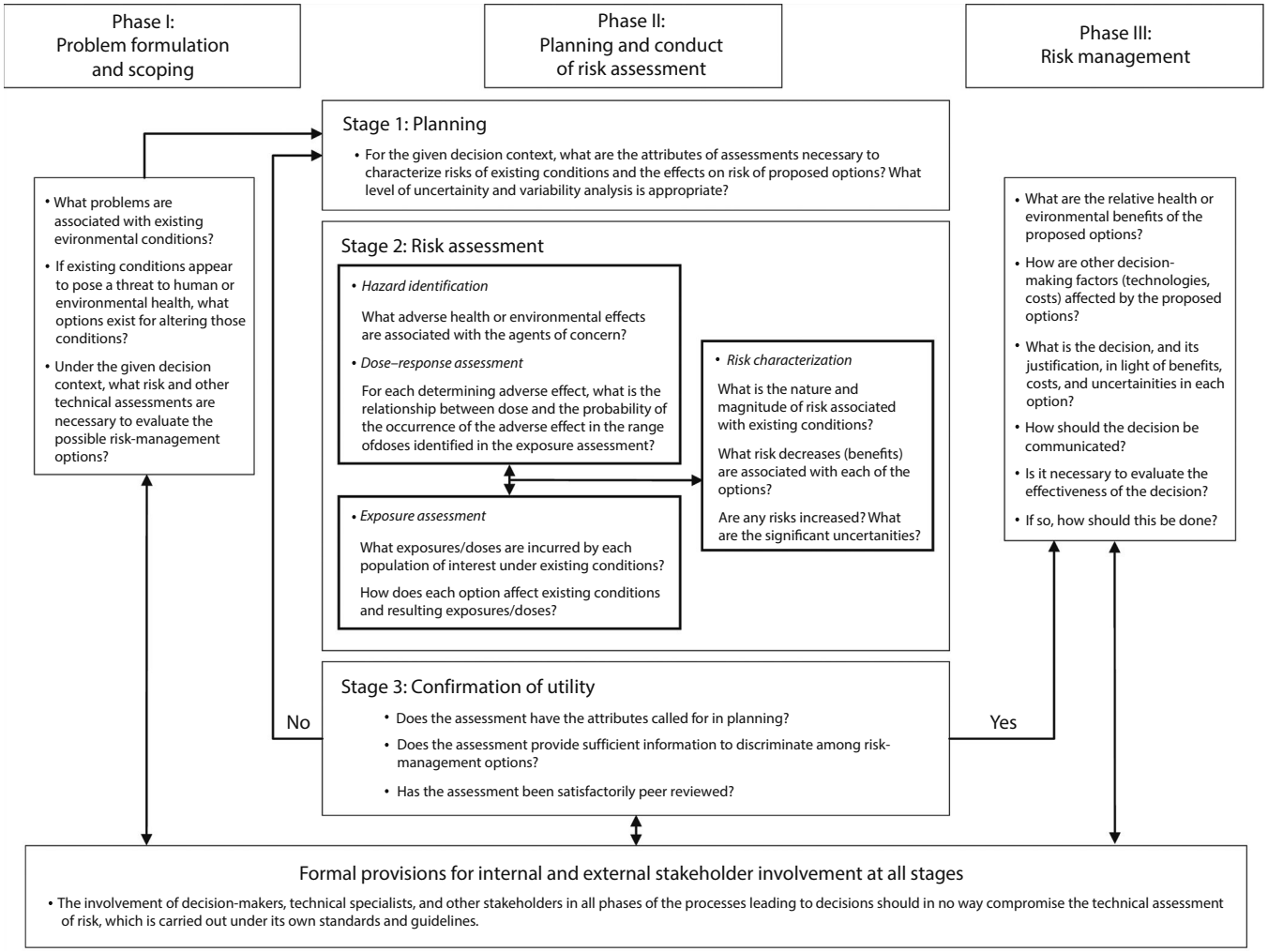


FIGURE 2.1 A framework for risk-based decision-making that maximizes the utility of risk assessment. (Reprinted from National Research Council, *Science and Decisions: Advancing Risk Assessment* [*The Silver Book*], National Academies Press, Committee on Improving Risk Analysis Approaches Used by the U.S. EPA, Washington, DC, Copyright 2009. With permission from National Academy of Sciences.)

As described earlier, risk assessment is commonly broken down into four components. The first component of risk assessment, *hazard identification*, involves an evaluation of whether a particular chemical can cause an adverse health effect in humans. The hazard identification process can be considered to be a qualitative risk assessment. It involves identifying the potential for exposure as well as the nature of the adverse effect expected. The types of information used in hazard identification include all categories described in the previous section. In hazard identification, the risk assessor must evaluate the quality of the studies (choice of appropriate control groups, sufficient numbers of animals, etc.), severity of the effect described, relevance of the toxic mechanisms in animals to those in humans, and many other factors.

The result is a scientific judgment that the chemical can, at some exposure concentrations, cause a particular adverse health effect in humans. The result is not a simple yes-or-no evaluation but a weight-of-evidence estimation of the likelihood that the particular chemical has the potential to cause the particular effect. For example, a recent animal study[42] demonstrated that intratracheal instillation (vs. inhalation) of diacetyl can result in lesions similar to bronchiolitis obliterans, a serious lung disease associated with workplace exposure to butter flavorings. Because diacetyl is a constituent in butter flavorings, the animal study added to the weight of evidence that it may be the relevant constituent for bronchiolitis obliterans in humans exposed to butter flavorings.

The hazard identification process has been codified mainly for carcinogens, as exemplified in the classification schemes from a variety of agencies, including the IARC,[43] EPA,[9] and OSHA.[44] These schemes are discussed in more detail later in this chapter.

Dose–response evaluation, the second component of the risk assessment process, involves quantitative characterization of chemical potency. In other words, the relationship between the dose of a chemical administered or received and the incidence or severity of an adverse health effect in the exposed population is evaluated. Characterizing the dose–response relationship involves understanding the importance of the intensity of exposure, the concentration × time relationship, whether a chemical has a threshold, and the shape of the dose–response curve. The metabolism of a chemical at different doses, its persistence over time, and an estimate of the similarities in disposition of a chemical between humans and animals are also important aspects of a dose–response evaluation. While the 1983 NAS report considered dose–response estimates mostly in terms of carcinogens, the evaluation of the dose–response relationships has long been a key component of pharmacology and toxicology for many chemicals.[32]

In *exposure assessment*, the third component of the risk assessment process, a determination is made as to the amount of a chemical to which humans are exposed. Data can be very limited for exposure assessment. Measures of chemicals in environmental media (such as air or soil) or in food may be available; however, the extrapolation of those levels to a dose received by humans has many uncertainties. Models exist that can describe the movement of chemicals through a particular medium, and assumptions can be made regarding inhalation, ingestion, or dermal contact rates and the bioavailability* of the chemical. This information can then be used to derive an estimate of the dose taken up by humans. Host factors, such as exercise, the use of certain consumer products, or the consumption of particular foodstuffs, will complicate the exposure assessment.

The use of biological monitoring—measurement of volatile organic chemicals in exhaled breath, for example[45]—as well as personal sampling devices, such as respirable particulate monitors,[46] represents ways in which the uncertainties of exposure assessment can be reduced. As noted earlier in this chapter, the CDC NHANES biomonitoring study provides significant new information on hundreds of chemicals measured in blood and in serum. In some cases, as with blood lead testing, such information can help reduce the uncertainty in quantifying exposure and extrapolating from exposure to dose. However, for other chemicals with more limited toxicological and epidemiological information, the biomonitoring information is difficult to interpret from the perspective of individual- or population-level risk.

The last stage of the risk assessment process, *risk characterization*, involves a prediction of the frequency and severity of effects in the exposed population. That is, the information from the dose–response evaluation (what dose is necessary to cause the effect?) is combined with the information from the exposure assessment (what dose is the population receiving?) to produce an estimate of the likelihood of observing the effect in the population being studied. Many risk assessments, particularly for cancer, performed in the regulatory arena produce a single-number estimate of risk (e.g., lung cancer risk of one in a million). These are often designed to represent the risk to the reasonable maximally exposed (RME) individual in a potentially exposed population.

Within any potentially exposed population, substantial *variability* exists in exposure rates, intake and uptake rates, and sensitivity to the effect. This variability is such that the risk to the most highly exposed and sensitive portion of the population may be orders of magnitude higher than the risks to the majority of the population. For example, some individuals in a given population may never eat locally caught fish, while other individuals may subsist on it. The fish intakes of these respective individuals will consequently vary by orders of magnitude. Information should generally be provided on both the risk to individuals and the aggregate risk of the exposed population. Point estimates of risk to a single individual in the population can be misleading when no information is provided to indicate whether that individual's exposure is typical of 50% or 0.001% of the exposed population.

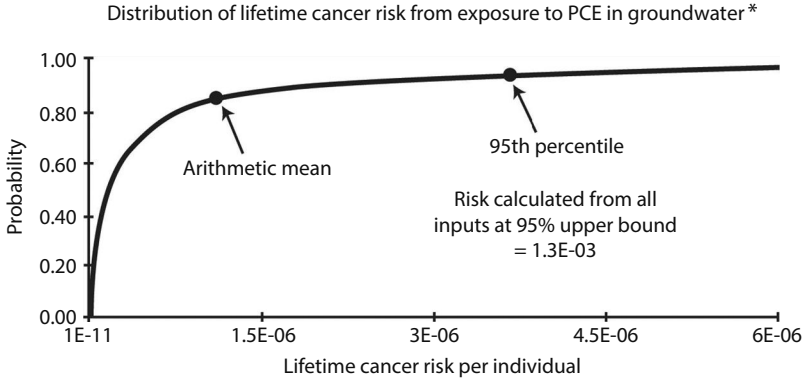
In addition to population variability, there is also significant *uncertainty* present in risk estimates, due to uncertainty in many of the risk assessment components (e.g., model and measurement error). It is critical that the

* In other words, absorption of a chemical in the relevant exposure medium as compared to absorption in the medium from the underlying toxicity study.

risk characterization step of the risk assessment process describes the biological and statistical uncertainties in the final estimation and identifies which component of the risk assessment process (hazard identification, dose–response, or exposure) involved the greatest degree of uncertainty. For example, the dose–response evaluation is generally highly uncertain. This is often due to the model error in extrapolating from animals to humans or short-term to lifetime exposures. Information may not be available to characterize the active species, mechanism of effect, effective dose, or absorption, metabolism, and excretion rates. Because the degree of uncertainty varies greatly among risk assessments for different chemicals, lack of consideration of uncertainty can lead to inappropriate levels of concern for different chemicals.

The Monte Carlo uncertainty analysis techniques have been applied to the risk assessment process as one method of attempting to more fully characterize the distribution of potential risks in a population. Rather than using single values to represent input parameters such as contaminant ingestion rates, body weights, and chemical potencies, the Monte Carlo analysis uses probability density functions to characterize the input parameters and produce a probability density function for risk.[48–51] Figure 2.2 shows a probabilistic exposure and risk model[52] produced by a Monte Carlo analysis for chloroform exposure in swimming pools. This analysis indicated that the 95% estimated cancer risk for males was nearly 3×10^{-4}, with the inhalation pathway being responsible for the bulk of the estimated risk. In contrast, the median risk was approximately tenfold lower, demonstrating a relatively skewed distribution of risks. Although these techniques provide more information on the distribution of potential risk than a single number risk estimate, they are limited by the availability of information with which to characterize the input probability density functions. Particularly uncertain are estimates of chemical potency, which can vary by orders of magnitude depending upon different interpretations regarding carcinogenic mechanisms.

The Committee on Risk Characterization, convened by the NRC, made recommendations for improving the risk characterization process in its 1996 book titled *Understanding Risk, Informing Decisions in a Democratic Society*.[53] Rather than simply presenting numerical risk results and associated uncertainties, a risk characterization also should convey the information in a clear and easily understandable way that is useful to risk managers in making informed decisions. In addition, risk characterization should address the concerns of interested and affected parties. Therefore, the rigorous scientific analyses involved in risk characterization must be performed in conjunction with frequent deliberations with all stakeholders. As explained by the NRC committee, "developing an accurate, balanced, and informative synthesis" involves "getting the science right,…getting the right science,…getting the right participation, [and] getting the participation right."[53] The EPA adopted similar values in its 1995 risk characterization guidance.[54]



* Based on the uncertainity in data used to calculate the source concentration, exposure, metabolized dose, and cancer potency

Selected input parameters and distributions

Parameter	Units	Distribution type	Min	Max	Arithmetic mean	Arithmetic std. dev.	Geometric std. dev.
PCE concentration in water	mg/L	Empirical			0.0003	0.00035	2.5
Breathing rate per unit body weight	m^3/kg-day	Lognormal			0.4	0.5	2.7
PCE metabolized cancer potency	kg-day/mg	Empirical			0.11	0.14	4.8
Exposure time in house	h/day	Uniform	8	20	14		
Skin permeability	m/h	Uniform	0.004	0.01	0.007		

FIGURE 2.2 Monte Carlo analysis of risk: tetrachloroethylene (PCE) in groundwater. (Reprinted with permission from McKone, T.E. and Bogen, K.T., Predicting the uncertainties in risk assessment: A California groundwater case study, *Environ. Sci. Technol*., 25, 1674. Copyright 1991 American Chemical Society.)

TOXICOLOGY INFORMATION USED IN THE REGULATORY PROCESS

Three main categories of scientific information are employed by agencies in the evaluation and regulation of toxic chemicals in the environment: (1) epidemiology, (2) controlled clinical exposures, and (3) animal toxicology. In vitro and in silico (i.e., computer-based such as structure–activity relationships) studies are typically used by regulatory agencies to support the interpretation of information from the three major categories and are only used occasionally as a primary source of information. However, with the need to generate data on more and more chemicals, it is expected that in vitro and in silico information may be used increasingly as a source of information, perhaps even of a primary nature, in risk assessment and risk management. This approach was examined in the 2007 NRC report, *Toxicity Testing in the Twenty-first Century: A Vision and a Strategy*.[55] This groundbreaking analysis describes identification and understanding of cellular response networks (as reflected in Figure 2.3) and how such networks are perturbed by chemical exposure as critical to enhancing the testing of chemicals and interpretation of such tests. High-throughput tests of cells and cell lines are expected to become more critical to the advancement of toxicology, with targeted testing in animals complementing such findings.

Epidemiology, studies of clinical exposures, and animal toxicology provide qualitatively different information, with unique advantages and limitations. Environmental epidemiology studies, which attempt to associate disease or other adverse health outcomes with an environmental exposure, have the advantage of measuring an effect in humans at exposure conditions that are by definition realistic. The first demonstration that benzene was a carcinogen came from epidemiological studies of rubber workers.[7] It was not until several years after these studies[57] that benzene was shown to cause cancer in animal studies. Studies of the London smog pollution episode in 1952 demonstrated that high levels of pollution from coal combustion could cause mortality, particularly in the very young, the elderly, and those individuals with preexisting cardiopulmonary disease.[58] Evaluation of similar effects in animal studies would be difficult, given the complexity of the exposure in London and the lack of good animal models for susceptible populations, such as asthmatics. In general, epidemiology has been particularly helpful in the evaluation of working environments or other environments where exposure concentrations are relatively high.

Several factors limit the use of epidemiological studies by regulatory agencies. One of the major limitations is the lack of well-defined exposure information, for both chemical species and actual concentrations. For example, the lack of accurate total exposure information limits the ability to quantify the effects of ambient air pollution in the United States. As an example, the Total Exposure Assessment, a Columbia and Harvard (TEACH) study quantified indoor, outdoor, and personal exposures of inner-city residents to a number of pollutants, including formaldehyde, dichlorobenzene, and benzene.[59] This study demonstrated that, depending upon the contaminant, outdoor exposure levels may underestimate, overestimate, or relatively well predict personal exposures. For example, due to the importance of indoor sources, outdoor measures of formaldehyde typically underestimated personal exposures. As discussed earlier in this chapter (e.g., the CDC biomonitoring study), the use of biological markers of exposure, such as measurements of arsenic levels in

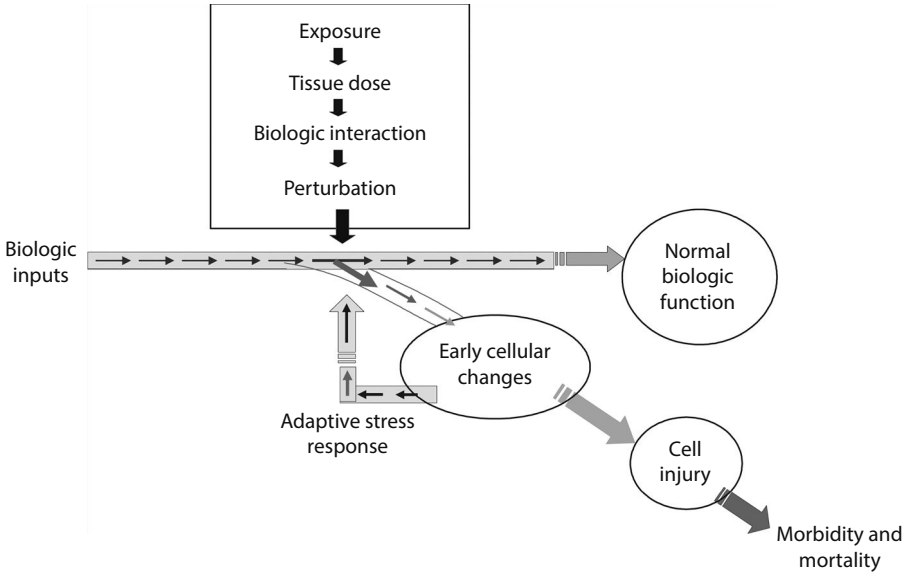


FIGURE 2.3 Biologic responses viewed as results of an intersection of exposure and biologic function. (Reprinted from National Research Council, *Toxicity Testing in the Twenty-first Century: A Vision and a Strategy*, National Academies Press, Washington, DC, Copyright 2007. With permission from National Academy of Sciences; Adapted from *Trends Biotechnol*., 23/3, Andersen, M.E., Dennison, J.E., Thomas, R.S., and Conolly, R.B., New directions in incidence-dose modeling, 122–127, Copyright 2005, with permission from Elsevier.)

urine or pesticides levels in blood, can provide more accurate information about exposure and help reduce uncertainties in the results of epidemiological studies. Still, the linking of some findings to risk may be limited by lack of adequate health information.

It is also difficult to define the causal element in epidemiological investigations, particularly when complex exposures are involved. For example, several indicators of pollution were measured during the London smog episodes that occurred between 1958 and 1972. Initial evaluations focused on the role of total particulate and SO_2 as causative agents for the elevated mortality levels; however, subsequent analyses of the London studies, as well as studies from other cities, indicate the importance of acid sulfates on mortality.[60] Large-scale epidemiological studies have demonstrated associations between various indicators of air quality, in particular particulate matter of 2.5 μm or less ($PM_{2.5}$), but debate continues regarding the nature and magnitude of causality due to the complexity of PM composition and difficulties in replicating the findings in animal studies (see, e.g., Valberg).[61]

Another limitation is that epidemiological studies are frequently of worker populations, and such studies can be difficult to apply to prediction of health effects in the general population. Occupational studies, in general, focus on healthy adult male workers. The general population is more heterogeneous than the worker population and, for some pollutants, may exhibit a greater range in susceptibility. In general, only more recent epidemiology studies have considered adverse health effects of chemicals specific to women and children, such as developmental, reproductive, or hormonally mediated effects, including cancer. An example of the limitations of occupational epidemiology involves studies of peripheral nerve function in lead-exposed workers that underestimates risk of lead exposure in young children, for whom the primary concern is neurobehavioral effects resulting from relatively low-level exposures.[62,63]

Epidemiology studies are frequently limited by the need for a relatively large increase in disease incidence (twofold or more), given the sample sizes generally available for such investigations. Enterline[64] notes that it would require a large population (1000 deaths, using the Peto model) to detect a 50% excess in deaths from lung cancer at an asbestos level of 2 fibers/cm^3 air.

Controlled clinical studies of humans exposed to pollutants address some of the difficulties of epidemiology studies. The exposures can be controlled and quantified, effects are observed in humans, and exposed populations can be chosen to consist of susceptible individuals, such as asthmatics or exercising individuals. Thus, changes in airway resistance in asthmatics exposed to SO_2 during exercise[65,66] have been important in the EPA's evaluation of the National Ambient Air Quality Standard (NAAQS) for SO_2,[67] because these effects reflect the response of the susceptible population, using an appropriate exposure concentration and a relevant averaging time. Given the subtlety of these changes (nonsymptomatic bronchoconstrictions) and the fact that they occur only in a selected subset of the general population (asthmatics constitute about 4% of the total population), these effects would not have been detectable in the general population.

One of the advantages of controlled clinical exposure studies—that they are performed with humans—is also a major limitation. Since these studies must be limited to short-term effects that are readily reversible, they cannot be used to evaluate the potential of a chemical to cause chronic disease. There is debate within the scientific and regulatory communities as to the circumstances under which data from controlled human studies, even considering that such studies present negligible risk to the participants, may be used by agencies in decision-making. For example, intentional dosing studies in human subjects (e.g., cholinesterase inhibition tests in individuals exposed to organophosphate pesticides) were considered by the NAS[68] to be acceptable under some circumstances (e.g., when there was a *reasonable certainty* of no adverse effects to the participants and the studies addressed an important question that could not be answered with animal studies); others[69] have questioned the appropriateness of such testing, particularly because of concerns over testing on children. In addition, because of the nature of the changes observed in clinical exposure studies, the health significance of the indicators is open to discussion. For example, perchlorate, a chemical used in treatment of certain thyroid disease and also found as a groundwater contaminant, inhibits iodide uptake at the thyroid. Studies in humans[70] have demonstrated that doses greater than 0.007 milligrams of chemical per kilogram of body weight per day (mg/kg-day) are required to inhibit iodide uptake. However, the inhibition of iodide uptake must be of sufficient magnitude and for sufficient duration (e.g., in adults, up to 75% inhibition over several months may be required to inhibit thyroid hormone synthesis) before impacts on thyroid hormone synthesis (the physiologically relevant effect) may occur.[71] A recent controlled human exposure by Braverman and coworkers of volunteers exposed to perchlorate at daily doses up to 3 mg for up to 6 months demonstrated no impact on thyroid function. Thus, the clinical significance of low-level inhibition of iodide uptake for a modest period of time may be limited.

Another issue with the use of clinical studies is that, although some susceptible populations (e.g., mild asthmatics) can be tested, individuals with a greater degree of impairment (e.g., asthmatics who require continual medication) are usually not considered to be appropriate subjects for these studies because of the greater potential for harm during exposure. Later sections in this chapter address the questions of severity of effect on susceptible populations in greater detail.

Animal toxicology studies constitute the third major source of information for assessing the toxicity of chemicals. Animal toxicology studies allow the investigator the greatest degree of control over the exposure conditions, population exposed, and effects measured. One can readily evaluate

subtle effects of acute and chronic exposure using this type of study. For example, hexavalent chromium (CrVI) in water has been shown to induce gastrointestinal tumors in rodents via the ingestion pathway.[72] Recent studies of rodents have yielded important insights on initial events in tumor production, including evaluating the role of oxidative injury and assessing progression of histopathological lesions in relationship to changes in gene transcription. These mechanistic findings would be very difficult to identify with other experimental approaches, and they provide relevant information in the extrapolation from the high-dose rodent studies to much lower human exposure levels.[73] In animal experiments, the ability to manipulate the experimental conditions permits the evaluation of many variables on the response to toxic chemicals. For example, the role of metabolism in susceptibility to polycyclic aromatic hydrocarbon-induced carcinogenesis has been evaluated in studies of genetic variants in mice.[74,75] Such studies can be important in predicting modifiers of toxicity in humans and identifying susceptible human populations.

The use of *omics*, including toxicogenomics (i.e., changes in gene transcription, protein synthesis) and metabolomics (i.e., changes in metabolite profiles) as a consequence of toxicant exposure in animals and cells in vitro can be quantified and measured even at a cellular level, allows for even more refined understanding of molecular responses to chemicals in animal models (see, e.g., NAS[12]). Toxicogenomics may allow for a better characterization of the linkage between chemical exposure and toxicological effects on a number of levels, such as identifying biomarkers of susceptibility, improving the understanding of a chemical's MoA, and identifying changes prior to histopathological events. For example, transcriptional changes have been observed after low- and high-dose acetaminophen exposure, prior to the development of histopathological changes.[76,77] Recent efforts, as exemplified in the aforementioned 2007 NRC report, seek to further the use of such methodologies not only in toxicity testing but also in the risk assessment process itself. An example of such an application is the work by Bercu and coworkers.[78] These investigators identified a point of departure (POD) (i.e., the most sensitive effect dose or no-effect dose) for two nongenotoxic carcinogens based on genomic changes in rodents treated for seven days or less. The changes, which were consistent with the proposed MoA, identified threshold doses potentially applicable to a risk assessment framework yet within a much shorter time frame than associated with animal bioassays.

The limitations of animal studies fall into two broad categories: (1) those due to uncertainties in extrapolating from animals to humans and (2) those due to uncertainties in extrapolating from the high exposures in animal studies to the lower exposures typically experienced by humans. Interspecies extrapolation is complicated by the greater homogeneity of laboratory animals than humans, controlled conditions of housing and diet, innate genetic factors, and other variables. The relevance of di-2-ethylhexyl phthalate-induced hepatocarcinogenesis in rodents to humans has been questioned on the basis of differences in peroxisomal proliferation in the liver in the two species as a consequence of differences in peroxisome proliferator-activated receptor (PPAR) alpha binding.[79,80] Similarly, high exposure concentrations typically used in animal studies may result in saturation of detoxification pathways and thus may produce effects that are not relevant to effects produced at ambient exposure concentrations, where detoxification pathways are not saturated. Increased numbers of macrophages and impairment of alveolar clearance are observed in rats exposed to relatively high concentrations of diesel particulates.[81] The significance of such particulate overload to humans, who are exposed to ambient levels of diesel particulates much lower than those employed in the animal studies, remains a source of discussion and debate.[82]

Historically, in vitro studies, analysis of structure activity relationships, and other short-term test procedures have been used to help set priorities for chemical testing. For example, structure activity relationships have been used to predict mutagenicity, lethality, and carcinogenicity.[83–85] This type of information can be useful, for example, in selecting compounds for longer-term testing in animals or eliminating chemicals being considered for potential industrial or pharmaceutical applications due to toxicological concerns.

Short-term tests have typically been used indirectly in the regulatory process to support decision-making rather than as a decision-making basis per se. For example, evidence that a chemical is a point mutagen in an in vitro test system might be used to support the classification of a chemical as a possible human carcinogen or the use of a linear dose–response model for carcinogenesis. More recent efforts, as reflected in the 2007 NRC report *Toxicity Testing in the Twenty-first Century*, seek to use short-term tests, such as characterizing toxicity pathways, more directly in the risk assessment process.

Metabolism, pharmacokinetic, and mechanistic studies can also provide information to reduce uncertainties in the use of toxicology information. Metabolic studies showing that a critical reactive metabolite in rodents is also formed in humans could reduce uncertainties in extrapolating from animals to humans, while mechanistic studies could indicate whether a subtle effect observed in a clinical study is a precursor for later, more serious health endpoints—and therefore of concern as a biomarker of effect.

A summary comparing the differences between epidemiology, controlled clinical exposure, and animal toxicology studies is provided in Tables 2.2 through 2.4.

It can be concluded from the preceding discussion that there is no *best* source of information for regulatory agencies. The rational approach, therefore, is to examine all available sources of reliable information in the evaluation of toxic chemicals. Some kinds of information may be especially useful in *hazard identification*, the likelihood that a chemical will be toxic to humans, whereas other types of information will be more appropriately applied to the estimation of the *dose–response relationship.*

TABLE 2.3
Advantages and Disadvantages of Controlled Clinical Studies

Advantages	Disadvantages
Well-defined, controlled exposure conditions	Costly
Responses measured in humans	Relatively low exposure concentrations and short-term exposures
Potential to study subpopulations (e.g., asthmatics)	Limited to relatively small groups (usually <50 individuals)
Ability to measure relatively subtle effects	Limited to short-term, minor, reversible effects
	Usually most susceptible group not appropriate for study

TABLE 2.4
Advantages and Disadvantages of Animal Toxicological Studies

Advantages	Disadvantages
Readily manipulated exposure conditions	Uncertainties in relevance of animal response to human exposure
Ability to measure many types of responses	Controlled housing, diet, etc., of questionable relevance to humans
Ability to assess effect of host characteristics (e.g., gender, age, genetics) and other modifiers (e.g., diet) of response	Exposure concentrations and time frames often very different from those experienced by humans
Potential to evaluate mechanisms	

EVALUATION OF CARCINOGENS

Background

The public demand for zero risk has made the regulation of carcinogens a formidable challenge. Within the scientific community, there is ongoing debate on how to define a potential human carcinogen, as well as on how to estimate cancer risks under practical conditions of chemical exposure. This uncertainty is due largely to the fact that mechanisms of carcinogenesis for many chemicals are still poorly understood, and different carcinogens act in different ways to induce cancer. The task of regulating carcinogens has been complicated, rather than simplified, by many of the mechanistic discoveries of recent years. The simple picture of the 1950s, when only a relatively small number of chemicals were thought to be carcinogens, has been replaced by the realization that chemical carcinogenesis takes place in multiple stages, some with reversible steps, which have different dose–response relationships. Essential nutrients and hormones can be carcinogenic in some circumstances. The same chemical can promote or inhibit carcinogenesis, depending on the circumstances of exposure.[86,87] Public pressure to regulate carcinogens, even where very little toxicological information exists, has in many instances compelled regulatory agencies to treat carcinogens as though they all act by the same mechanisms, even as it has become apparent that they do not.

From a public health standpoint, regulatory agencies have generally regulated carcinogens at exposure levels that reflect a very low probability of tumor production (e.g., excess cancer risk of 1–100 per million exposed). However, for practical reasons, it is impossible to conduct animal studies of a size that would allow observation of effects following treatment at such low doses. The practice has therefore been to conduct animal studies at relatively high dose levels and then extrapolate the results from high to low dose and from animals to humans. Thus, the chronic animal bioassay results, extrapolation from high to low doses, and extrapolation across species are used to derive potency factors (i.e., indicators of carcinogenic potency) for carcinogens. These potency factors enable one to relate an estimated chemical dose in humans to an upper bound probability of tumor occurring as a result of that dose. Even with established human carcinogens, extrapolation procedures must still be used to extrapolate carcinogenic response from high to low dose (e.g., workplace to ambient) or from one type of exposure condition (e.g., intermittent, subchronic) to another (e.g., continuous, chronic).

This section on carcinogens first provides some basic information on mechanisms of carcinogenesis, then it describes some of the key issues that agencies address in the interpretation and application of scientific data on carcinogens. These issues fall into the categories of *hazard identification* and *dose–response assessment*.[32] Hazard identification for carcinogens addresses two questions: (1) What is the evidence that a particular chemical is an animal carcinogen? and (2) What is the likelihood that an animal carcinogen is a human carcinogen (and under what circumstances of exposure pathway and dose)? Dose–response assessment has traditionally attempted to determine the probability of tumor production given a particular exposure or dose level (i.e., assuming a low-dose, linear response).* The dose–response assessment section of this chapter discusses mathematical models used to extrapolate from high to low doses, physiologically based pharmacokinetic (PBPK) modeling to relate administered and effective doses in animals and humans, and issues concerning the relationship between effective dose and response.

Mechanisms of Carcinogenesis

Carcinogenesis is generally understood to be a multistage process that has, until recently, been described as a relatively linear process involving the initiation, promotion, and progression of normal cells into neoplastic cells, although, as discussed later in this section, this framework is likely over simplistic in a number of cases. In the initiation, promotion, and progression model, chemicals can act at one or more of

* As discussed in later sections of this chapter, for carcinogens that act through a threshold mechanism, such probabilistic low-dose models are not appropriate.

these stages; action can be through direct (e.g., mutagen) or indirect (e.g., immune suppression) mechanisms. Initiation is the first step in the process of carcinogenesis and is generally understood to be a permanent and irreversible event involving DNA mutation. Some genotoxic agents are considered to be capable of initiating activity and thus having the potential to begin the transition from normal to cancer cells. Thus, genotoxic (particularly mutagenic) agents have been considered to act via a nonthreshold mechanism; this belief has formed the basis for linear extrapolation of effects seen at high doses down to low doses. Inferences as to the absence of a threshold for initiating agents come from the study of mutations that result from these agents. In addition, some studies investigating the number of preneoplastic focal lesions induced by an initiating agent did not find a measurable threshold.[88] Certain chemicals (e.g., aflatoxin B_1, diethylnitrosamine, tobacco smoke) are considered to be complete carcinogens—in other words, capable of initiation, promotion, and progression. Potential factors modifying the efficiency of initiation include rates of cell division and DNA synthesis, rate of metabolism of a chemical to its active form, or rate of metabolic detoxification. (It should be noted that, due to the existence of repair mechanisms and other factors that reduce or eliminate responses at low exposure levels, even the no-threshold concept may not be applicable to all mutagenic carcinogens. This point is discussed subsequently.)

The second stage of carcinogenesis, promotion, has been characterized by clonal expansion of the initiated cells. Promoting agents can act by various mechanisms to increase rates of cell proliferation or decrease rates of cell death. For example, cell proliferation can be induced by cytotoxic agents or mitotic agents. Interference with intercellular communication may also be involved in clonal expansion of initiated cells.[89] An important feature of this stage is its reversibility and, in some cases, the existence of a threshold for the effect. In the standard tumor initiation/promotion model, withdrawal of the promoting agent halts the development of tumors. The promotion stage can also be modulated by environmental factors, including frequency of dosing, age of test animal, and diet.[88] Promoting agents are generally thought to exhibit a threshold (or inflection point) in the dose–response curve. Examples of promoting agents include hormones, alcohol, and dietary fat.

More recent studies indicate that the earlier paradigm is, as noted earlier, likely to be overly simplistic in a number of cases. There are situations where available data on certain tumors do not comport with the linear initiation–promotion–progression model. For example, as described by Cohen and Arnold,[90] evidence from cancer studies in animals and humans indicates that there are many malignancies that do not occur with an intermediate stage of benign proliferation. While some chemical carcinogens may be characterized as DNA reactive (i.e., initiators in the paradigm described earlier) and others as non-DNA reactive (i.e., promoters in the paradigm), in actuality, DNA-reactive chemicals can, at certain doses, induce cell proliferation. In some cases, as with the example of formaldehyde, it is this cell proliferation that is more relevant to carcinogenicity.[90] Other chemicals may act primarily through non-DNA-reactive mechanisms that, by virtue of increasing the actual number of cell replications in a relevant target cell population, result in increased mutational events simply by increasing the size of the cell population.[90] For example, the rat-specific bladder carcinogen, dimethylarsinic acid (DMA), induces cytotoxicity to the urothelial cells of the bladder followed by necrosis, cell regeneration, proliferation, and hyperplasia, leading to tumor production. In this case, genotoxicity occurs subsequent to cytotoxicity in both dose and time and appears to have a limited, if any, relationship to carcinogenicity. Thus, with respect to DMA, genotoxicity would not be indicative of a linear dose–response relationship.[91] In the case of hormonal carcinogens, tumors may arise as a consequence of prolonged stimulation of cell division in which genetic damage occurs as a secondary event.[92,93]

Hazard Identification

The question of how to decide whether a particular chemical is a potential human carcinogen is currently the subject of considerable scientific debate. It is an important question because the act of labeling some chemicals, but not others, *carcinogens*, can have important regulatory and societal implications.[94] The regulatory paradigm, whereby chemicals are regulated either as carcinogens or as noncarcinogens, requires that the question of whether a particular chemical is a carcinogen typically be answered with a *yes* or *no*. In the United States, most regulatory agencies have historically regulated all carcinogens as though they operate via the same no-threshold mechanism. However, the various mechanisms of tumor formation are not all consistent with the mechanistic assumptions that form the basis of the regulatory framework for carcinogens. A chemical may be carcinogenic via certain routes of exposure and not others or only above certain dose levels. More flexible classification approaches have been developed[95] that allow the incorporation of greater understanding of MoA into the classification process.

The next section describes current classification approaches, but the reader is reminded that current scientific debate on these schemes continues to fuel new approaches. Regulatory agencies generally classify potential carcinogens based on an evaluation of both human and animal studies, as well as supporting information from short-term tests for mutagenicity and structure–activity relationships. Because human evidence exists for relatively few chemicals, animal studies typically provide most of the available information about the potential of a chemical to be carcinogenic to humans.

Animal Studies

The evidence that a chemical is an animal carcinogen frequently derives from long-term animal bioassays. Such studies usually consist of exposing groups of about 50 animals (typically rats or mice) to two to three concentrations of a chemical over the lifetime of the animals. Sex- and

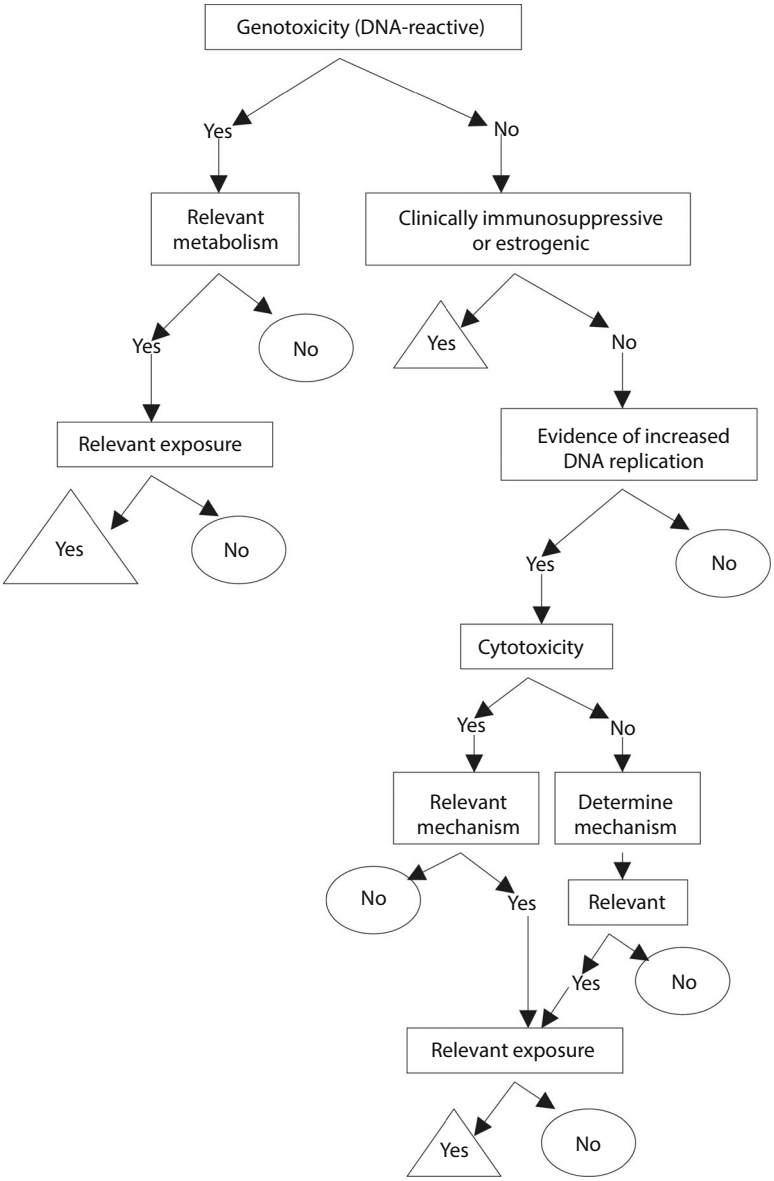


FIGURE 2.4 A proposed guide for evaluating the potential carcinogenicity of chemicals. Each box poses an evaluation to be performed. If the sequence results ultimately in a *no* that is in a circle, there is no (or negligible) carcinogenic risk in humans. If the sequence results ultimately in a *yes* that is in a triangle, it poses a presumptive human carcinogenic risk. (From Cohen, S.M., *Toxicol. Sci*., 80(2), 225, 2004. With permission of Society of Toxicology.)

age-matched unexposed animals constitute the control group. At the termination of the bioassay, the animals are killed and the number of tumor-bearing animals and the number and type of tumors per animal are quantified. All tumors are recorded, including those that are present as a consequence of spontaneous processes. Interim examinations may be performed, particularly on animals that appear moribund. Alternatives to the standard bioassay are being developed. For example, genetically engineered strains of mice in which tumor suppressor genes are inactivated (knockouts) or activated oncogenes are introduced (transgenics)[96] may allow detection of carcinogens in shorter periods of time than the standard bioassay; however, use of transgenics and knockouts remains limited for a number of reasons, including limited histopathological analysis. More recently, based on MoA considerations within the multistage model of carcinogenesis concept, Cohen has proposed a testing framework, described in Figure 2.4, that uses shorter-term exposure. This framework also evaluates key parameters for carcinogenesis, such as DNA reactivity and cell proliferation.[97] This and similar approaches, particularly when combined with tissue-specific markers, have the potential to act as an initial screen for carcinogenicity under much shorter time frames than the typical 2-year bioassay.

Maximum Tolerated Dose

Dose selection plays a key role in the design and interpretation of the animal bioassay. Animals are typically exposed

at two dose levels: the maximum tolerated dose (MTD) and one-half the MTD. In recent years, one-quarter of the MTD has also been used. The MTD is predicted from subchronic toxicity studies as the dose "that causes no more than a 10% weight decrement, as compared to the appropriate control groups, and does not produce mortality, clinical signs of toxicity or pathologic lesions (other than those related to a neoplastic response) that would be predicted [in the long-term bioassay] to shorten an animal's natural lifespan."[98] The MTD is not a nontoxic dose, but is expected to produce some level of acceptable toxicity to indicate that the animals were sufficiently challenged by the chemical. The MTD has been justified as a means of increasing the sensitivity of an animal bioassay involving limited numbers of animals so as to be able to predict risks in large numbers of humans.[99]

An objection to the use of MTDs has been that metabolic overloading may occur at high dose levels, leading to an abnormal handling of the test compound.[100] For example, toxic metabolites could be produced as a consequence of saturation of detoxification pathways. Organ toxicity could occur that might not happen at lower concentrations of the chemical,[101] particularly at those concentrations to which humans are typically exposed. Thus, it has been argued that nongenotoxic agents that are determined to be positive in rodent carcinogenicity bioassays may exert their carcinogenicity via target organ toxicity and subsequent cell proliferation and should not be assumed to be carcinogenic at low doses.[102]

Ames and coworkers[103,104] have suggested that target organ toxicity and subsequent mitogenesis is responsible for the fact that over half of all chemicals tested in chronic bioassays at the MTD are determined to be carcinogens in rodents. They observed that both genotoxic and nongenotoxic agents tested at the MTD cause increased rates of mitogenesis, thus increasing the rate of mutation. For several chemicals, induction of tumors was more strongly correlated with cell division than with DNA adducts or mutagenic activity. Others have reported that cancer potency and MTD are inversely correlated and, consequently, the potency estimate is simply an artifact of the experimental design.[105]

Gaylor[106] noted that, given sufficient animals (e.g., about 200 per group), it is estimated that about 92% of all chemicals tested at the MTD would yield a positive response at one or more tumor sites in rats or mice. Gaylor observes that "this MTD bioassay screen is not distinguishing between true carcinogens and non-carcinogens." The author further suggests a common mechanistic explanation for this result—that is, for nongenotoxic carcinogens in particular, the MoA involves cytotoxicity followed by regenerative hyperplasia. Thus, the relevant question is not so much whether a chemical causes cancer at the MTD (i.e., is a chemical a carcinogen) but at what dose does the chemical induce cancer.

The EPA[95] cancer guidelines note that bioassay results at doses that exceed the MTD can be rejected if toxic damage to target organs compromises study interpretation. The reason is that dosing above the MTD in a study may result in tumor production secondary to tissue damage, rather than a direct carcinogenic influence of the agent tested. Thus, use of information from testing at fractional doses of the MTD is expected to yield results that are more relevant to human risk. Importantly, the use of information on MoA, metabolism, and other biological processes is being used as a more scientifically grounded approach to dose selection.[107]

Other Issues in Hazard Identification

Another key issue in the evaluation of animal bioassays is the analysis of the tumors themselves. Considerations include the categorization of benign tumors and whether tumor analysis should be site-specific or based on all sites. The weight to be ascribed to benign tumors is based on a number of factors. For example, the IARC considers whether benign tumors have the same cellular origin as malignant tumors arising from the same organ, which may represent an earlier stage in progression to malignancy.[108] In this example, benign tumors would be given greater weight than in a situation where such tumors occur in the absence of any malignancy. It should be noted that findings of benign-only tumors may merit further investigation nonetheless.

It should be emphasized that chemical carcinogens produce specific types of tumors that are characteristic of that chemical, exposure route, and dose. There is no convincing evidence of a chemical agent that, in animal bioassays, increases overall tumor incidence, rather than increases at specific sites. Thus, the classification of 2,3,7,8-tetrachlorodibenzodioxin as a human carcinogen based on increase in all cancers as observed in some epidemiology studies[109–111] has been questioned on the basis of biological plausibility.[112]

Interestingly, reductions in tumor incidence are frequently observed in the same cancer bioassays in which tumor increases are observed. Linkov and coworkers[87] concluded that the anticarcinogenic effects observed in rodent bioassays are not explained by random effects. The basis for the reduction in tumors is not known and could be a consequent perturbation in the animal's physiology. These observations lend credence to the concept that animal bioassays must be interpreted with special attention as to whether biological phenomena are induced at high doses that may not occur (or occur with a relatively much lower frequency) at low doses. A similar observation is found in the evaluation of some human carcinogens, in particular those that act through hormonal processes. For example, oral contraceptives are associated with an increased risk of breast cancer but a decreased risk of ovarian and endometrial cancer (Table 2.5). Anticarcinogenic properties of carcinogens are typically not considered as part of the regulatory process for carcinogens.

Approaches to Interpretation of Carcinogenicity Findings

Agency Classification Schemes

The IARC, EU, EPA, NTP, German Commission for Investigation of Health Hazards, Health Canada, and ACGIH have developed classification schemes for carcinogens based on a weight-of-evidence or strength-of-evidence evaluation of available human and animal studies. These seven classification systems are shown in Table 2.6.

TABLE 2.5
Chemicals, Industrial Processes, and Environmental Factors Associated with Cancer Induction in Humans: Target Organs and Main Routes of Exposure in Humans and Degree of Supporting Evidence in Animals, According to IARC

	Humans		Animals
Chemical or Industrial Process	**Main Type of Exposure[a]**	**Target Organ(s)/Cancer Type[b]**	**Degree of Evidence for Carcinogenicity**
Acetaldehyde associated with the consumption of alcoholic beverages; and the consumption of alcoholic beverages[115]	Cultural	Esophagus, upper aerodigestive tract combined (acetaldehyde), oral cavity, pharynx, larynx, colorectum, liver, female breast; lack of carcinogenicity for kidney and non-Hodgkin's lymphoma	Sufficient for ethanol and acetaldehyde
Aflatoxins[116]	Environmental, occupational	Liver	Sufficient; limited evidence for alflatoxin B2; inadequate for alflatoxin G2
Aluminum production[116]	Occupational	Lung, bladder	Sufficient for the carcinogenicity of airborne particulate polynuclear organic matter
4-Aminobiphenyl[116]	Occupational	Bladder	Sufficient
Arsenic, arsenic compounds, and gallium arsenide[c,117]	Occupational, medicinal, and environmental	Bladder, skin, lung (liver, hematopoietic system, gastrointestinal tract, prostate, kidney)	Sufficient for DMA, calcium arsenite, and sodium arsenate; limited or inadequate for certain other forms of arsenic
Asbestos (all forms)[117]	Occupational	Lung, mesothelioma, larynx, ovary (pharynx, stomach, colorectum)	Sufficient
Auramine manufacture and auramine production[116]	Occupational	Bladder	Sufficient (auramine); no data for auramine production
Azathioprine[118]	Medicinal	Non-Hodgkin's lymphoma, skin	Sufficient
Benzene[116]	Occupational, environmental	Acute myeloid and acute nonlymphocytic leukemia (acute lymphocytic lymphoma, chronic lymphocytic lymphoma, multiple myeloma, non-Hodgkin's lymphoma)	Sufficient
Benzidine[116]	Occupational	Bladder	Sufficient
Benzidine, dyes metabolized to[116]	Occupational	(Bladder)[b,e]	Sufficient
Benzo[*a*]pyrene[116]	Occupational, environmental	(Lung)[d]	Sufficient
Beryllium and beryllium compounds[117]	Occupational	Lung	Sufficient
Betel quid (with and without tobacco), areca nut[115]	Cultural	Oral cavity, esophagus (with and without tobacco), pharynx (with tobacco)	Sufficient for betel quid with and without tobacco and areca nut; limited evidence for pan masala; evidence suggesting lack of carcinogenicity for betel leaf
Bis(chloromethyl) ether and chloromethyl methyl ether (technical grade)[116]	Occupational	Lung	Sufficient for bis(chloromethyl) ether; limited for chloromethyl methyl ether
Busulfan[118]	Medicinal	Acute myeloid leukemia	Limited
1,3-Butadiene[116]	Occupational	Hematolymphatic organs	Sufficient for 1,3-butadiene and diepoxybutane metabolite
Cadmium and cadmium compounds[117]	Occupational	Lung (kidney, prostate)	Sufficient for cadmium compounds; limited for cadmium metal
Chlorambucil[118]	Medicinal	Acute myeloid leukemia	Sufficient
Chlornaphazine (*N,N*-Bis(2-chloroethyl)-2-naphthylamine)[118]	Medicinal	Bladder	Limited
1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (Methyl-CCNU; semustine)[118]	Medicinal	Acute myeloid leukemia	Limited
Chromium (VI) compounds[117]	Occupational	Lung	Sufficient
Ciclosporin[118]	Medicinal	Skin, non-Hodgkin's lymphoma, and cancer at multiple other sites	Limited
Clonorchis sinensis (infection with)[119]	Environmental	Cholangiocarcinoma	Limited

TABLE 2.5 (continued)
Chemicals, Industrial Processes, and Environmental Factors Associated with Cancer Induction in Humans: Target Organs and Main Routes of Exposure in Humans and Degree of Supporting Evidence in Animals, According to IARC

	Humans		Animals
Chemical or Industrial Process	**Main Type of Exposure[a]**	**Target Organ(s)/Cancer Type[b]**	**Degree of Evidence for Carcinogenicity**
Coal, indoor emissions from household combustion of[115]	Environmental, cultural	Lung	Sufficient for combustion of coal and coal-derived soot extract
Coal gasification[116]	Occupational	Lung	Sufficient
Coal-tar distillation[116]	Occupational	Skin, including cancer of scrotum (lung)[b,e]	Sufficient
Coal-tar pitch[116]	Occupational, environmental	Lung, bladder (limited)	Sufficient
Coke production[116]	Occupational	Lung	Sufficient
Cyclophosphamide[118]	Medicinal	Bladder, acute myeloid leukemia	Sufficient
Diethylstilboestrol[118]	Medicinal	Cervix/vagina, breast (testis, endometrium)	Sufficient
Epstein–Barr virus[119]	Environmental	Burkitt lymphoma, non-Hodgkin's lymphoma, Hodgkin's lymphoma, extranodal NK/T-cell lymphoma (nasal type), and cancer of the nasopharynx (lymphoepithelioma-like carcinoma, gastric cancer)	No data
Erionite[119]	Environmental	Mesothelioma	Sufficient
Estrogen-only menopausal therapy[118]	Medicinal	Endometrium, ovary (breast), colorectum (protective)	Sufficient
Estrogen–progestogen menopausal therapy (combined)[118]	Medicinal	Breast, endometrium	Limited for estradiol plus progesterone and for conjugated equine estrogens plus medroxyprogesterone acetate
Estrogens-progestogen oral contraceptives[118]	Medicinal	Endometrium (protective effect), breast, cervix, liver, ovary (protective), colorectum (protective)	Sufficient
Ethanol in alcoholic beverages[115]	Cultural	Esophagus, oral cavity, pharynx, larynx	Sufficient
Ethylene oxide[116]	Occupational	Lymphatic and hematopoietic systems (limited evidence)	Sufficient
Etoposide (with cisplatin and bleomycin)[118]	Medicinal	Acute myeloid leukemia	No data for combination; inadequate evidence for etopside alone
Formaldehyde[116]	Occupational, environmental	Nasopharyngeal, leukemia[f] (sinonasal—limited evidence)	Sufficient
Helicobacter pylori[119]	Environmental	Stomach (esophagus—protective effect)	Sufficient
Hematite mining (underground; exposure to radon; listed under internalized α-particle emitting radionuclides)[120]	Occupational	Lung	No data
Hepatitis B virus (HBV)[119]	Environmental	Liver (biliary tract, non-Hodgkin's lymphoma)	No data
Hepatitis C virus (HCV)[119]	Environmental	Liver, non-Hodgkin's lymphoma (biliary tract)	No data
Human immunodeficiency virus type 1[119]	Environmental	Cervix, anus, and conjunctiva; Kaposi sarcoma, non-Hodgkin's lymphoma, and Hodgkin's lymphoma (vulva, vagina, penis, liver, skin [nonmelanoma])	No data
Human papillomaviruses[c,119]	Environmental	Cervix, vulva, vagina, penis, anus, oral cavity, oropharynx, tonsil (larynx)	None
Human T-cell lymphotropic virus Type 1[119]	Environmental	Adult T-cell leukemia/lymphoma	Sufficient
Internally deposited α-particle emitting radionuclides (including individually listed Group 1: radium-224, radium-226, radium-228, radon-222, plutonium-239, thorium-232, and their decay products)[c,120]	Environmental, occupational	Lung, bone, liver, leukemia, bile ducts, gall bladder, paranasal sinuses and mastoid process (pancreas, prostate)	Sufficient

(*continued*)

TABLE 2.5 (continued)
Chemicals, Industrial Processes, and Environmental Factors Associated with Cancer Induction in Humans: Target Organs and Main Routes of Exposure in Humans and Degree of Supporting Evidence in Animals, According to IARC

	Humans		Animals
Chemical or Industrial Process	**Main Type of Exposure[a]**	**Target Organ(s)/Cancer Type[b]**	**Degree of Evidence for Carcinogenicity**
Internally deposited β-particle emitting radionuclides[c] (including individually listed fission products [including strontium-90] phosphorus-32 as phosphate, and iodine-131)[120]	Environmental, occupational	Thyroid, leukemia (digestive tract, salivary gland, bone and soft tissue sarcoma)	Sufficient for following β-emitting radionuclides: ^{3}H, ^{32}P, ^{90}Sr, ^{90}Y, ^{91}Y, ^{131}I, ^{137}Cs, ^{144}Ce, ^{147}Pm, ^{228}Ra; limited for calcium-45 and lutetium-177
Iron and steel founding[116]	Occupational	Lung	No data
Isopropyl alcohol manufacture (strong acid process)[116]	Occupational	Nasal cavity	No data
Kaposi sarcoma herpesvirus (KSHV)[119]	Environmental	Kaposi sarcoma, primary effusion lymphoma (multicentric Castleman disease), multiple myeloma (protective effect)	No data
Leather dust[117]	Occupational	Nasal cavity, paranasal sinuses	No data
Magenta and magenta production[116]	Occupational	Bladder	Sufficient for CI Basic Red 9
Melphalan[118]	Medicinal	Acute myeloid leukemia	Sufficient
8-Methoxypsoralen (methoxsalen) plus UVA radiation[118]	Medicinal	Skin	Sufficient; limited evidence for methoxsalen alone
4,4-Methylenebis(2-chloroaniline) (MOCA)[116]	Occupational	(Bladder)[e]	Sufficient
Mineral oils, untreated or mildly treated[116]	Occupational	Skin (observed in the scrotum)	Sufficient for untreated vacuum distillates, acid-treated oils, and aromatic oils; mildly hydrotreated oils [class 4]; and used gasoline-engine oil
MOPP and other combined chemotherapy including alkylating agents[118]	Medicinal	Lung, acute myeloid leukemia	No data
Mustard gas (sulfur mustard)[116]	Occupational	Lung (larynx—limited)	Limited
N′-nitrosonornicotine (NNN) and 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)[117]	Environmental, cultural	(Lung)	Sufficient
2-Naphthylamine[116]	Occupational	Bladder	Sufficient
Neutron radiation[120]	Medicinal, Occupational, Environmental	Unclear[g]	Sufficient
Nickel and nickel compounds[c,117]	Occupational	Nasal sinus, nose, lung	Sufficient
Opisthorchis viverrini[119]	Environmental	Biliary tract	Limited
ortho-Toluidine[116]	Occupational, environmental	Bladder	Sufficient
Painters (occupational exposures as)[116]	Occupational	Lung, bladder, mesothelioma (childhood leukemia)	No data
Phenacetin (in analgesic mixtures)[118]	Medicinal	Renal pelvis, ureter (phenacetin and mixtures containing phenacetin)	Sufficient for phenacetin; limited for analgesic mixtures containing phenacetin
Plants containing aristolochic acid[118]	Medicinal	Renal pelvis, ureter	Sufficient
Radon and its decay products (listed in monograph for internally deposited α-particle emitting radionuclides)[120]	Environmental	Lung (leukemia)	Sufficient
Rubber industry[116]	Occupational	Bladder, leukemia, lymphoma, lung, stomach (prostate, esophagus, larynx)	No data
Salted fish (Chinese-style)[115]	Environmental	Nasopharynx (stomach)	Sufficient
Schistosoma haematobium[119]	Environmental	Bladder	Limited
Shale oils[116]	Occupational	Skin (observed in scrotum)	Sufficient
Silica dust, crystalline, in the form of quartz or cristobalite[117]	Occupational	Lung	Sufficient for quartz dust; limited for tridymite and cristobalite dust

TABLE 2.5 (continued)
Chemicals, Industrial Processes, and Environmental Factors Associated with Cancer Induction in Humans: Target Organs and Main Routes of Exposure in Humans and Degree of Supporting Evidence in Animals, According to IARC

	Humans		Animals
Chemical or Industrial Process	**Main Type of Exposure[a]**	**Target Organ(s)/Cancer Type[b]**	**Degree of Evidence for Carcinogenicity**
Solar radiation[h,120]	Environmental	Cutaneous malignant melanoma, squamous cell carcinoma of the skin, basal cell carcinoma of the skin (lip, conjunctival squamous cell carcinoma and ocular melanoma)	Sufficient
Soot, as found in occupational exposure of chimney sweeps[116]	Occupational	Skin (observed in scrotum), lung (bladder)	Inadequate evidence of soot alone; sufficient evidence of soot extracts
Strong inorganic acid mists[116]	Occupational	Larynx (lung—limited)	No data
Talc containing asbestos fibers[117]	Occupational	Lung, mesothelioma	Inadequate
Tamoxifen[118]	Medicinal	Endometrium (reduces risk for contralateral breast cancer in women with previous diagnosis of breast cancer)	Sufficient
2,3,7,8-Tetrachlorodibenzo-para-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran, and 3,3,4,4,5-pentachlorobiphenyl (PCB 126)[116]	Occupational	Multisite with no site predominating (soft tissue sarcoma, non-Hodgkin's lymphoma, and lung for TCDD)	Sufficient for all three compounds
Thiotepa[118]	Medicinal	Leukemia	Sufficient
Tobacco products, smokeless[115]	Environmental, cultural	Oral cavity, esophagus, pancreas	Sufficient
Tobacco smoke, secondhand[115]	Environmental, cultural	Lung (larynx, pharynx)	Sufficient for mixtures of mainstream and sidestream tobacco smoke and sidestream tobacco smoke condensates
Tobacco smoking[115]	Environmental, cultural	Lung, oral cavity, naso-, oro-, and hypopharynx, nasal cavity and paranasal sinuses, larynx, esophagus, stomach, pancreas, colorectum, liver, kidney, ureter, bladder, cervix and ovary, myeloid leukemia, breast, childhood hepatoblastoma and acute lymphocytic leukemia; reduced risk for endometrium (postmenopausal) and thyroid	Sufficient
Treosulphan[118]	Medicinal	Acute myeloid leukemia	No data
Vinyl chloride[116]	Occupational	Liver (connective and soft tissue—contradictory)	Sufficient
Wood dust[117]	Occupational	Nasal cavity, paranasal sinus, nasopharynx	Inadequate
X- and γ-radiation[120]	Medicinal, occupational, environmental	Salivary gland, esophagus, stomach, colon, lung, bone, basal cell of the skin, female breast, urinary bladder, brain and CNS, thyroid, kidney, and leukemia (rectum, liver, pancreas, ovary, prostate, non-Hodgkin's lymphoma, multiple myeloma)	Sufficient

[a] The main types of exposure mentioned are those by which the association has been demonstrated; exposures other than those mentioned may also occur.
[b] Suspected target organs in parentheses.
[c] The evaluation of carcinogenicity to humans applies to the group of chemicals as a whole and not necessarily to all individual chemicals within the group.
[d] There are no epidemiological studies of exposure to B[*a*]P (only in mixtures of polycyclic aromatic hydrocarbons); classification is based on animal and mechanistic data in human lung tissue.
[e] No adequate epidemiological studies were available; classification based on animal carcinogenicity data and/or mechanistic data in animal and/or human cells.
[f] The working group was not in full agreement on the evaluation of formaldehyde causing leukemias in humans, with a small majority viewing the evidence as sufficient of carcinogenicity and the minority viewing the evidence as limited.
[g] The specific cancer types were not discussed for humans but likely mimic those seen for x- and γ-radiation.
[h] Solar radiation is *carcinogenic to humans (Group 1)*; use of UV-emitting tanning devices is *carcinogenic to humans (Group 1)*; ultraviolet radiation (bandwidth 100s nm^3, encompassing UVC, UVB, and UVA) is *carcinogenic to humans (Group 1).*

TABLE 2.6
Summary of the Classification Schemes for Carcinogens

Germany (DFG/MAK)[a]	1	Carcinogenic to humans.
	2	Carcinogenic in animal studies.
	3	Suspected carcinogenic potential.
	4	Nongenotoxic carcinogens.
	5	Weak potency genotoxic carcinogens.
EU[121]	1	Carcinogenic to humans.
	2	Should be regarded as if carcinogenic to humans.
	3	Cause for concern in humans.
		3A. Substances that are well investigated
		3B. Substances that are insufficiently investigated
IARC[108]	1	Carcinogenic to humans.
	2A	Probably carcinogenic in humans; limited human evidence; sufficient animal evidence.
	2B	Possibly carcinogenic in humans; limited human evidence; less than sufficient animal evidence.
	3	Not classifiable.
	4	Probably not carcinogenic to humans.
ACGIH[122]	A1	Confirmed human carcinogen.
	A2	Suspected human carcinogen, limited human evidence and sufficient relevant animal evidence.
	A3	Confirmed animal carcinogen with unknown relevance to human; epidemiological studies do not confirm risk to humans.
	A4	Not classifiable.
	A5	Not suspected as human carcinogen, based on properly conducted epidemiological studies or evidence in animal studies.
Health Canada[123]	Group I	Carcinogenic to humans.
	Group II	Probably carcinogenic to humans. Inadequate epidemiological evidence; sufficient evidence in animal species.
	Group III	Possibly carcinogenic to humans. Inadequate or flawed epidemiological studies. Limited animal evidence, or adequate animal evidence, but involves epigenetic mechanisms.
	Group IV	Unlikely to be carcinogenic in humans. No evidence in adequate epidemiological studies; positive animal studies of limited or unlikely relevance to humans.
	Group V	Probably not carcinogenic in humans. No evidence in adequate epidemiological studies. No evidence or inadequate evidence in animal studies.
U.S. EPA[95]		Carcinogenic to humans.
		Likely to be carcinogenic to humans.
		Suggestive evidence of carcinogenic potential.
		Inadequate information to assess carcinogenic potential.
		Not likely to be carcinogenic in humans.
NTP[124]	1	Known to be a carcinogen.
	2	Reasonably anticipated to be a carcinogen.
		A. Limited evidence in human studies indicating credible causal relationship evidence in human studies.
		B. Sufficient evidence in animal studies.

[a] DFG/MAK, Deutsche Forschungsgemeinschaft/maximale arbeitsplatz-Konzentration (German Commission for the Investigation of Health Hazards of Chemical compounds in the work area), as discussed in Seeley et al.[40]

Some agencies, including the IARC,[43] typically conclude that a chemical demonstrating *sufficient evidence of carcinogenicity* from animal experiments is a potential human carcinogen. To some degree, this conclusion is consistent with the evaluation of known human carcinogens in animal bioassays. About 2/3 of the 87 chemicals, processes, or environmental factors associated with cancer indication in humans by the IARC[113,114] have also been considered by that agency to be positive in animal bioassays (Table 2.5). More current understanding of carcinogenesis indicates that, based on MoA and other considerations, this assumption is not appropriate for all animal carcinogens.

A more sophisticated understanding of carcinogenicity with an emphasis on understanding the biology of carcinogenesis on MoA was presented in the groundbreaking 2005 EPA guidelines.[95] These guidelines, in their flexibility and incorporation of new science, represent a significant advance in carcinogen classification schemes. There are a number of

important features in the guidelines; in particular, the *MoA** forms the underpinning of other elements wherever possible.

The guidelines use five standard narrative descriptors to assess the carcinogenic hazard to humans: (1) carcinogenic to humans, (2) likely to be carcinogenic to humans, (3) suggestive evidence of carcinogenic potential, (4) inadequate information to assess carcinogenic potential, and (5) not likely to be carcinogenic to humans. The guidelines take a weight-of-evidence approach in which all human, animal, and other relevant toxicological information is evaluated. As part of this evaluation, the quality of individual studies and overall consistency across studies is considered. Confidence that a specific chemical is the cause of cancer in human studies is enhanced by positive findings at the same organ site in multiple studies with well-characterized exposures. In contrast to the 1986 EPA guidelines,[125] other evidence relevant to carcinogenicity—importantly, the MoA information in animals that attests to the relevance (or lack of relevance) of a particular tumor response—is considered explicitly. Data from human, animal, and other sources are combined to weigh the totality of evidence to classify the human carcinogenic potential of a particular chemical. Using this framework, some animal carcinogens such as DMA,[126] captan,[127] and chloroform[128] have been identified by the EPA as associated with a threshold MoA; that is, at exposures less than the threshold dose, the risk of the chemical's potential as a human carcinogen is expected to be negligible—if not zero.

The 2005 guidelines allow for multiple descriptors of carcinogenicity—that is, a chemical may be classified as *not likely to be carcinogenic* by one route of exposure but *likely to be carcinogenic* by another route. Dose may also be an element of the descriptor in which a chemical may be carcinogenic only above a specified dose (which is equivalent to assuming a threshold dose–response relationship).

In situations where there is not enough information to make a determination on a chemical's carcinogenic hazard to humans, the EPA has specified default assumptions; for example, positive findings in animals are assumed to be relevant to humans. However, as noted earlier, well-founded MoA information (such as findings that a kidney tumor response in rodents is a consequence of alpha-2-μ globulin accumulation) can be used to avoid use of such a default assumption.

Recent Approaches

There continues to be important developments in the evaluation of chemical carcinogens, providing for fuller incorporation of biological understanding into interpretation of carcinogenicity findings and bringing greater rigor to evaluations conducted by regulatory agencies. As a practical matter, such advances can influence the classification of chemical carcinogens and identify the most appropriate dose–response relationship for risk assessment purposes.

Building on earlier EPA and International Programme on Chemical Safety efforts, an expert panel organized by the International Life Sciences Institute Risk Science Institute (ILSI RSI) expanded upon the *human relevance framework* for chemical carcinogens. The framework addressed the sufficiency of evidence for a particular MoA in animals, the plausibility of key events in the MoA in humans (i.e., can the MoA occur in humans?), and, considering kinetic and dynamic factors, the plausibility of the animal MoA in humans (i.e., is it plausible that, under relevant exposure conditions, the animal MoA could actually occur in humans?). In this framework, an overall conclusion, along with a statement of confidence in the findings, is developed regarding likelihood of carcinogenicity in humans and under what circumstance(s). Key to the framework is the comparison of multiple plausible MoAs for an individual chemical to assess which MoA best fits the available data. The applicability of the framework was reflected in several case studies. For example, the MoA of melamine bladder cancer (accumulation of calculi) in rodents was considered plausible in humans under the framework, but such accumulation was considered unlikely to occur in humans under any realistic exposure conditions. This yielded the overall conclusion that melamine would not present a human cancer hazard.

The hypothesis-based weight-of-evidence (HBWoE) method provides a complementary approach to the ILSI RSI human relevance framework described earlier. With HBWoE, multiple hypotheses to explain a particular carcinogenic are identified. Findings in target and nontarget tissues and species are then presented, consistency with the proposed hypotheses is evaluated, and the need to invoke alternative assumptions is considered. Using this approach, Rhomberg and coworkers[129] concluded that the weight of evidence supporting a cytotoxic or dual cytotoxic/genotoxic MoA for naphthalene was stronger than that for an initiating genotoxic MoA.

Dose–Response Assessment

One of the most contentious aspects of the evaluation of animal carcinogens by regulatory agencies is characterizing the dose–response relationship at the exposure levels to which humans are likely to be exposed. Animals are typically exposed to carcinogens at levels orders of magnitude greater than those likely to be encountered in the environment by humans. It would be impossible to perform animal experiments with large enough numbers to directly estimate the level of risk at low exposure levels. Thus, to obtain a quantitative estimate of the risks humans are likely to encounter at ambient exposures requires the extrapolation of effects

* Mode of action is defined by EPA as a sequence of biochemical and cellular event resulting in tumor formation. It may be contrasted with a mechanism of action that implies a detailed understanding of the carcinogenic process, often at the molecular level.

observed at high doses to low doses and from effects observed in animals to humans. Even the use of carcinogenicity data from human studies (mostly occupational studies) frequently requires the use of extrapolation models to estimate risks to humans exposed at lower ambient levels.

Mechanistic models are being developed to assist in dose–response assessment. Pharmacokinetic models attempt to describe the relationship between exposure and biologically relevant dose to the target tissue. These models characterize absorption, distribution, metabolism, and excretion of chemicals. Pharmacodynamics models attempt to describe the relationship between the dose to target tissue and response. Both of these types of models can assist in extrapolation from high to low doses and across species.

Low-Dose Extrapolation

Extrapolation from high to low dose is done using models that are hypothesized to characterize the dose–response relationship of carcinogens at both the high dose and response levels observed in animal or human occupational studies and the low dose and response levels of interest for human exposures. The choice of mathematical model depends on two factors: (1) the hypothesis for the mechanism of carcinogenesis for a particular chemical and (2) the science policy decision to choose, in the absence of data firmly supporting one model or another, the more conservative model (of several biologically plausible models) or to present results from a range of plausible models.

Threshold versus Nonthreshold Mechanisms

The determination of whether carcinogenesis is a threshold or nonthreshold phenomenon is a key consideration in the choice of model to characterize the dose–response relationship. Chemical carcinogenesis has historically been considered as a nonthreshold phenomenon, particularly for certain types of genotoxic agents (i.e., those that interact directly with DNA to cause mutations). For example, trichloropropane was classified by the EPA as having a mutagenic MoA, supporting a linear no-threshold dose–response model.[130]

In the case of the linear no-threshold model, the measure of a chemical's carcinogenic potency is typically determined by fitting a model to the observed data and then linearly extrapolating to low doses, often from a POD (i.e., the highest dose associated with no tumors) or, in some cases, a precursor indicator such as stimulation of cell proliferation.[126] For chemicals that cause cell damage at high doses, or for chemicals for which detoxification pathways become saturated at high doses, it is likely that a different dose–response relationship will be observed at high and low doses, even for those chemicals where a nonzero slope is plausible at any dose. As discussed in "Maximum Tolerated Dose" section, work by scientists such as Cohen and Arnold[90] highlights the predominant role of cell proliferation in carcinogenicity of many chemicals that yield positive tumor responses at the MTD.

A striking example of different dose–response relationships for a single carcinogen is 2-acetyl-aminofluorene (2-AAF).[131] 2-AAF is a potent mutagenic carcinogen. The dose–response relationship for 2-AAF-induced liver cancer exhibits the expected (for a genotoxic carcinogen) linear dose–response relationship, whereas the dose–response relationship for bladder cancer is highly nonlinear—demonstrating an apparent threshold. The mechanistic basis for the different dose–response relationships appears to involve differences in the relative importance of genetic damage (the likely key event in liver cancer) versus genetic damage *and* hyperplasia of the bladder urothelium (the likely key events in bladder cancer). Thus, the selection of the appropriate shape of the dose–response relationship for *any* chemical requires understanding of the mechanism by which tumors are induced.

Models

The choice of the low-dose extrapolation model can have a major impact on the estimate of risk at low exposure levels, as presented by Bickis and Krewski.[132] The authors estimated risk from 2-AAF at low exposure levels using different models. The level of risk varied by many orders of magnitude at the same exposure level depending on the model chosen to characterize the dose–response curve in the unobservable region.

Because of the uncertainties in dose–response modeling for low-dose risk and an increased emphasis on MoA in cancer risk assessment, efforts are being made to incorporate greater biological understanding of tumorigenesis into cancer dose–response assessment. Some of the most comprehensive efforts in this area have been conducted by scientists at the Chemical Industry Institute of Toxicology (now the Hamner Institute) with respect to the development of a biologically motivated computational model for formaldehyde in the F344 rat. Investigators have developed a model that incorporates information on nasal dosimetry, cell replication, and DNA cross-links into a two-stage clonal growth model (see Figure 2.5).[133] This modeling yields a j-shaped dose–response relationship that reflects the highly nonlinear dose–response relationship for tumorigenicity. The biologically based modeling for nasal tumors from formaldehyde results in maximum likelihood estimates for cancer risk at 0.1 ppm formaldehyde in air that are, in some cases, as much as 1000-fold lower than the values used by the EPA in its Integrated Risk Information System. Analysis of formaldehyde-dG adduct data, an indicator of internal dose at the molecular target, has provided evidence that the EPA risk values may be overestimated, in some cases by several orders of magnitude.[134]

In the 2005 cancer risk assessment guidelines, the EPA revised its approach to dose–response assessment.[95] If sufficient data are available, a biologically based dose–response

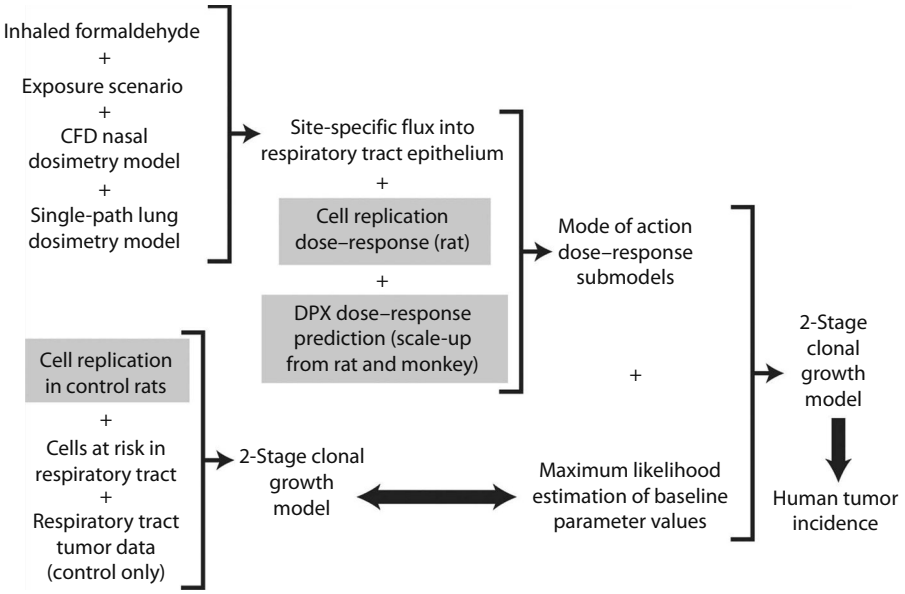


FIGURE 2.5 Interrelationships of the major components of the human dose–response model. (From Conolly, R.B. et al., *Toxicol. Sci*., 82(1), 279, 2004. With permission of Society of Toxicology.)

model (such as the one described earlier for formaldehyde) is chosen as the most appropriate method for evaluating the observed data and extrapolating to exposures below the observed dose range. However, the more likely case is that sufficient data are not available for development of biologically based models. In this situation, a *point-of-departure* approach is recommended. The POD represents a dose, within the range of observed data, associated with a specified extra tumor risk. The POD is developed using mathematical models, such as the linearized multistage model (although other models can be used), and it is typically expressed as the lower limit on effective dose (LED). The LED is the lower 95% confidence limit on the dose associated with specified extra risk (e.g., LED_{10} would be the lower 95% confidence limit on the dose associated with 10% extra risk above background). Risks below the LED_{10} are characterized either through linear extrapolation (for chemicals believed to act via a linear dose–response relationship [e.g., genotoxic carcinogens]) or through a margin-of-exposure analysis (for chemicals for which dose–response relationships are likely to be either threshold or nonlinear). For chemicals where data might support either linear extrapolation or a margin-of-exposure analysis, both analyses are presented.

EVALUATION OF NONCANCER EFFECTS

Noncancer effects are generally postulated to occur through a threshold mechanism. That is, there is a level of exposure below which there is minimal, if any, chance for an adverse effect. Noncancer effects can range from acute skin and eye irritation to subchronic or chronic damage to an organ system, such as pulmonary fibrosis. The underlying hypothesis for the threshold model for systemic toxicants is that multiple cells must be injured before an adverse effect occurs, and the injury must occur at a rate that exceeds the rate of repair. This is in contrast to the regulatory paradigm for carcinogens, in which a genotoxic insult involving direct DNA damage to a single cell is theoretically sufficient to allow that cell to grow to a malignant tumor.[135] (As discussed earlier in this chapter, it should be noted that this model for carcinogenesis is now viewed as too limiting.) Pulmonary fibrosis due to mineral dust exposure provides an example of a threshold-type injury. Fibrotic areas may be present and observed as radiographic or histopathological changes in the lungs of miners as a consequence of mineral dust exposure in the absence of any physiological impairment such as reduced lung function. Physiological impairment will occur as the fibrosis increases and the fibrotic areas begin to coalesce.[136]

For noncancer effects that may involve genotoxic mechanisms, such as developmental effects, a threshold model may still be the most appropriate choice of dose–response model. This is because multiple cells must be injured before an effect can be manifested. For example, the prenatal death of a single retinal cell, even through genetic damage, would not result in blindness because of the existence of many other retinal cells.

Acceptable Exposure Level

The general approach for setting exposure limits for noncancer effects is based on developing a reference value, or acceptable exposure level (AEL), which is associated with

minimal or no risk of adverse effects in humans. For ingestion and dermal exposures, the AEL is expressed in terms of mg/kg-day[137]; for inhalation exposure, the AEL is typically expressed as an air concentration (e.g., mg/m^3).

AELs are based on a POD from either an epidemiology or animal toxicology study, which could be a no observed adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) or a benchmark exposure level. Uncertainty factors (UFs) are then applied to the POD to account for the uncertainty inherent in extrapolating from the adverse health effect observed in the human epidemiology or animal toxicology study to the likelihood of observing an adverse health effect in the general population.

The methodology for deriving an AEL is widely used or recommended by organizations such as the EPA, ATSDR, FDA, Joint Food and Agriculture Organization of the United Nations (FAO)/WHO Expert Committee on Food Additives (JECFA), Joint FAO/WHO Meeting on Pesticide Residues (JMPR), and NAS/NRC.[32,138] Table 2.7 lists AELs used by different governmental organizations.

Identifying a Point of Departure

The traditional approach for identifying a POD involves identifying a NOAEL or LOAEL for the most sensitive endpoint, in the most sensitive species. Ideally, AELs should be developed from studies in a susceptible population of humans. Because suitable human studies of sufficient quality are not available for most chemicals of concern, most AELs are based on animal studies. Although it is preferable to use a NOAEL from an animal study, NOAELs cannot be identified from all studies, in which case a LOAEL is used. Similarly, the study duration should correspond with the time frame for the AEL, although shorter durations can also be used.

There are several limitations in using a NOAEL or LOAEL as a POD, with the net result that chemicals with the same AEL are not necessarily associated with the same level of risk. This is because NOAELs/LOAELs do not represent a consistent response level across studies. Importantly, NOAELs do not always represent an exposure level at which there is no risk of an adverse effect; rather, these values correspond with an average response rate of 5%–20% above control. This is because NOAELs depend on both experimental exposure levels and sample size. Specifically, the choice of a NOAEL or a LOAEL does not take into consideration the greater experimental confidence associated with, for example, studies using more animals. An exposure level defined as a NOAEL in one experiment could turn out to be a LOAEL had more experimental animals been used (i.e., an effect may have been detected if more animals had been studied). As a result, poor-quality experiments may yield anticonservative AELs, since studies using fewer animals may result in a higher AEL than studies using larger numbers of animals.[144] In addition, the use of NOAEL or LOAEL as a POD does not account for the shape of the exposure–response relationship, which is a key determinant in assessing the likelihood of effects. That is, a chemical with a steep exposure–response curve would be associated with a greater likelihood of effects as exposure increased above the AEL (and a smaller likelihood of effects with exposures below the AEL) than would a chemical with a more shallow exposure–response curve.[145]

A preferred alternative to the NOAEL/LOAEL, when suitable studies are available, is the benchmark dose (BMD)/benchmark concentration (BMC). BMDs/BMCs are exposure levels (typically a dose or an air concentration) corresponding to a specific response near the low end of exposure–response curve. These values are derived using exposure–response modeling and statistics, thereby addressing issues such as experimental quality and shape of the exposure–response relationship in a manner similar to that used for developing cancer potency factors (as discussed in "Evaluation of Carcinogens" section of this chapter). Developing a BMD or BMC generally requires data showing a graded monotonic response at increasing exposure levels, with a significant exposure-related trend. If available, data should be modeled using a biologically based model; if not, the model that best fits the data is selected.[144]

For quantal data, the EPA recommends using a benchmark response (BMR) of 10%, which is close to the limit of sensitivity for most cancer bioassays and also for some noncancer bioassays. For continuous data, the EPA recommends identifying a BMR that corresponds to the level of change at which an effect is considered to be biologically significant. If individual-level data are available, continuous data can also be dichotomized for identifying a cutoff value associated with an adverse response. Alternatively, the response can be selected as a change equal to one standard deviation from the mean control response. For effects that are normally distributed, this response corresponds with an excess risk of approximately 10% for individuals either below the 1.4th percentile or above the 98.6th percentile. In the case of either quantal or continuous data, either higher or lower BMRs can be used, depending on statistical or biological considerations.[144] The BMD/BMC corresponding with a BMR provides a central estimate of an exposure level associated with a specific response. For the POD, the EPA recommends use of the 95% lower bound on a BMD/BMC (i.e., a BMDL/BMCL) to account for uncertainty associated with the study design, such as the number of animals per exposure group and the selection of exposure levels.[144]

Figure 2.6 illustrates derivation of a BMDL, in comparison to a NOAEL, as a function of number of animals per exposure group.

The benchmark approach overcomes many of the weaknesses of the AEL approach.[146,147] Because benchmark values are determined based on statistical modeling of exposure–response data, the approach accounts for the sample size and the slope of the exposure–response curve. Unlike NOAELs or LOAELs, benchmark values are not constrained to be one of the experimental exposure levels and are less dependent on the study design. Overall, the benchmark approach allows for greater consistency between values derived for different chemicals. Although BMDs/BMCs are preferred for identifying a POD, there are instances where a NOAEL/LOAEL would be used. This is because some chemicals

TABLE 2.7
Acceptable Exposure Levels Used by Different Agencies

Value	Description	Use	POD	UFs
RfD U.S. EPA[139]	"An estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious effects during a lifetime."	Support regulatory activities such as remediation	NOAEL, LOAEL or BMDL	Interspecies. Intraspecies. LOAEL/NOAEL. Subchronic/chronic. Database deficiencies.
RfC U.S. EPA[139]	"An estimate (with uncertainty spanning perhaps an order of magnitude) of a continuous inhalation exposure to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious effects during a lifetime."	Support regulatory activities such as remediation	NOAEL, LOAEL or BMCL	Interspecies. Intraspecies. LOAEL/NOAEL. Subchronic/chronic. Database deficiencies.
MRL ATSDR[140,141]	"An MRL is an estimate of the daily human exposure to a hazardous substance that is likely to be without appreciable risk of adverse non-cancer health effects over a specified duration of exposure."	Screening levels for identifying contaminants associated with hazardous waste sites that may be a concern for the general population	NOAEL or LOAEL	Interspecies. Intraspecies. LOAEL/NOAEL. Subchronic/chronic. Database deficiencies (referred to by ATSDR as an MF).
Acceptable Daily Intake (ADI) U.S. FDA[142]	"A food additive generally is considered safe for its intended use if the estimated daily intake (EDI) of the additive is less than, or approximates, the ADI. Because the ADI is calculated to protect against the most sensitive adverse effect, it also protects against other adverse effects occurring at higher exposures to the ingredient."	Define safe levels for food additives	NOEL or NOAEL	U.S. FDA applies a safety factor of 100 for ADIs derived from animal studies. This safety factor may be modified to account for potentially sensitive subpopulations.
Acceptable Daily Intake (ADI) WHO[138,143]	"The ADI of a chemical is the estimate of the amount of a substance in food or drinking-water, expressed on a body-weight basis, that can be ingested daily over a lifetime without appreciable health risk to the consumer on the basis of all the known facts at the time of the evaluation. It is expressed in milligrams of the chemical per kilogram of body weight."	Established for food additives and pesticide residues	NOAEL, LOAEL or BMDL	Interspecies. Intraspecies. Adequacy of studies or database. Nature and severity of effect.
Tolerable Daily Intake (TDI) WHO[138]	"The TDI is an estimate of the amount of a substance in food and drinking-water, expressed on a body weight basis (milligram or microgram per kilogram of body weight), that can be ingested over a lifetime without appreciable health risk, and with a margin of safety."	Established for chemical contaminants	NOAEL, LOAEL or BMDL	Interspecies. Intraspecies. Adequacy of studies or database. Nature and severity of effect.

Notes: BMDL, lower 95% confidence limit on BMD; LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level; NOEL, no observed effect level.

may lack the minimum exposure–response data required to identify a BMD/BMC.[144]

Table 2.8 lists chemicals for which the EPA has derived noncancer toxicity criteria based on a BMR between 2000 and 2012. During this time, approximately 60% of reference doses (RfDs) and 50% of reference concentrations (RfCs) were derived using BMR rather than a NOAEL or LOAEL. A BMR of 10% is most common, although BMRs of 5% and 1%, those equal to one standard deviation from the mean or those equal to twofold greater than control response, have also been used. For example, a BMR of 5% was used for acrylamide. This response was considered to represent a

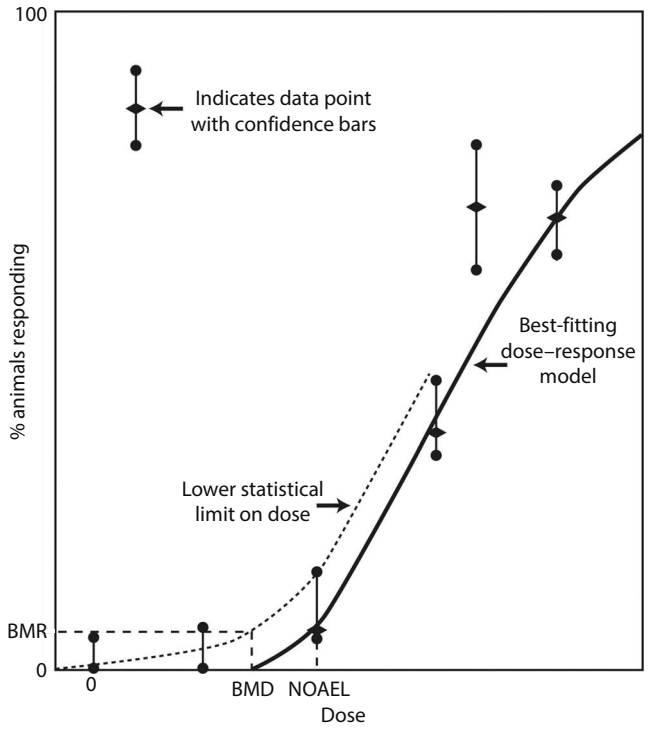


FIGURE 2.6 Relative magnitude of BMDL versus NOAEL as a function of number of animals per dose group (hypothetical data). (From U.S. Environmental Protection Agency, *The Use of the Benchmark Dose Approach in Health Risk Assessment*, Risk Assessment Forum, Office of Research and Development, Washington, DC, EPA/630/R-94/007, 1995.)

change that was of minimal biological significance, which was also near the range of observed responses, and it was further supported given that principal underlying studies used a relatively large number of animals.[148] A 5% response was also used for deriving the RfD for methylmercury, which was based on developmental neurological effects in humans. The response of 5% results in a doubling of the number of children responding below the 5th percentile of abilities for the normal population, which represents a significant level of compromise.[149] The EPA used a response of 1% for TCE due to the severity of the heart malformations considered as the critical effect.[150] Chemicals assessed prior to 2000 for which U.S. EPA also used a BMR as the POD include beryllium, tributyltin oxide, antimony trioxide, CrVI particulates, carbon disulfide, methylene diphenyl diisocyanate, methyl methacrylate, phosphoric acid, and 1,1,1,2-tetrafluoroethane. The EPA used a BMR of 10% for all of these chemicals.[151]

Selecting Uncertainty Factors

There are five possible UFs that can be applied to a POD, each with a default value of 10.[138,139,141] These are described in Table 2.9.

In the past, the EPA has also applied a modifying factor (MF); in practice, an MF is similar to the currently used database UF. This factor has been applied in deriving toxicity criteria for only a small number of chemicals, including RfDs for CrIII, CrVI, nitrite, 1,1-biphenyl, and manganese, as well as RfCs for methyl ethyl ketone and acetonitrile. In 2002, the EPA's RfD/RfC Technical Panel recommended against using an MF on the basis that the database UF should account for potential issues that the MF is intended to address.[139]

UFs are used multiplicatively. To derive an AEL, the POD is divided by the product of the individual UFs. Mathematically, this is represented as

$$\frac{\text{RfD}}{\text{RfC}} = \frac{\text{POD}}{\text{UF}_1 \times \text{UF}_2 \times \text{UF}_n} \tag{2.1}$$

The use of all five UFs (UF_H, UF_A, UF_S, UF_L, UF_D), each representing an order of magnitude, could in theory lead to a total UF of 100,000. This would occur if data were from a subchronic animal study that identified only a LOAEL and the database was limited. However, the EPA's RfD/RfC Technical Panel recommended against deriving a reference value using the full tenfold value for four or more UFs, as it is likely in such cases that the database is insufficient for deriving a reference value. The technical panel hence recommended using a maximum total UF of 3000.[139]

Although the basis for an initial choice of 10 as a default value for UFs was never defined, subsequent analyses provide experimental support that a value of 10 is adequate in most, but not all, cases.[154] Evaluation of data regarding toxicokinetics and toxicodynamics indicates that the tenfold factor for intraindividual variability is sufficient in most cases.[155] This evaluation, however, did not address children; thus, some scientist have contended that the tenfold interspecies UF may not account for potential increased susceptibility in that subpopulation.[139] Another instance for which a intraindividual UF of 10 may not be sufficient is in the case of genetic polymorphisms that may result in large variations in susceptibility.[139] Dorne and coworkers[156] observed an overall interindividual variability of 31% for compounds eliminated via glucuronidation. Walton and coworkers[157] evaluated interspecies toxicokinetic differences specifically for compounds eliminated by CYP1A2, including caffeine, theobromine, theophylline, and paraxanthine. They determined that that interspecies toxicokinetic UFs of 10.6, 5.4, 2.6, and 1.6 for mice, rats, rabbits, and dogs, respectively, would account for differences in the route of metabolism and metabolic enzymes. Because the variability observed for metabolism related to CYP1A2 and glucuronide does not account for differences in toxicodynamics, the default UF of 10 may not be adequately protective for all compounds metabolized by these enzymes; however, it would be necessary to assess the implications of such polymorphisms on dose to target tissue (considering factors such as absorption and distribution in addition to metabolism) before drawing confirmed conclusions as to the overall impact of polymorphisms on susceptibility.

There is also support for use of a tenfold factor to account for insufficient study duration. For example, the ratio between the subchronic and chronic NOAEL or LOAEL for 52

TABLE 2.8
U.S. EPA Reference Doses and Reference Concentrations Based on Benchmark Responses (2000–2012)

Chemical	Criteria	Endpoint	POD
Acrylamide	RfD/RfC	Degenerative nerve changes (rats)	$BMDL_{05}$[a]
Barium/Barium compounds	RfD	Nephropathy (mice)	$BMDL_{05}$
Benzene	RfD/RfC	Decreased lymphocyte count (humans)	$BMDL_{1SD}$[b]
Boron	RfD	Decreased fetal weight (rats)	$BMDL_{05}$
Bromobenzene	RfD	Hepatocellular cytomegaly (mice)	$BMDL_{10}$
	RfC	Hepatocellular cytomegaly (mice)	$BMCL_{10}$
1,3-Butadiene	RfC	Ovarian atrophy (mice)	$BMCL_{10}$
Carbon tetrachloride	RfD	Elevated activity of sorbitol dehydrogenase (rats)	$BMDL_{2X}$
	RfC	Fatty changes in the liver (rats)	$BMCL_{10}$
Cerium oxide and cerium compounds	RfC	Alveolar epithelial hyperplasia (rats)	$BMCL_{10}$
Chlordecone	RfD	Glomerulosclerosis (rats)	$BMDL_{10}$
Chloroprene	RfC	Olfactory atrophy (rats, male)	$BMCL_{10}$
		Alveolar hyperplasia (rats, female)	
		Splenic hematopoietic proliferation (mice)	
Cyanide/hydrogen cyanide	RfD	Decreased weight of cauda epididymis (rats)	$BMCL_{1SD}$
Cyclohexane	RfC	Reduced pup weight (rats)	$BMCL_{1SD}$
1,2-Dibromomethane	RfC	Nasal inflammation (mice)	$BMCL_{10}$
cis-1,2-Dichloroethane	RfD	Increased relative kidney weight (rats)	$BMDL_{10}$
trans-1,2-Dichloroethane	RfD	Decreased antibody forming cells against sheep RBCs (mice)	$BMDL_{1SD}$
1,1-Dichloroethylene	RfD	Fatty liver (rats)	$BMDL_{10}$
Dichloromethane	RfD	Hepatic vacuolization, liver foci (rats)	$BMDL_{10}$
	RfC	Hepatic vacuolization (rats)	$BMCL_{10}$
1,3-Dichloropropene	RfD	Basal cell hyperplasia of forestomach mucosal cells	$BMDL_{10}$
	RfC	Hypertrophy/hyperplasia of nasal respiratory epithelium (mice)	$BMCL_{10}$
Ethylene glycol monobutyl ether (2-Butoxyethanol)	RfC	Hemosiderin deposition in the liver (rats)	$BMCL_{10}$
Hexachlorocyclopentadiene	RfD	Hyperplasia, acanthosis, hyperkeratosis of forestomach epithelium (rats)	$BMDL_{10}$
Hexachloroethane	RfD	Renal tubule atrophy and degeneration (rats)	$BMDL_{10}$
n-Hexane	RfC	Peripheral neuropathy (rats)	$BMCL_{10}$
2-Hexanone	RfD	Axonal swelling of peripheral nerve (rats)	$BMDL_{10}$
	RfC	Reduced motor conduction velocity (monkeys)	$BMCL_{05}$
Methylmercury	RfD	Developmental neuropsychological impairment (humans)	$BMDL_{05}$
2-Methylnaphthalene	RfD	Pulmonary alveolar proteinosis (mice)	$BMDL_{05}$
Nitrobenzene	RfD	Increased MetHb (rats)	$BMDL_{1SD}$
	RfC	Alveolar bronchiolization and olfactory degeneration (mice)	$BMCL_{10}$
2,2′,4,4′5-Pentabromodeiphenylether (BDE-99)	RfD	Neurobehavioral effects (mice)	$BMDL_{1SD}$
Phenol	RfD	Decreased maternal weight gain (rats)	$BMDL_{1SD}$
Phosgene	RfC	Lung fibrosis (rats)	$BMCL_{10}$
Propionaldehyde	RfC	Olfactory epithelium atrophy (rats)	$BMCL_{10}$
2,2′,4,4′-Tetrabromodiphenylether (BDE-47)	RfD	Neurobehavioral effects (mice)	$BMDL_{1SD}$
1,1,2,2-Tetrachloroethane	RfD	Increased relative liver weight (rats)	$BMDL_{1SD}$
Tetrahydrofuran	RfD	Decreased pup body weight gain (rats)	$BMDL_{10}$
	RfC	Increased liver weight and centrilobular cytomegaly (mice)	$BMCL_{10}$
		CNS effects–narcosis (mice)	
Toluene	RfD	Increased kidney weight (rats)	$BMDL_{1SD}$
Trichloroacetic acid	RfD	Hepatocellular necrosis	$BMDL_{10}$
1,1,1-Trichloroethane	RfD	Reduced body weight (mice)	$BMDL_{10}$
TCE	RfD/RfC	Cardiac malformations (rats)	$BMDL_{01}$[a]
1,2,3-Trichloropropane	RfD	Increased absolute liver weight (rats)	$BMDL_{10}$
	RfC	Peribronchial lymphoid hyperplasia (rats)	$BMCL_{10}$

Source: U.S. Environmental Protection Agency, Integrated Risk Information System (IRIS): A–Z list of substances, 2012, Electronic database available from http://cfpub.epa.gov/ncea/iris/index.cfm?fuseaction = iris.showSubstanceList&list_type = alpha&view = A.

Notes: $BMDL_{05}$, associated with a 5% response; $BMDL_{10}$/$BMCL_{10}$, associated with a 10% response; $BMDL_{1SD}$/$BMCL_{1SD}$, associated with a response 1 standard deviation from control response; $BMDL_{2X}$, associated with a twofold increase above control value.

[a] Converted to BMCL for deriving the RfC.

[b] Converted from BMDL for deriving RfD.

TABLE 2.9
Basis for UF Use

Term	Information
Intraindividual—UF_H	To account for variations in susceptibility among humans; comprised of a component to account for variability in toxicokinetics (TK), which relates an external exposure to an internal dose, and a component to account for variability in toxicodynamics (TD), which relates an internal dose to a toxicological response. Whereas U.S. EPA equally apportions TK and TD components into default values of $10^{0.5}$ (3.16), which is sometimes rounded to 3; IPCS recommends values of $10^{0.6}$ (4) and $10^{0.4}$ (2.5).[139,152] • If a POD was identified from a long-term study in humans, this would be the only UF applied. In a later section of this chapter we discuss in detail the variability in human responsiveness to environmental pollutants and its relevance to the regulatory process. • A UF less than 10 can be used if the available data capture exposure–response relationships for susceptible subpopulations.
Interspecies—UF_A	To extrapolate from responses observed in animal data to expected responses in humans; comprised of a component to account for variability in toxicokinetics (TK), which relates an external exposure to an internal dose, and a component to account for variability in toxicodynamics (TD), which relates an internal dose to a toxicological response. Both U.S. EPA and IPCS equally apportion TK and TD components into equal values of $10^{0.5}$ (3.16), which sometimes rounded to 3.[139,152] • This factor is used when the POD is identified from an animal study, based on the assumption that humans may be more susceptible than experimental animals to a particular chemical. • U.S. EPA's RfC methodology applies a dosimetric adjustment factor (DAF) to the POD address interspecies toxicokinetic differences, for calculating a HEC. To account for interspecies differences in toxicodynamics, U.S. EPA uses a partial UF of $10^{0.5}$ (which is typically rounded to 3.0).[139] (Additional description of the RfC methodology is provided in the chapter by Rees and Hattis.)
Subchronic/chronic—UF_S	To extrapolate from a subchronic exposure to a chronic exposure. • This factor is used when the POD for a chronic AEL is identified from a study that involves less than lifetime exposure, and is based on the assumption that effects are proportional to both exposure level and exposure duration, with effects observed at lower exposure levels with increasing exposure durations.
LOAEL/NOAEL—UF_L	To extrapolate from a LOAEL to a NOAEL. • This factor is used for studies in which a NOAEL was not identified. The default assumption is that an exposure at 1/10 the LOAEL would result in a NOAEL.
Database—UF_D	To account for an incomplete database. • This factor is applied when the database is incomplete, based on the assumption that there is uncertainty as to whether the POD might be significantly lower if other studies were performed or if additional health endpoints (e.g., immunotoxicity, neurobehavioral toxicity, reproductive toxicity) had been evaluated.[153] A complete database is defined as having two chronic mammalian studies, one mammalian multigeneration study, and two mammalian developmental toxicity studies. If these five studies are available, and there is no reason to expect that the chemical would cause toxicity for other specific endpoints, such as immunotoxicity or neurobehavioral toxicity, then there is a high degree of confidence that one has approximated the lowest POD. • U.S. EPA uses a UF of 3 if RfD/RfC is based on animal data, in absence of data on prenatal toxicity or a two-generation reproductive study; UF of 10 may be applied if both prenatal and two-generation reproductive data are missing.[139]

chemicals was less than 10 in 96% of the cases, as described in the analysis by Dourson and Stara.[154] Thus, the UF of 10 would be an underestimate for only 4% of these chemicals. A similar analysis was performed by Lewis,[158] who observed that for 18 chemicals, the ratio of the subchronic to chronic NOAEL was 3.5 or less for 14 chemicals; only one had a ratio of greater than 10.[158] If the chemical with a ratio greater than 10 were excluded from the analysis, the mean subchronic to chronic NOAEL ratio was 3.3. Thus, the default UF of 10 for extrapolating from subchronic to chronic exposures would be very protective for most chemicals, and a UF of 3 may be more appropriate than the default value of 10 for many chemicals.

To account for interspecies toxicokinetic differences, as well as some aspects of toxicodynamic differences, the EPA now recommends allometric scaling, which involves scaling

physiological processes to growth and size. The EPA recommends using body weight to the ¾ power ($BW^{3/4}$), as follows:

$$\text{Dosimetric Adjustment Factor (DAF)} = \left(\frac{BW_a}{BW_h}\right)^{-1/4}$$

The EPA recommends applying this DAF along with an interspecies UF of 3, for both systemic and local effects.[159]

Use of allometric scaling to account for interspecies differences, which is consistent with the approach used for deriving cancer slope factors (CSFs), results in a human equivalent dose (HED). The HED is also analogous to the human equivalent concentration (HEC), which the EPA uses to derive an RfC (see Table 2.9).[159] As discussed by Rhomberg and Lewandowski, use of allometric scaling for physiological processes underlying toxicokinetics, such as metabolism, cardiac output, breathing rate, and glomerular filtration, is premised on similar anatomical features and biochemical processes operating at rates inversely proportional to size.[160] As such, allometric scaling is best applied to chemicals for which toxicity is related either to a parent compound or stable metabolite and which can be cleared by first-order processes.[159]

Table 2.10 shows DAFs for different species and the resulting interspecies UF, based on a partial UF of 3 to account for toxicodynamic differences. As this table shows, an interspecies UF of 10 may be insufficient for AELs based on studies in mice.

The process of identifying appropriate UFs is continually undergoing refinements, with increasing use of chemical-specific factors derived using PBPK models. Chemical-specific PBPK models are most often used to address the toxicokinetic component of the interspecies UF, although they can also be used to address the toxicokinetic component of the intraindividual UF. The next section of this chapter discusses the use of PBPK models for deriving chemical-specific UFs in more detail. Although much less common, chemical-specific factors can also be derived for the toxicodynamic component of the intraindividual and interspecies UFs, using in vivo, ex vivo, or in vitro data.[152] Table 2.11 lists chemicals assessed by the EPA for which PBPK models were used to address interspecies and/or intraindividual uncertainty and variability.

TABLE 2.10
Dosimetric Adjustment Factors and Interspecies Uncertainty Factors as a Function of Body Weight

Species	Weight (kg)	DAF	Interspecies UF
Mouse	0.03	7	21
Rat	0.25	4	12
Guinea pig	0.5	3	9
Rabbit	2.5	2	6

Notes: Values for weight and DAF are from U.S. EPA[139]; DAF calculated as $[BW_{animal}]^{-0.25}/[BW_{human}]^{-0.25}$.

TABLE 2.11
Subdivision of Uncertainty Factors for Inter- and Intraspecies Differences[162]

Species	UF_{AH}	UF_A / UF_H	Subdivision PK × PD
Mice	150	38	9.0 × 4.3
		4	2 × 2
Hamsters	100	25	7.0 × 3.6
Rabbits		4	2 × 2
Rabbits	40	10	4.0 × 2.5
Monkeys		4	2 × 2
Dogs			

Source: Reprinted from *Regul. Toxicol. Pharmacol*., 58/2, Hasegawa, R., Hirata-Koizumi, M., Dourson, M.L., Parker, A., Sweeney, L.M., Nishikawa, A., Yoshida, M., Ono, A., Hirose, A., Proposal of new uncertainty factor application to derive tolerable daily intake, 237–242, Copyright 2010, with permission from Elsevier.

Notes: UF_{AH}, composite UF accounting for inter-individual variability and interspecies uncertainty; UF_A, UF for interspecies uncertainty; UF_H, UF for intraindividual variability; PK, pharmacokinetics; PD, pharmacodynamics.

The EPA used chemical-specific information to replace the default values for the toxicokinetic components of the intraindividual and interspecies UFs for developing an RfD for boron.[161] The endpoint of concern was the developmental effects in rodents; hence, the pregnant female was considered the sensitive population and the basis for the NOAEL. Data were available for differences in clearance rates of boron across species (the toxicokinetic component of the UF_A), allowing a UF_A for toxicokinetic of 3.3—only slightly higher than the default value of 3.16. For the toxicokinetic component of the UF_H, data on differences in glomerular filtration rate among women resulted in the use of value of 2.0 (vs. the default value of 3.16). Default values of 3.16 were used for the toxicodynamic component for the UF_A and for the UF_H. The total UFs in this analysis were 66, rather than the default of 100. Thus, the use of chemical-specific UFs for boron resulted in a more scientifically founded RfD that was somewhat lower than would have resulted from use of default values.

Another approach, to account for both intraindividual variability and interspecies uncertainty, has been proposed by Hasegawa and coworkers.[162] They identified distributions for both intraindividual variability and interspecies uncertainty and combined them using probabilistic methods to derive species-specific UFs, shown in Table 2.11, that account for both interindividual variability and interspecies uncertainty. Similar to the EPA's recommended approach of calculating an HED based on $BW^{3/4}$ scaling, these combined UFs are consistent with the use of the default UF of 10 for AELs based on studies in rats. The findings suggest that the default UFs may not be sufficient for AELS based on studies in mice.

Alternative Approaches to the Risk Reference Dose

For compounds that have been well studied, particularly in humans (both in terms of exposure and toxicity), distributional population approaches have been used to evaluate toxicity and provide input into the decision-making process. Such approaches have been applied mainly to evaluation of the NAAQS for criteria air pollutants (e.g., carbon monoxide [CO], lead, nitrogen dioxide [NO_2], ozone, PM, SO_2). Much of the basis for the selection of an NAAQS is based on data from human epidemiology and controlled exposure studies, although animal data are used in a supporting role.

An example of a distributional population approach can be seen in the evaluation of CO toxicity and exposure by the EPA.[163] As part of this assessment, the EPA reviewed several studies that evaluated the relationship between exposure to CO, using carboxyhemoglobin (COHb) in blood as an indicator, and percent decrease in time to angina or pain in the chest, as an indicator of effect. Most of the studies showed an impact of low COHb levels on angina. However, there was no consistent dose–response relationship when studies were analyzed in the aggregate. This may have been due to differences in study design, study populations, and other factors. Because of the lack of a clear dose relationship, the EPA evaluated the impact of different concentrations of CO in air upon various *cutoff* points of COHb—from 2.1% to 3.0%. These cutoff points are conceptually similar to the LOAEL used in RfD development. Levels of CO that result in COHb of 2.9%–3.0% or higher might constitute frank effect levels (FELs). This is because levels of COHb of 2.9%–3.0% or higher in persons with heart disease are considered as possibly increasing the risk of myocardial ischemia and diminishing blood flow to the heart.

The risk of CO exposure to people with heart disease in Denver (36,345 individuals at the time) was estimated under different CO levels.[163] The number of person-days where individuals might have at least one hourly COHb level greater than or equal to a defined percent COHb was estimated. Table 2.12 presents some of the results of this analysis. For example, under conditions at the time (considering both indoor and outdoor sources of CO), there were approximately 488 person-days in which the Denver population with preexisting heart disease would experience COHb levels greater than 2.1%. If only ambient air is considered, the person-days drop to 72. If the NAAQS for CO is attained, the person-days drop to 457 for all sources and 0 for ambient air only. This type of analysis is useful in showing the benefits of CO reduction, as well as identifying the significance of different sources.

Distributional population approaches to evaluating environmental chemicals provide a more comprehensive evaluation of risks than the RfD approach. Rather than focusing on point estimates (e.g., above or below the RfD), this method allows one to more fully characterize variability in responsiveness to chemicals and variability in exposure levels among defined populations. However, this approach is feasible only for a limited number of chemicals and is quite resource intensive.

TABLE 2.12
Heart Person-Days with at Least One Hourly COHb Estimate ≥ Value for Four Alternative Scenarios

	As Is Air Quality		*Just Attain* Air Quality	
Exposure Indicators	*Ambient Air* Plus Internal Sources	*Ambient Air* without Internal Sources	*Ambient Air* Plus Internal Sources	*Ambient Air* without Internal Sources
COHb ≥ 2.1%	488	72	457	0
COHb ≥ 3.0%	37	0	24	0

Source: Adapted from U.S. Environmental Protection Agency, *Review of the National Ambient Air Quality Standards for Carbon Monoxide 1992. Reassessment of Scientific and Technical Information*, Office of Air Quality Planning and Standards, Research Triangle Park, NC, EPA-450/5-84-004, 1992.

Incorporating Information on Severity of Effect

A critical difference in evaluating risks for carcinogenicity versus risks for noncancer effects is that, whereas from a regulatory perspective almost all types of cancer are considered equally severe, regulations based on noncancer effects can account for variations in severity. In general, regulatory agencies do not distinguish among carcinogens on the basis of malignancy of tumor type. Despite advances in earlier diagnosis and treatment, the fatality rate for cancer overall is still relatively high and, even when treatable, the treatment methods are not without adverse effects. For example, the relative 5-year survival rate for all cancers from 2001 to 2007, excluding nonmelanoma skin cancer, was 69% for whites and 59% for blacks,[164] meaning that 31% of whites and 41% of blacks did not survive 5 years past diagnosis. Nonmelanoma skin cancers such as squamous and basal cell carcinoma, which can be induced by agents such as ultraviolet (UV) light and arsenic, have relatively low (<10%) fatality rates, even when untreated.[165]

In contrast, target-organ effects range greatly in severity. For example, using the same target organ and susceptible population—namely, airways for individuals with asthma—responses may range from imperceptible mild bronchoconstriction induced by low levels of SO_2 to a fatal asthmatic response, as may have been due to acid sulfate pollution in the London smog episodes.[60,166] Table 2.13 lists effects, ranging from enzyme induction to severe organ function, and corresponding effect categories (e.g., NOAEL, LOAEL, FEL). Using these categories, the California EPA's Office of Environmental Health Hazard Assessment (CalEPA OEHHA) recommends using a LOAEL/NOAEL UF of 6 rather than 10 for mild effects (severity level grade 5 or less) for deriving acute AELs for inhalation exposure.[167]

TABLE 2.13
Relationship between Effect Categories and Severity

Severity Level	Effect Category	Effect
0	NOEL	No observed effects
1	NOAEL	Enzyme induction or other biochemical change (excluding signal transduction effects), consistent with possible mechanism of action, with no pathologic changes, no change in organ weights, and no downstream adverse developmental effects
2	NOAEL/LOAEL	Enzyme induction and subcellular proliferation or other changes in organelles, consistent with possible mechanism of action, but no other apparent effects
3	NOAEL/LOAEL	Hyperplasia, hypertrophy, or atrophy, but without changes in organ weight
4	NOAEL/LOAEL	Hyperplasia, hypertrophy, or atrophy, with changes in organ weight
5	LOAEL	Reversible cellular changes including cloudy swelling, hydropic change, or fatty changes
6	(LO)AEL	Degenerative or necrotic tissue changes with no apparent decrement in organ function
7	(LO)AEL/FEL	Reversible slight changes in organ function
8	FEL	Pathological changes with definite organ dysfunction, which are unlikely to be fully reversible
9	FEL	Pronounced pathological change with severe organ dysfunction and long-term sequelae; developmental dysfunction including biochemical changes affecting signal transduction that result in developmental defects or dysfunction
10	FEL	Life-shortening or death

Source: California Office of Environmental Health Hazard Assessment, Technical support document for the derivation of noncancer reference exposure levels, Air Toxicology and Epidemiology Branch, 2008.

Notes: AEL, adverse effect level; FEL, frank effect level; LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level; NOEL, no observed effect level.

Consideration of severity then becomes important for regulatory decision-making in several ways. For RfD development, is an effect such as a 2% decrease in weight a NOAEL or a LOAEL? Does an effect of sufficient severity warrant protection of 95% the population or 99% of the population? Several agencies and organizations have developed approaches to incorporate information on severity of effect into the risk assessment or risk management process for environmental chemicals.

Information on severity has been incorporated into the *reportable quantity* (RQ) definition under CERCLA. Under this statute, releases of chemicals in amounts greater than some predetermined level, defined as the RQ, require that the EPA be notified of the release.[168] The amount of release that triggers notification is based on an assessment of the potency of the chemical and severity of the effect at the dose level where the potency was quantified. The ranking of severity is shown in Table 2.13,[168] where it can be seen that effects range from slight biochemical changes through gross toxicity—including lethality. Unlike the RfD process, this scoring is not restricted to datasets containing information on mildly adverse effects from subchronic or chronic studies. The RQ process can result in development of scoring indicators from lower-quality datasets, involving shorter time periods of exposure and more severe toxicity. The RQ process demonstrates the use of severity information in both risk assessment (developing RQ indicators) and risk management (defining release levels requiring notification as associated with defined RQ values).

Efforts involving the use of categorical exposure–response modeling demonstrate additional approaches toward consideration of severity. Guth and coworkers[158] analyzed acute effects resulting from methyl isocyanate exposures of less than 8 h in duration (as seen in Figure 2.7). Effects were separated into three categories—NOAEL (circles), adverse effect level (triangles), and lethal (squares). Effect categories were then analyzed on the basis of concentration and time using logistic regression. The straight line in Figure 2.7 presents the level above which there is a 90% probability that the true NOAEL lies. This method allows the use of data from a range of severity endpoints and considers various combinations of exposure level and exposure duration. Conceivably, this type of approach could lead to the development of concentration-time nomograms for definition of NOAELs for different exposure durations.

PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS

PBPK models, which can be used to refine derivation of AELs and cancer potency factors, thus have implications for regulations. Rather than provide a detailed discussion of the structure and development of PBPK models, this chapter will focus on their regulatory impact. PBPK models are discussed in detail in Chapter 6.

PBPK models are essentially mechanistic models that describe quantitatively the pharmacokinetic processes affecting the disposition and metabolism of a chemical from the time it is absorbed through its elimination from the body, including its interaction with various body tissues. By incorporating basic physiologic and metabolic parameters, PBPK models are designed to predict

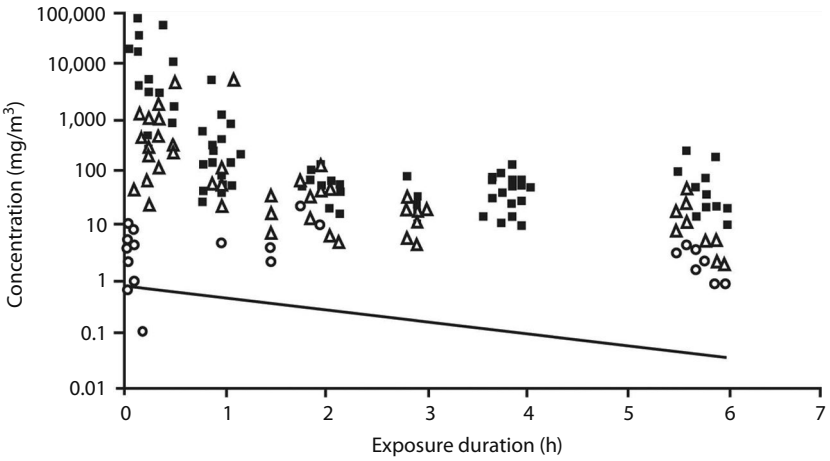


FIGURE 2.7 Categorical data from published results on methyl isocyanate.[158] Categorical data from published results on methyl isocyanate for exposures of less than 8 h in duration are shown as NOAEL (circles), AEL (triangles), or lethality (squares). The maximum likelihood model fit is shown by the line representing the model prediction of p = 0.1 that severity is greater than the NOAEL category at the corresponding exposure concentration and duration. (Reprinted from Beck, B.D. et al., *Fund. Appl. Toxicol*., 20/1, 1, Copyright 1993. With permission from Society of Toxicology.)

kinetic behavior over a wide range of doses and exposure conditions. PBPK models can also account for nonlinear processes, such as saturation of metabolic enzymes, enzyme induction or inactivation, and depletion of antioxidants.[169]

PBPK models involve multiple compartments defined by having similar physiology, with tissue weights and blood flow obtained from experimental data and compartmental rate constants based on physicochemical/biochemical properties of the modeled chemical.[170] Physiological processes such as absorption, metabolism, cardiac output, and clearance are described using a series of mathematical equations.[169] Tissues or compartments typically include target tissue and those involved in storage, metabolism, or clearance. PBPK models also consider transfer of chemical between blood and tissues, either via passive diffusion or active transport, and metabolism in liver or other tissues, either as linear (first-order) or saturable (Michaelis–Menten). PBPK models may consider other processes that can affect kinetics, such as protein binding in the blood or in tissues.[169,170]

PBPK models may be developed to quantify the magnitude and time course of exposure at the critical target site, for both the parent compound and metabolites. After the target tissue dose in the animal model has been estimated and validated, the information can be scaled to the human to obtain an estimate of target-organ dose in humans. This estimate may then be used to predict human cancer risk under different exposure conditions or develop more precise estimates of the AEL, enabling use of chemical-specific, rather than default, UFs. PBPK models can be used to extrapolate between and within species, between high and low exposure levels, and between exposure routes and durations.[169,170] The extent to which PBPK models are able to extrapolate toxicity data between or within species depends on the level of biological detail included in the model; the level of confidence in model predictions depends on the quality of data used for defining model parameters. Table 2.14 shows chemicals for which the EPA has used PBPK models to derive toxicity criteria. Figure 2.8 illustrates use of PBPK models for derivation of cancer potency factors for dichloromethane (methylene chloride). The EPA used a PBPK model to extrapolate target tissue doses from mice to humans and estimate toxicokinetic variability within humans.[171]

PBPK modeling can improve dose–response assessment by accounting for sources of change in the proportions of applied to delivered dose in animals versus humans and at high versus low doses. Although this approach does not account for the sensitivity of the target tissue to the delivered dose (which may differ in humans and animals or between high and low doses), it still addresses some major areas of uncertainty in risk assessment. In fact, many sources of potential nonlinearity in applied dose–response assessment involve saturation or induction of enzymatic processes at high doses or differences in toxification/detoxification pathways between humans and animals or across doses.

It is important to note that there are substantial uncertainties in PBPK modeling. For example, Hattis and coworkers[179] compared PBPK models for perchloroethylene developed by seven different authors and found appreciable differences among the model predictions. With respect to methylene chloride, Clewell[180] notes the importance of the tissue distribution of glutathione S-transferase (GST) enzyme activity across species, especially in humans, as a source of model uncertainty. Given identical exposure levels in humans, the range of values for metabolized perchloroethylene spans a 50-fold range, with six of the seven models having predictions with a 14-fold range. Studies to refine estimates of GST enzyme activity across species and within the human population will serve to provide more refined estimates of dose across humans and, hence, of potential differences in susceptibility.

It should be emphasized that although PBPK models can estimate dose at the target tissue, they do not provide information regarding the most appropriate dose–response relationship.[181,182] Furthermore, full validation of the model at

TABLE 2.14
Use of Physiologically Based Pharmacokinetic Models by U.S. EPA for Deriving Toxicity Criteria

Compound	PBPK Model Characteristics	Dose Metric	Critical Noncancer Endpoint	Application to Toxicity Criteria
Carbon tetrachloride[172]	*Rat/human model* • Four compartments: liver, fat muscle, viscera (richly perfused organs) • Saturable metabolism in liver • Estimates disposition of CCl_4 following inhalation	Mean rate of hepatic CCl_4 metabolism	Fatty liver changes in rats	• Estimate HEC for RfC and IUR • Replace PK component of interspecies UF for RfC
Dichloromethane[171]	*Mouse model* • Probabilistic • Five compartments: lung, fat, richly perfused organs, slowly perfused organs, liver • Saturable metabolism by P450 and first-order metabolism by GST in liver and lung • Estimates disposition of dichloromethane following inhalation *Human model* • Similar to mouse model, with addition of pathway modeling metabolism to carbon monoxide and formation of COHb	Amount of dichloromethane metabolized by GST pathway	Hepatocyte vacuolization in mice	• Estimate HED and HEC for RfD, CSF, RfC, and IUR • Replace PK component of interspecies UF for RfD and RfC • Estimate intraindividual PK variability • Replace PK component of interindividual UF for RfD and RfC
Ethylene glycol monobutyl ether (EGBE)[173]	*Human model* • Model for EGBE linked to model for EGBE metabolite 2-butoxyacetic acid • Eight compartments in EGBE model: lungs/arterial blood, rapidly and slowly perfused organs, fat, skin, muscle, liver, gastrointestinal tract • Model for 2-butoxyacetic acid includes kidney as additional compartment, to allow for saturable elimination pathway • First-order metabolism in liver: • EGBE to 2-butoxyacetic acid, as well as other metabolites and conjugates • 2-Butoxyacetic acid to carbon dioxide • Estimates disposition of EGBE, via metabolism to 2-butoxyacetic acid, following inhalation, dermal, and oral exposure.	AUC in blood of EGBE metabolite 2-butoxyacetic acid	Hemosiderin levels in rat liver	• Estimate HEC for RfC • Replace PK component of interspecies UF • Enable route-route extrapolation from HEC to HED, for deriving RfD • Replace PK component of interspecies UF

(*continued*)

TABLE 2.14 (continued)
Use of Physiologically Based Pharmacokinetic Models by U.S. EPA for Deriving Toxicity Criteria

Compound	PBPK Model Characteristics	Dose Metric	Critical Noncancer Endpoint	Application to Toxicity Criteria

Methanol[174]	*Rat model*
	• Six compartments: lung/blood, fat, liver, stomach, intestine, rest of the body
	• Saturable metabolism in liver via high affinity/low capacity and low affinity/high capacity pathways
	• Estimates disposition of methanol following inhalation or oral exposure
	Human model
	• Based on rat model, with exclusion of intestine, and inclusion of bladder
		AUC of methanol in blood	Decreased brain weight in neonatal rats	• Estimate HEC for RfC
				  • Replace PK component of interspecies UF
				• Enable route-to-route extrapolation from HEC to HED, for deriving RfD
				  • Replace PK component of interspecies UF
				• Provide support for value of 10 for interindividual UF

Tetrachloroethylene (PCE)[175]	*Rat/mouse/human model*
	• Comprised of main model for PCE, along with submodels for oxidative metabolism, conjugative metabolism (rat and human only), and trichloroacetic acid (TCA)
	  • Main PCE model includes nine compartments: respiratory tract, rapidly and slowly perfused tissues, fat, gut, liver, kidney, stomach, and duodenum
	  • Oxidative metabolism submodel estimates oxidation of PCE to TCA in liver, lung, and kidney
	  • Conjugative metabolism submodel estimates formation of conjugates in liver and kidney
	  • TCA submodel includes the liver and the rest of the body
	• Saturable and first-order metabolism
	  • Saturable oxidation and conjugation by mouse
	  • Saturable and first-order oxidation, first-order conjugation by human
	• Estimates disposition of PCE following inhalation, oral, or intravenous exposure
		AUC of PCE in blood	Neurological effects in humans	• Enable route-to-route extrapolation from inhalation to oral POD, for deriving RfD and CSF
				• Estimate HEC based on concentrations in mouse study for deriving IUR

1,1,1-trichloroethane[176]	*Human model* • Four compartments: liver, fat, rapidly and slowly perfused tissues • Saturable metabolism in liver • Estimates disposition of 1,1,1-trichloroethane following inhalation, intravenous, gavage, or drinking water exposure	Venous blood concentration	Neurobehavioral effects in humans	• Predict effect levels at 4, 8, 24 h, and 14 days, based on POD for a 1 h exposure

Trichloroethylene (TCE)[177]	*Rat/mouse/human model*
	• Comprised of main model for TCE, along with submodels for oxidative metabolism, conjugative metabolism (rat and human only), trichloroethanol (TCOH), trichloroethanol-glucuronide conjugate (TCOG), and trichloroacetic acid (TCA)
	  • Main TCE model includes nine compartments: respiratory tract, rapidly and slowly perfused tissues, fat, gut, liver, kidney, stomach, and duodenum
	  • Oxidative metabolism submodel estimates oxidation of TCE to TCOH, TCA, and other metabolites in lung and the rest of the body
	  • Conjugative metabolism submodel estimates formation of glutathione (GSH) conjugate in liver (humans only) and kidney (rats and humans), bioactivation of glutathione conjugate in kidney, and urinary excretion
	  • Submodels for TCOH, TCOG, and TCA include the liver and the rest of the body, with an additional bile compartment in the TCOG submodel
	• Saturable metabolism
	  • Oxidation of TCE to TCOH in liver and lung; GSH conjugation of TCE in liver and kidney
	  • Oxidation of TCOH to TCA and conjugation with glucuronide in liver
	• Estimates disposition of TCE following inhalation, oral or intravenous exposure
		Amount of TCE oxidized (per kg body weight)$^{3/4}$	Decreased thymus weight in mice
			Fetal heart malformations in rats
				• Estimate HED for RfD
				  • Replace PK component of intraindividual and interspecies UFs
				• Estimate HEC for RfC, based on route-to-route extrapolation
				  • Replace PK component of intraindividual and interspecies UFs
				• Enable route-to-route extrapolation from IUR to CSF

Vinyl chloride[178]	*Rat/human model*
	• Five compartments: lung, liver, fat, richly and slowly perfused tissues
	• Saturable metabolism in liver via high affinity/low capacity and low affinity/high capacity pathways
	• Estimates disposition of vinyl chloride following inhalation or oral exposure
		Amount of vinyl chloride metabolized per liver volume (for route–route extrapolation)	Liver cell polymorphisms and cysts in rats	• Estimate HED for RfD
				  • Replace PK component of interspecies UF
				• Estimate HEC for RfC, based on route-to-route extrapolation
				  • Replace PK component of interspecies UF
				• Estimate HED based on doses in rat study for deriving CSF
				• Estimate HEC based on concentrations in rat study for deriving IUR

Notes: AUC, area under the curve; CSF, cancer slope factor; COHb, carboxyhemoglobin; HEC, human equivalent concentration; GST, glutathione S-transferase; HED, human equivalent dose; IUR, inhalation unit risk; PK, pharmacokinetic; POD, point of departure; RfC, reference concentration; RfD, reference dose; UF, uncertainty factor.

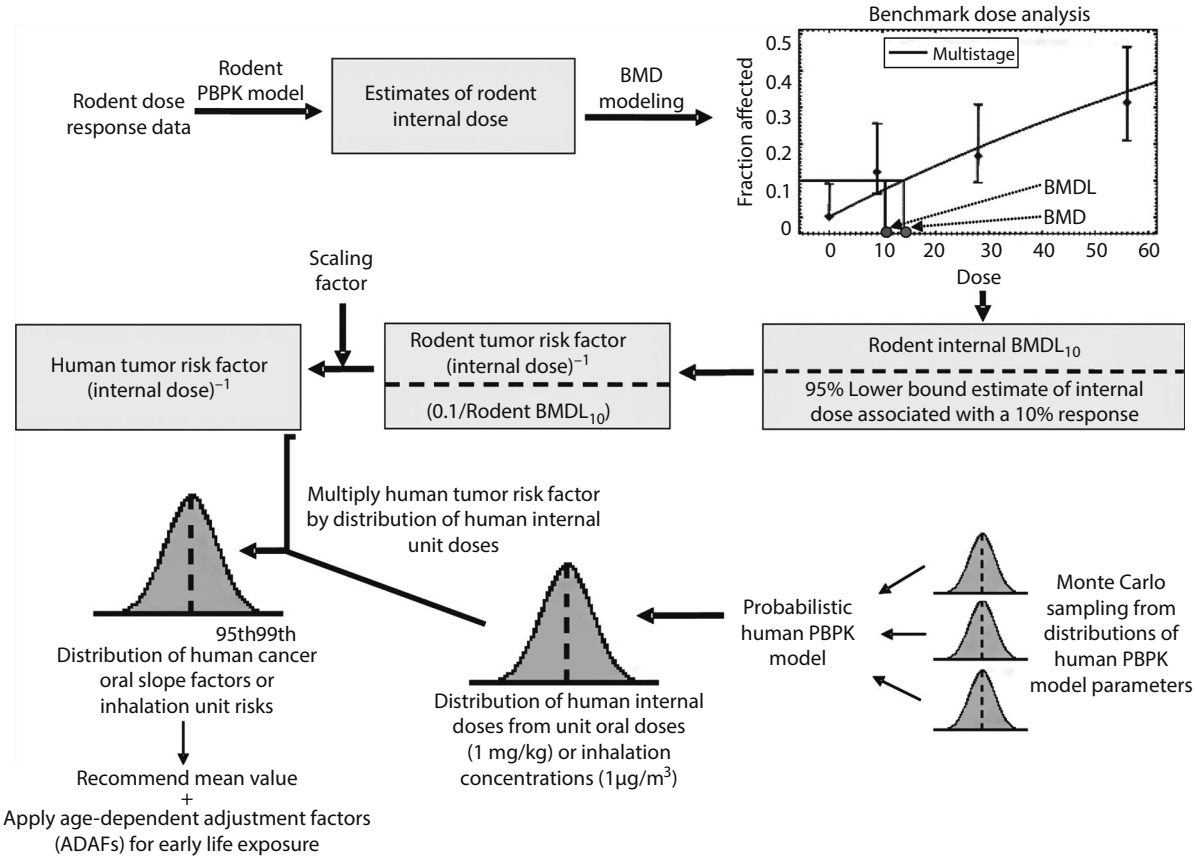


FIGURE 2.8 Use of PBPK models for derivation of CSF and IUR for dichloromethane. (From U.S. Environmental Protection Agency, Toxicological Review of Dichloromethane (Methylene Chloride) (CAS No. 75-09-2) in Support of Summary Information on the Integrated Risk Information System (IRIS) (Final), EPA/635/R-10/003F, 2011.)

the relatively low levels of environmental chemicals to which humans are typically exposed can be difficult. Another limitation of PBPK models is that, despite modeling processes that vary with life stages, such as metabolic clearance rates,[183,184] they are typically parameterized using adult values due to lack of availability of data for other life stages.[139]

ROLE OF HIGH-RISK GROUPS

The "Evaluation of Noncancer Effects" section described how AELs are used by regulatory agencies to estimate acceptable levels for noncancer effects. One of the UFs used for deriving AELs accounts for variations in population susceptibility. The purpose of this section is to expand upon that issue, describe the basis for variations in susceptibility and the magnitude of that variation, and demonstrate the relationship of this issue to the regulatory process. (For more detail, the reader is referred to references.[185–187])

There is a high degree of variability in the exposure level required to elicit a response in humans.[185,188] Knowing which groups of individuals are at high risk with respect to pollutants, for example, is very important, since these individuals will be the first to experience morbidity and mortality as pollutant levels increase. If the high-risk segments of a population are protected, the entire population is thus protected.

Consideration of Specific High-Risk Groups

A better approach than the use of generic UFs is to consider, when data are available, specific high-risk groups on a chemical-by-chemical basis. For certain chemicals, there are clear examples of groups more susceptible to adverse health effects (reviewed in Refs. [189–191]). These include the following:

- Individuals with genetic variations in metabolism. For example, a slow acetylator phenotype is associated with an increased risk of bladder cancer following exposure to aromatic amine dyes.[189]
- Individuals with inherited genetic defects. For example, individuals with xeroderma pigmentosum, a rare autosomal recessive disease, have defects in the nucleotide excision DNA repair pathway, which increases the risk of skin cancer by more than 1500-fold.[191,192]
- Individuals with preexisting illness. For example, individuals with asthma are more susceptible to air pollutants such as ozone and PM,[190,193] and those with hepatitis B are more susceptible to liver cancer.[189]

Other factors that can affect susceptibility to environmental chemicals include gender, age, lifestyle (e.g., cigarettes, alcohol, diet), and low socioeconomic status, which can be

associated with poor living conditions and inadequate health care.[27,194,195] The role of diet in the etiology of certain types of cancer is suggested in studies demonstrating an inverse relationship between the amount of vitamin A in the diet and susceptibility to hydrocarbon-induced epithelial cancers.[196] With respect to age, both young children and the elderly may be more susceptible to certain chemicals—children may be more susceptible due to reduced levels of xenobiotic-metabolizing enzymes, while the elderly may be more susceptible due to impaired cardiovascular and renal function.[184] Thus, it is likely that—even given the same exposure—individuals are not equally susceptible to the induction of cancer and other adverse health effects and, in many cases, the differential susceptibility could be large. In addition, certain subgroups may be at greater risk—not because of an inherent difference in toxicological susceptibility but because they are more likely to be exposed. For example, young children are at greater risk from soil contaminants because they tend to incidentally ingest more soil and dust than older children and adults due to significant hand-to-mouth activity.[197] Exposure to chemicals in the environment may also be higher in low-income communities, which are more likely to be located near one or more pollution sources.[194]

Regulatory agencies have focused in particular on the potential for children to be more susceptible to environmental chemicals. In 1996, the EPA emphasized its focus of protecting infants and children in a report titled *A National Agenda to Protect Children's Health from Environmental Threats*.[198] The 1996 FQPA requires use of an additional tenfold UF for pesticides to account for potential prenatal and postnatal developmental toxicity[199] for agents. The UF is not used if the agent has not been demonstrated to exhibit developmental toxicity in a reliable testing program. As noted by Roberts,[200] children may be more susceptible because many cells and organs are undergoing growth and development and have not yet matured. Metabolic capacity of the liver is also not fully developed when children are born, although most metabolic enzymes reach adult levels by 1 year of age.[183] A child's diet and physical environment, and therefore his or her exposure potential, may vary significantly from that of an adult. For many routes of exposure (air, food, water, and dermal exposures), chemical intake (on a per kilogram body weight basis) is generally greater for infants and children than adults.[201]

However, a subgroup at high risk for one chemical is not necessarily at high risk for other chemical exposures. For example, although children are often assumed to be more sensitive than adults, this is not always the case. Reactions to pharmaceuticals, since they are more widely studied than responses to environmental exposures, can be considered as examples. Acute overdoses of acetaminophen result in less hepatotoxicity in children than adults with comparable plasma concentrations, possibly due to differences in metabolism.[202]

There is currently debate about whether existing risk cancer assessment methods adequately account for more highly susceptible groups. Only recently has evaluation of carcinogen exposure addressed the role of population variability in susceptibility to carcinogens. As discussed in the following, the EPA now has guidelines to account for increased susceptibility of children to mutagenic carcinogens.[203] While differential susceptibility has generally not been addressed in cancer risk assessment, the conservatism of the process has been assumed to result in adequate protection of high-risk groups.

For noncancer effects, there is debate over the appropriate UFs to account for high-risk subgroups. At a conference organized by the ILSI and EPA,[204] it was suggested that "in many cases, genetic variation in human susceptibility may be greater than an order of magnitude when comparing differences between children and adults." A coalition of farm food, manufacturing, and pest management organizations concluded that it was not necessary to use an additional UF of 10 (as required by the FQPA) *across the board* to protect infants and children.[205] The coalition also concluded that the standard default UFs are adequate for a pesticide with a complete and reliable database, and an additional UF "should only be applied to an endpoint that is relevant to protection of fetuses, infants, and/or children."[205] The EPA is looking into establishing criteria for appropriate use of the tenfold additional FQPA UF.[199,206] In 2002, the EPA's RfD/RfC Technical Panel concluded that the intraspecies UF and, if necessary, the database UF should account for uncertainty regarding differences in susceptibility for perinatal exposures, such that for most chemicals an additional UF of 10 to protect infants children should not be necessary.[139] Overall, the best approach is to consider susceptible subgroups on a case-by-case basis when data are available, for both carcinogens and noncarcinogens.

Regulatory Implications

The role of population variability should be considered by regulatory agencies in risk assessments for both carcinogens and noncarcinogens. Identification and quantitative characterization of susceptible populations could provide decision-makers with a theoretical framework on which to base regulatory action. For example, Tamplin and Gofman[207] have employed knowledge of susceptible populations in predicting the incidence of cancer from radiation pollution in drinking water to help define acceptable levels of exposure. They assumed that the latency period is shorter for in utero exposure than for all radiation exposure beyond birth (i.e., 5 vs. 15 years). Consideration of the increased susceptibility of the fetus to radiation-induced cancer resulted in greater estimates of cancer risk.[208]

The EPA has specifically evaluated the increased sensitivity of high-risk groups in setting NAAQS for the criteria air pollutants (i.e., CO, lead, NO_2, ozone, particulates, and sulfur dioxide) and establishing drinking water standards for some environmental chemicals. Examples of the high-risk groups considered are shown in Table 2.15. For instance, the NAAQS for lead considers high-risk populations in a quantitative way by estimating the fraction of the susceptible subpopulation (children) that would be protected at different air levels of lead.[209] A detailed description of the EPA's consideration of high-risk groups in the derivation of drinking water standards for nitrates and cadmium (Cd) is presented in the following.

TABLE 2.15
High-Risk Groups in the Derivation of Standards by the EPA

A. Drinking Water Standards

Substance	High-Risk Condition Considered
Arsenic	None
Barium	No specific groups, but a safety factor of two incorporated to account for variation (or increased susceptibility) within the human population
Cadmium	None
Fluoride	Children—to prevent mottling of teeth
Lead	Children—to prevent neurological disorders
Nitrate	Infants—to protect against methemoglobinemia
Selenium	None
Sodium (no standard)	Individuals with heart and kidney disease
Chlorinated hydrocarbon insecticides (noncarcinogenic)	None
Chlorophenoxy herbicides (noncarcinogenic)	None

B. National Ambient Air Quality Standard

Substance	Original Group	Primary Groups Currently Considered
Carbon monoxide	Individuals with neurological or visual impairment	Adults with heart disease (angina, coronary artery disease)
Lead	Children—to protect against neurological and hematological impairment	Same
Nitrogen dioxide	Children—to protect against respiratory infections; also concern for changes in lung structure	Same
Ozone	Asthmatics	Exercisers, individuals with preexisting disease
Particulates	Elderly, individuals with cardiopulmonary disease	Same
Sulfur dioxide	Elderly, individuals with cardiopulmonary disease	Asthmatics

Nitrates in Drinking Water

The drinking water standard of 10 mg nitrate (NO_3^{-2}) as mg nitrogen per liter is designed to prevent the formation of elevated levels of methemoglobin (MetHb) in infants. In the presence of nitrite (NO_2^{-1}) formed from NO_3^{-2}, hemoglobin is oxidized to MetHb, which is not able to reversibly combine with oxygen. Levels of 1%–2% and 2%–5% MetHb are typical in the blood of adults and infants, respectively. When concentrations of MetHb are less than 5%, there are no obvious indications of toxicity. However, with levels of MetHb from 5% to 10%, clinical signs of toxicity (e.g., cyanosis) may appear.[185]

Compared to adults, infants are at considerable risk for nitrate-related toxicity. Factors that predispose infants to the development of MetHb formation include the following:

1. The incompletely developed ability to secrete gastric acid. This permits the gastric pH to be high enough (5–7 pH) to permit the growth of nitrate-reducing bacteria in the gastrointestinal tract, which facilitates conversion of NO_3^{-2} to NO_2^{-1} before absorption into the circulation.[210]
2. The higher levels of fetal hemoglobin in infants. This form of hemoglobin is more susceptible than adult hemoglobin to oxidation to MetHb.[211]
3. The diminished enzymatic capability of infants to reduce MetHb to hemoglobin.[212]

Research has revealed that levels of NO_3^{-2} beyond 20 mg/L result in a marked increase in the frequency of methemoglobinemia in infants but not adults.[185] Consequently, a drinking water standard of 10 mg/L is principally designed to prevent the occurrence of elevated levels of MetHb in infants, whereas a concentration of 20 mg/L would still protect adults.

Cadmium

Studies with rats show that renal damage is initiated at a kidney concentration of 200 ppm Cd. The EPA calculated that humans would need to ingest 50 g Cd/day for 50 years to reach this level of 200 ppm in their kidneys. In the derivation of the Cd drinking water standard, the EPA assumed a daily Cd exposure of 75 μg from the diet and 20 μg from water. This 20 μg Cd/day from drinking water would occur at a level

of 0.01 mg/L. The total daily Cd exposure is therefore approximately 95 µg Cd/day; thus, a safety factor of 4 was assumed.

In proposing its drinking water standard for Cd, the EPA requested feedback from the public as to whether the standard should include additional protection for cigarette smokers (smoking is a source of appreciable Cd exposure; approximately 1.5 µg Cd/cigarette).[185] Of the 52 comments received by the EPA on this issue, only three suggested the standard be modified to include protection for cigarette smokers. The EPA decided not to incorporate additional safety factors to protect smokers,[125] demonstrating a situation in which protection of a high-risk group was not taken into account in derivation of a standard.

Susceptible Groups and Early-Life-Stage Exposure to Carcinogens

Recent analyses by Ginsberg[213] and others (e.g., see Preston)[214] have evaluated whether children might represent a susceptible population with respect to carcinogen exposure. Answering this question is complicated by limitations in the available data. Relevant human data are derived primarily from epidemiological studies of radiation exposure and cancer development in atomic bomb survivors. Such studies provide evidence for increased risk of certain cancers from early-life exposure; however, it is difficult to extrapolate from studies of ionizing radiation, a direct-acting mutagen that can induce mutation at any stage of the life cycle, to chemical carcinogens, the majority of which are not direct-acting mutagens and require cell proliferation for indication of mutation (often via indirect mechanisms).[214]

Nonetheless, animal bioassays provide some insights on early-life-stage exposures to carcinogenic agents. For example, a greater tumorigenic response from early-life-stage than from later-life-stage exposures has been observed following acute exposure to mutagenic agents.[203,213] Mechanistic information also suggests a greater susceptibility to developing cancer for early-life-stage exposure from mutagenic agents. The high proliferation rate of cells early in life may increase the likelihood that a cell containing damaged DNA could replicate before the DNA is repaired. Studies of carcinogens with other MoAs (e.g., hormonally mediated) or genotoxic agents that are not direct acting but include genetic damage through other means (such as generation of reactive oxygen species) provide a more complicated picture, with evidence for and against early-life-stage susceptibility.

In response to concerns that children may be more susceptible to certain carcinogens than adults, the EPA developed guidance for early-life-stage exposures to carcinogens. Figure 2.9[203] provides a schematic for this approach. As with the 2005 EPA cancer guidelines (discussed in "Approaches to Interpretation of Carcinogenicity Findings" section), an understanding of the MoA is an important component of the approach. For agents with either a nonlinear or linear but nonmutagenic MoA, the dose–response approach is unchanged. However, for agents that the EPA concluded were likely to be linear due to a mutagenic MoA, the first approach recommended, where feasible, is chemical-specific adjustment. Where such an adjustment is not feasible, age-dependent adjustment factors are proposed—a tenfold increase in the potency factor for ages up to 2 years and a threefold increase in potency for ages 2 to <16 years of age.[203] The underlying premise is that direct-acting mutagens are likely to exhibit a linear, no-threshold dose–response; as discussed earlier in this chapter, this assumption considers neither repair mechanisms nor the potential for hormetic responses and, thus, may be especially conservative. The implications of these recommendations for regulatory decision-making remain to be seen.

Increased susceptibility of children to mutagenic carcinogens is directly incorporated in the EPA's CSF for vinyl chloride, for which there is evidence from studies in laboratory animals that early-life exposures differ both quantitatively and qualitatively from exposures occurring later in life.[178] To account for increased susceptibility of children, the EPA recommends a slope factor of 1.4 (per mg/kg-day) for lifetime exposure starting at birth, rather than the slope factor of 0.72 (per mg/kg-day) recommended for exposure during adulthood.[215]

IMPLICATIONS OF CHEMICAL INTERACTIONS FOR THE REGULATORY PROCESS

One of the major difficulties in current environmental public health practice is that the focus is on a limited number of environmental contaminants, with little consideration of interactive effects among pollutants. In fact, the number of environmental pollutants in different media is large, making it difficult to estimate the degree of public health protection afforded by the current regulatory paradigm (which largely focuses on single pollutants).

Recognition of the importance of considering potential interactions in the regulatory arena is not new. In 1996, the Safe Drinking Water Act Amendments required the EPA to address the potential for chemical interactions; efforts to date have focused primarily on disinfection by-products.[195] Also in 1996, the FQPA required the EPA to evaluate cumulative exposure to pesticides sharing a common mechanism of toxicity.[216] To date, cumulative risk assessments have been conducted for four groups of pesticides—organophosphates, N-methyl carbamates, triazines, and chloroacetanlides.[217]

Still, it is clear that, in many cases, the scientific and regulatory communities can do more to address the issue of multiple and/or interactive chemical exposures. In fact, animal models and human epidemiological studies show that interactions do occur among chemicals and that this can result, under certain circumstances, in greater-than-additive effects. For example, uranium miners who do not smoke have a fourfold greater risk of cancer than nonsmokers in the general population. However, uranium miners who smoke display a 40-fold greater cancer risk than the general population of nonsmokers.[218]

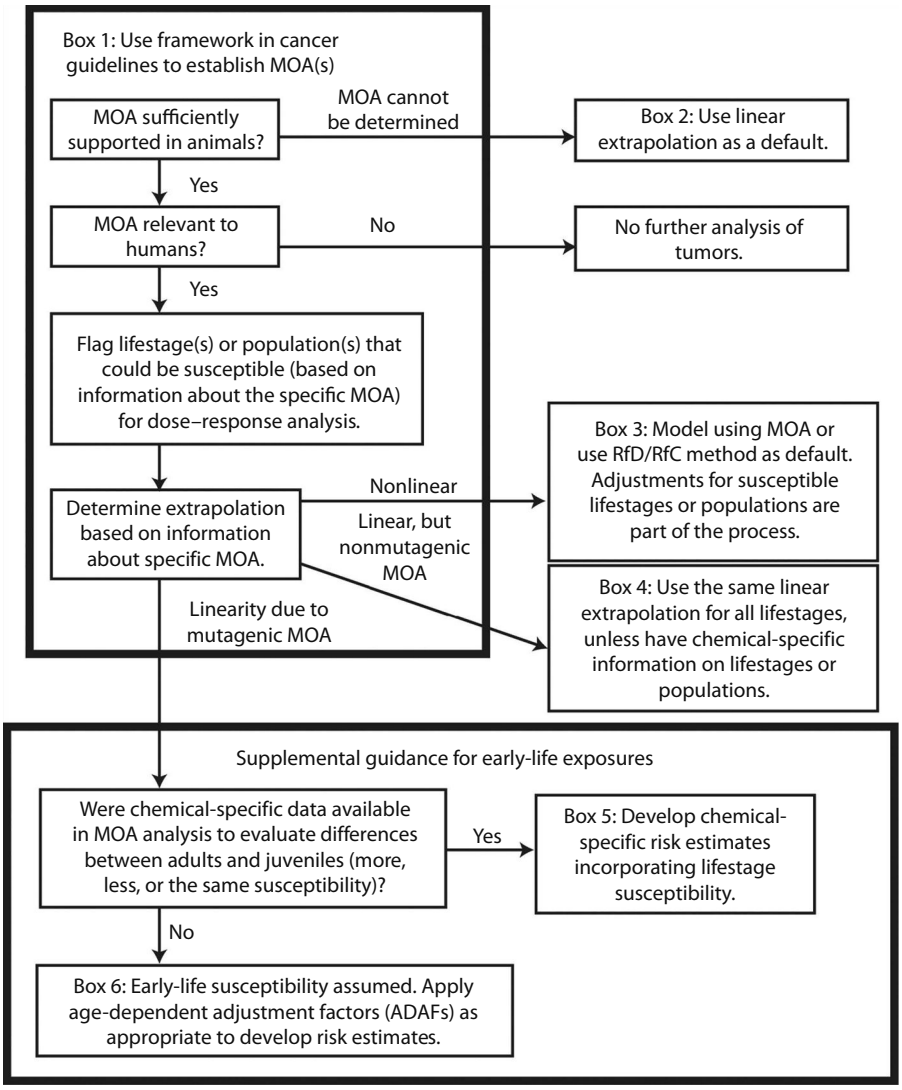


FIGURE 2.9 Flowchart for early-life risk assessment using MoA framework. (From U.S. Environmental Protection Agency, *Supplemental Guidance for Assessing Susceptibility from Early Life Stage Exposure to Carcinogens*, Risk Assessment Forum, Washington, DC, EPA/630/R-03/003F, 2005.)

Interactions have been studied for many years by the drug industry, insecticide manufacturers, and forensic/clinical toxicologists. Given the widespread use of multiple drug therapy, the need to anticipate possible adverse interactions has been essential. Thus, much of the basis of our current understanding of toxicological interactions is derived from the pharmaceutical industry. It should be recognized that there is uncertainty in extrapolating from drug exposures where doses are relatively high (i.e., by definition, at pharmacologically active doses) to environmental exposures, which are typically much lower than doses with established effects.

Chemical interactions have been broadly classified by three general terms: *addition* (additivity), when the toxic effect produced by two or more chemicals in combination is equivalent to that expected by simple summation of their individual effects; *antagonism*, when the effect of a combination is less than the sum of the individual effects; and *synergism*, when the effect of the combination is greater than would be predicted by summation of the individual effects. Other terms have been used, such as indifference and potentiation, which represent specialized aspects of antagonism and synergism, respectively.

Additive interactions for chemical mixtures can be characterized further as being either dose or response additive. The underlying assumption of dose additivity is that the individual chemicals in the mixture are toxicologically similar; therefore, the potency of the mixture is a function of the sum of the potencies of the individual chemicals. This approach is used for noncancer hazards, by way of the hazard index, which sums hazard quotients across chemicals and pathways.[219,220] An implication of this approach, as reflected in Figure 2.10, is that even though chemicals individually cause no toxicological response, they may result in toxicity when multiple chemicals (all below their NOAELs) are present in a mixture. In contrast, response additivity

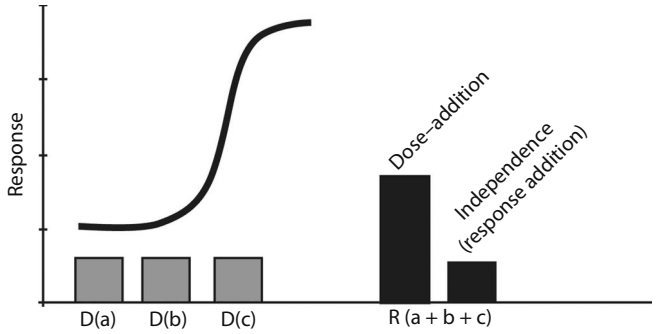


FIGURE 2.10 A model dose–response curve for an effect with a threshold.[222] Below the curve, bars indicate doses D(a), D(b), and D(c), each of which is one-half the toxic threshold for chemicals a, b, and c, respectively. The calculated combined response R (a + b+ c) would be greater or less than the threshold depending on whether a dose addition or independence model was used to predict the combined action of chemicals a, b, and c in a mixture. (Reprinted from *Toxicol. Appl. Pharmacol*., 201(2), Borgert, C.J., Quill, T.F., McCarty, L.S., and Mason, A.M., Can mode of action predict mixture toxicity for risk assessment?, 85–96, Copyright 2004, with permission from Elsevier.)

assumes that each chemical in a mixture acts independently to cause a similar health endpoint; thus, the toxicity of a mixture is a function of the toxic effects of the individual compounds.[220] In the example in Figure 2.10, response additivity would imply that no toxicity would ensue, since no individual chemical is associated with any toxicological responses (see also Monosson).[221] The EPA uses the response-addition approach for evaluating cancer risks, by first estimating cancer risk for each individual chemical in a mixture and then summing the individual cancer risks across chemicals.[220]

Implications of Chemical Interactions

Frequently, little information is available on toxic mechanisms. Nonetheless, regulatory agencies need to develop approaches in such situations when reasonable mechanistic predictions cannot be made. To this end, Finney[223] developed a theoretical mathematical approach for predicting the degree of toxicity derived from various types of chemical interactions. Of the 36 pairs of mixtures of industrial vapors tested for acute toxicity in rats, Pozzani and coworkers[224] found that only two deviated significantly from the calculations of Finney's theoretical approach for additive joint toxicity. According to Smyth et al.,[225] the study by Pozzani and coworkers[224] supported the hypothesis that the acute toxicity of randomly chosen chemical mixtures has a high likelihood of being accurately predicted by Finney's theoretical formula for additive joint toxicity. In an attempt "to evaluate the overall confidence that can be placed on the prediction of the joint toxicity of many chemical pairs," Smyth et al.[225] studied the toxicity of 27 industrial chemicals in all possible pairs to rats. Their results were consistent with Finney's[223] prediction that most interactions should be considered as additive until proven otherwise. Smyth et al.,[225] in agreement with the general findings of Pozzani and coworkers,[224] concluded that approximately 5% of the various combinations tested exhibited effects that were either less than or greater than additive.

However, it should be noted that recent studies of chemicals in mixtures at doses below the NOAEL provide evidence that dose additivity may overpredict the toxicity of a mixture (in which case, response additivity is a more appropriate characterization of the interaction). For example, Feron and coworkers[226] observed that exposure to a mixture of nephrotoxic chemicals, each below the NOAEL, did not result in a toxic response, suggesting that dose additivity may not appropriately describe interaction potential at low doses. A similar conclusion was reached by Borgert et al.[222] who postulated that the lack of additivity may reflect a different MoA at low doses.

Thus, it is possible that synergistic interactions—the type of interactions of greatest regulatory concern—may be less likely to occur at environmentally relevant exposure levels than at higher (e.g., pharmaceutical or some occupational) exposure levels.

Approaches Used by Regulatory Agencies to Assess Interactions

Despite the frequent lack of a clear mechanistic understanding of how chemicals may interact, regulatory agencies have developed interim approaches to facilitate the decision-making process. In this section, some of the typical approaches used by agencies are highlighted with illustrative examples.

Hazard Index Approach

Perhaps the simplest approach is the assumption of additivity of hazard. In this approach (applied to noncarcinogens), hazard indices (the ratio between the estimated dose and the AEL; see "Acceptable Exposure Level" section) are summed across chemicals and routes of exposure to obtain a total hazard index for a particular exposure setting.[227] If the decision criterion of a total hazard index of 1 is exceeded, further review is performed to determine which chemicals act at the same target organ. A subsequent summation is performed for chemicals grouped together by target organ. While this approach is useful as a screening approach, it has several limitations that must be considered when interpreting the results. These include (but are not limited to) the following:

- AELs (the denominator in the hazard index) for different chemicals contain different types and magnitudes of UFs. Thus, differences in the magnitude of a hazard index for a particular chemical or a pathway of exposure may reflect intrinsic differences in hazard as well as differences in the uncertainty of a particular toxicity value. This limitation can be addressed by use of the BMRs and PBPK models for deriving AELs.

- Different types of interactive effects are possible, even for chemicals that act at the same target organ. For example, organophosphates, which act via the inhibition of acetylcholinesterase at nerve endings, would generally be presumed to act in an additive manner.[228] In contrast, when consumed simultaneously, TCE and alcohol, both of which affect the central nervous system, can act synergistically (e.g., producing *degreaser's flush*); however, chronic alcohol consumption can, by induction of metabolizing enzymes, diminish the response to TCE.[80]

Both the EPA and ATSDR recommend using the hazard index approach as a default for most mixtures.[219,220] The hazard index approach, which assumes that greater-than-additive interactions are not likely at AELs, is likely appropriate in most situations. However, the possibility for greater-than-additive interaction effects due to cumulative, low-level chronic exposure cannot be completely ruled out.[229]

Toxicity Equivalency Factor Approach

The toxicity equivalency factor (TEF) approach has been applied to mixtures that contain structurally and toxicologically similar chemicals. Perhaps one of the best-known examples of the TEF approach was developed by the EPA for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other dioxin-like compounds, including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and PCBs.[230] This approach is based on the assumption that PCDDs and PCDFs exert toxicity through binding to the aryl hydrocarbon receptor (AhR) with subsequent effects on transcription and translational events responsible for toxicity. The most potent PCDD, 2,3,7,8-TCDD, has the greatest affinity for the AhR; hence, it is assigned a TEF of 1.0. TEFs are developed for individual PCDDs and PCDFs and expressed as a fraction (typically in orders of magnitude) of that of TCDD. Thus, RfDs and CSFs for PCDDs/PCDFs are calculated as a ratio to the RfD and CSF for 2,3,7,8-TCDD. While there is experimental support for this approach based on mixtures of PCDDs and PCDFs, there are few, if any, data from long-term studies.[231] Other uncertainties in this approach include the choice of a particular TEF value, which can be influenced by selection of endpoint, exposure duration, and dose, as well as the assumption of additivity, where competitive interactions may occur at sufficiently high doses.[232]

Cumulative Risk Assessment

There is a growing recognition, both in the United States and Europe, regarding the potential for cumulative risks from combined exposure to chemical as well as nonchemical stressors, such as poverty, poor diet, living conditions, and inadequate health care.[27,195] Specifically, low-income communities, many of which have a high percentage of minority populations, are more likely to be located near multiple pollution sources. Furthermore, results from some studies indicate that individuals with low income and education levels may be more sensitive to pollutant exposures.[194] In a study regarding ways to improve how the EPA conducts risk assessment, the NRC recommended that the EPA expand its efforts at assessing cumulative risks from chemical and nonchemical stressors, with the short-term goals of developing default approaches and databases and long-term goals of sponsoring research, including epidemiology and PBPK modeling, to evaluate interactions.[27]

Several states have initiated efforts to evaluate cumulative risks from chemical and nonchemical stressors. The New Jersey Department of Environmental Protection (NJDEP) developed a screening tool to integrate measures of exposure to chemicals in the environment with demographic and socioeconomic factors. For evaluating chemical exposures, NJDEP uses a variety of data sources, including the EPA's cancer risk and diesel data from the National-Scale Air Toxics Assessments (NATA), state emissions inventories, traffic density, and density of contaminated sites, dry cleaners, and junk yards.[233] In 2010, the CalEPA OEHHA published a framework for assessing cumulative impacts of chemical and nonchemical stressors.[194]

Complex Mixture Approach

There are certain classes of chemicals for which toxicological data exist primarily for the complex mixture itself, with limited data for individual constituents. An example of this type of mixture is PCBs. Historically, in the United States, PCBs were manufactured under the trade name of Aroclors for use in electrical capacitors. Different Aroclor mixtures contained different percentages of chlorine. For example, Aroclor 1242 contained approximately 42% chlorine.[234] Much of the toxicity testing of PCBs consists of studies of different Aroclor mixtures.[234,235] As a result, toxicity criteria for PCBs are typically expressed as Aroclor-specific values, although there are also congener-specific toxicity criteria for certain dioxin-like PCB congeners.[236] For example, ATSDR developed a chronic minimal risk level (MRL, a value conceptually comparable to the RfD), for Aroclor 1254 based on immunological effects in monkeys exposed to Aroclor 1254 in feed for 23 months.[232] While this approach does not require assumptions on how individual constituents will interact, it does assume that the characteristics of the mixture in the environment are the same as in laboratory studies. Unfortunately, this assumption is not always correct; complex mixtures frequently undergo chemical transformations in the environment. Moreover, the individual constituents may partition differently in the environment. In the case of PCBs, for example, the more chlorinated forms bioaccumulate in fish more readily than the less chlorinated forms.[180] Table 2.16 lists chemical mixtures for which toxicity criteria have been developed.

TABLE 2.16
Toxicity Criteria Based on Chemical Mixtures

Chemical	Description of Mixture	Toxicity Criteria	Basis
Aroclor 1016[237]	Mixture of PCB congeners	RfD	Reduced birth weight (monkeys)
Coke oven emissions[238]	Complex mixture produced from carbonization of bituminous coal, contains gases and respirable particulate matter, including polycyclic organic matter, aromatic compounds (e.g., beta-naphthylamine, benzene), trace metals (e.g., arsenic, beryllium, cadmium, chromium, lead, nickel), and gases (e.g., nitric oxide, sulfur dioxide)[239]	IUR	Respiratory cancer (humans, occupational)
Diesel engine exhaust[240]	Complex mixture of hundreds of constituents in gas or particulate form. Key gaseous components of toxicological concern including formaldehyde, acetaldehyde, acrolein, benzene, 1,3-butadiene, and PAHs[241]	RfC	Pulmonary inflammation and histopathology (rats)
Fuel oils[242]	Mixtures of aliphatic and aromatic hydrocarbons	MRL (inhalation, acute)	Ataxia, disturbed gait (mice)
Jet fuels (JP-4/JP-7)[243]	Refined crude petroleum oil blended with other chemicals per specifications of the U.S. Air Force	MRL (inhalation, intermediate) MRL (inhalation, chronic)	Hepatic toxicity (mice) Hepatic toxicity (rats)
Polybrominated biphenyls[244]	Similar to PCBs, with bromine rather than chlorine substituted on phenyl rings	MRL (oral, acute)	Decreased serum thyroid T4 levels (rats)

Notes: RfC, reference concentration; RfD, reference dose; IUR, inhalation unit risk; MRL, minimal risk level.

Conclusions

Consideration of interactive effects in the regulatory arena is an evolving process. Because, in many cases, data are limited, simplifying assumptions are often used (e.g., the assumption of additivity for chemicals that act via the same target organ). As scientists acquire greater mechanistic understanding of interactive effects in complex mixtures, approaches that better reflect molecular events, such as the TEF approach, can be developed that can be used for chemicals that are structurally and toxicologically similar. It must be recognized, however, that uncertainty remains regarding the extent to which such effects occur at environmentally relevant exposure levels and under exposure conditions that do not mimic those tested in the laboratory (e.g., intermittent vs. chronic exposures).

CONCLUSIONS

This chapter has demonstrated the multiple applications of toxicology to the regulatory process. Applications include developing and evaluating chemical testing protocols, such as for endocrine disruptors, developing classification schemes (to date, mainly for carcinogens) aimed at characterizing the types of toxic effects that might be observed in humans, and developing health-based criteria for chemicals in various media (food, water, air, soil) or notification levels for release of chemicals under accidental circumstances.

In addition, toxicology is used in the regulatory process to help assess potential risk associated with defined exposure levels. The traditional paradigm is different for assessing risks related to carcinogens versus noncarcinogens: for carcinogens, potential risk is defined as an upper bound estimate of excess cancer risk based on cancer incidence at high dose levels; for noncarcinogens, potential risk is defined as the ratio of the estimated exposure to an exposure level associated with negligible, if any, risk. Advances in the understanding of toxicological mechanisms indicate that these methodologies are not appropriate in all circumstances. Some carcinogens, such as those that operate by receptor-mediated or cytotoxic mechanisms, may exhibit a threshold or nonlinear dose–response relationship; thus, exposure levels associated with virtually zero risk might be defined. Examples of chemicals with these types of dose–response relationships are saccharin and phenobarbital. The EPA's recent cancer risk assessment guidelines, which consider different dose–response relationships for different carcinogens, represent an important development in this area.

The use of BMRs and PBPK models for developing AELs is an example of recent advances in noncarcinogenic risk assessment. Our understanding of certain noncarcinogenic effects, such as angina associated with CO exposure, has been reasonably advanced; in this example, risks from CO are more fully described in terms of the number of individuals with heart disease who might be expected to exceed defined COHb levels under certain exposure conditions.

Toxicology is frequently applied in the regulatory context of developing permissible exposure levels in different exposure media, such as ambient air, drinking water, or food. As discussed in this chapter, defining a permissible health-based exposure level is only one part of developing a regulatory standard. Other important factors in the regulatory process include risk

management issues (such as the definition of acceptable risk), the weighing of the costs and technical feasibility of reducing risk, the availability of alternatives, and the consideration of potential new risks created by reducing the original risk (e.g., use of a less well-tested substitute chemical). Issues of equity and whether certain members of the population are unfairly burdened by chemical exposure represent other considerations.

In order to provide risk managers with the full information needed for making sound decisions, it is critical that toxicologists participating in the regulatory process effectively communicate not only the results of a risk assessment but the associated uncertainties as well. In addition, despite pressure to employ older methods for the sake of consistency, toxicologists must work to develop and encourage the use of new methodologies reflecting the advances in our understanding of toxicological mechanisms. May this chapter serve as a useful guide to the use of better science in the regulatory process.

QUESTIONS

2.1 How do different approaches used for noncancer risk assessment (e.g., BMD, RfD, and the distributional population approach) address susceptible populations?

2.2 What is MoA, and how is it used in carcinogen classification and in selecting dose–response models for cancer risk assessment?

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KEYWORDS

Mode of action, Cancer risk assessment, Noncancer risk assessment, Susceptibility, Chemical interactions, Carcinogen classification

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3 Dose–Response
A Fundamental Concept in Toxicology

Edward J. Calabrese

CONTENTS

INTRODUCTION

What is the most fundamental concept in toxicology? Is there a most fundamental concept in toxicology? If there is, would it affect how toxicology is taught, how textbooks are written and organized, how toxicological research is conducted, and how hazard assessments are designed and executed?

Within this context, if one were to name some of the most significant discoveries in toxicology, what would they be? Would these discoveries assist in clarifying and defining the most fundamental concept in toxicology?

While there has been no published survey on this conjectural topic of the most important discoveries in toxicology, some possibilities might include xenobiotic metabolism, including cytochrome P450, phase 2 detoxification, the induction of mutations and cancer with ionizing radiation and chemicals, the linkage of mutation with cancer, DNA repair, biostatistical assessment of experimental data, receptor biology and cell signaling, epigenetics, intercellular communication, and apoptosis, among others. Each important toxicological discovery is expressed within a dose–response framework that affects critical dose-dependent transitions via physiological, pharmacological, and toxicological mechanisms that account for when, why, and how agents induce effects on biological systems.

The shape of the dose–response guides clinical medicine and therapeutics. It also affects the strategy and design of the hazard assessment, risk assessment processes, and regulatory decisions. The toxicology of the twenty-first-century report of the U.S. National Academy of Sciences [1] is set within a cellular and systems biology framework to derive reliable mechanistic understandings of the dose–response, especially in the low-dose zone. The long-stated euphemism that all roads lead to Rome has its toxicological equivalent. All toxicological discoveries and emerging concepts will lead to improved understandings of dose–response relationships.

Getting the dose–response right is at the core of toxicology. Failure to do so has profound implications for environmental risk assessment, regulatory decisions, as well as drug discovery and success in the clinical trial, therapeutics, and health care. Getting the dose right also affects environmental health with concerns over the effects of agents, such as pesticides on nontarget species, as well as their persistence and capacity to interact with other agents. The dose–response question is therefore an important one, dominating biomedical themes and social concerns.

The study of the dose–response is broad and complex. To understand the how's and why's of the dose–response requires the capacity to integrate findings from a wide range of highly specialized areas of research. It is very interdisciplinary by nature.

The dose–response is not simply a tool of toxicology; it is much more, being even more expansive than the concept of toxicity per se. Within this framework, knowledge of the dose–response is basic to the assessment of interspecies differences in response to xenobiotics and the survival strategies adopted within the plant and animal domains. Dose–response assessments are, therefore, at the core of not only toxic responses but also adaptive strategies.

The dose–response concept is of such fundamental importance to toxicology that it provides a framework by which toxicology may be defined. The concept of the dose–response implies the need to assess the entire dose–response continuum from very low doses that are seemingly without demonstrable effect to higher doses that cause frank toxic effects and mortality and the underlying mechanisms of these effects. Thus, contrary to what may be broadly believed and typically taught, toxicology is not a discipline that is exclusively concerned with adverse effects. It should be concerned with all processes that affect life, including adaptive responses. Ignoring adaptive responses in the study of toxicology impairs the capacity to develop accurate and more fundamental understandings of the dose–response.

Adaptive responses occur within a dose–response framework. What are adaptive responses? When are they induced? Are there molecular detection systems for the discernment of biological alterations/injuries that affect the upregulation of adaptive responses? How does the induction of the adaptive response, or the failure to do so, affect the dose–response for toxic endpoints? It is not, therefore, possible to separate the concept of adaptive response from that of the toxicological dose–response. These differential concepts are part of an integrated dose–response continuum.

Within this dose–response context, the risk assessment definition of EPA purposefully and explicitly excludes the concept of adaptation and repair. Yet, the concept of risk assessment is entirely dependent upon an understanding of the dose–response, its underlying mechanisms, and how they can be used to predict and estimate responses to toxic substances within the population [2].

This chapter therefore argues for a new and more expansive concept of toxicology, framed within a dose–response perspective that is at its core. The truncating of toxicology into those responses that occur at the so-called high doses, above the traditional threshold, and that deal only with induced pathologies has been its history. This history has suffered from a limited view of the dose–response, a perspective fueled by an incorrect understanding of the dose–response concept and its evolution. With the expansion of the dose–response concept into the area of the biological effects of low-dose exposures, including below-threshold effects, toxicology will interface in new and more integrative ways with related subdisciplines within the biological and biomedical sciences. This perspective offers a broader and more inclusive view while building upon past toxicological research achievements and understandings of dose–response relationships and mechanisms.

Toxicology is a fundamental component of systems biology as essentially all biological disciplines are inherently dependent on the dose–response. The dose–response is at the foundation of cellular messaging, biological regulation, plasticity, cellular and tissue repair, health, dysregulation, and disease. The notion that toxicology is a discipline that is only, or even principally, concerned with adverse health effects is

too limited. Toxicology needs to encompass the entire dose–response continuum.

The dose–response concept is therefore one that unifies and merges long-held conceptual differences between the disciplines of toxicology and its historical parents of pharmacology and physiology. These once distinct disciplines are being progressively seen differently in light of mechanistic advances and the tools by which they are studied. Toxicology is no longer simply a descriptive discipline; it has become transformed into a mechanistically focused discipline within a dose–response context setting where the lines of former disciplinary demarcations are blurring with striking rapidity.

All biological and biomedical disciplines have been built upon the dose–response concept. The dose–response concept emerged from and has been continuously used by researchers in many fields, including physiology, microbiology, pharmacology, ecology, entomology, biostatistics, radiation biology, radiation medical therapies, genetics, and botany [3–9]. Thus, researchers have historically developed toxicological concepts and frameworks based upon the issues in their own discipline. Such individuals have therefore entered the field of so-called modern toxicology from a broad range of scientific disciplines.

Despite the fact that all of the previous fields utilized the concept of dose–response, toxicology tends to see itself historically as an outgrowth of pharmacology during the middle decades of the twentieth century. Yet, the dose–response was emerging as quickly and often with a stronger dose–response basis in the areas of microbiology, plant biology, and entomology. These areas were important not only because of practical societal consequences, but they could also address dose–response issues more effectively (i.e., more doses, greater sample sizes, more replications) because of the ease and cost-effectiveness of more efficient experimental systems. Such areas were able to accommodate the possibility of developing and exploring broad dose–response relationships. This was not something that the area of whole-animal studies could readily accommodate.

Attempts to develop in vitro testing were initiated as early as the first decade of the twentieth century by Alexis Carrel (Figure 3.1) since he needed to avoid excessive variability in the responses of outbred animals in wound-healing studies [10]. Since there were no inbred rodent strains available at that time, Carrel opted to explore the novel yet imposing area of cell culture. While reliable findings were not easily obtained, these efforts were to play a determining historical role in the success of in vitro research today. These achievements also were important in the historic success of the Salk polio vaccine [11]. Carrel was initially interested in the search for agents that could significantly accelerate wound healing and then later applied these in vitro methods to assess ways in which life span could be increased within a dose–response context.

The challenging issues with whole-animal testing would continue to be the case throughout most of the twentieth century as whole-animal mammalian systems have been expensive, space demanding, and often yielding highly variable

FIGURE 3.1 French surgeon and biologist Alexis Carrel (1873–1944). (From Commons.wikimedia.org/wiki/File:Alexis_Carrel_02.jpg [PD-U.S.].)

results. So challenging has been the demands of whole-animal systems, it contributed significantly to the in vitro revolution of the 1980s and the incorporation of high-throughput chemical screenings, in an effort to more cost-effectively prioritize agents for further evaluation. These more recent efforts would have their origin with the extensive work of Carrel and his inspired followers such as Robert Parker and his remarkable work on the polio vaccine.

The dose–response therefore had detailed formulation and evaluation from other *nontoxicological* disciplines, well before the so-called transition of toxicology had been made from pharmacology. In fact, the development of early statistical assessment methods occurred in the first two decades of the twentieth century with the publication of William Gosset's (Figure 3.2) Student's T-test and Sir Ronald Fisher's the analysis of variance [12], both of which had an important role in the analysis of toxicological data in the second half of the twentieth century. Yet, their important advances were neither inspired by nor directly related to toxicology per se. The dose–response relationships for the use of disinfectants starting with the applications of Joseph Lister for the sterilizing of surgical activities in the 1860s–1870s were based on the discoveries of Louis Pasteur and expanded by the discoveries of Robert Koch that permitted the isolation and culturing of specific bacteria and other microbial species. Koch [13] was the first to assess the capacity of a range of chemical disinfectants in a concentration-dependent manner using pure cultures of bacteria. These findings accelerated developments in the areas of chemical assessment of disinfectants with their applications to public health practices from community drinking water disinfection starting in the first decade of the twentieth century. Detailed dose–response

FIGURE 3.2 William Sealy Gosset, British statistician. (From Commons.wikimedia.org/wiki/File:William_Sealy_Gosset.jpg [PD-U.S.-not renewed].)

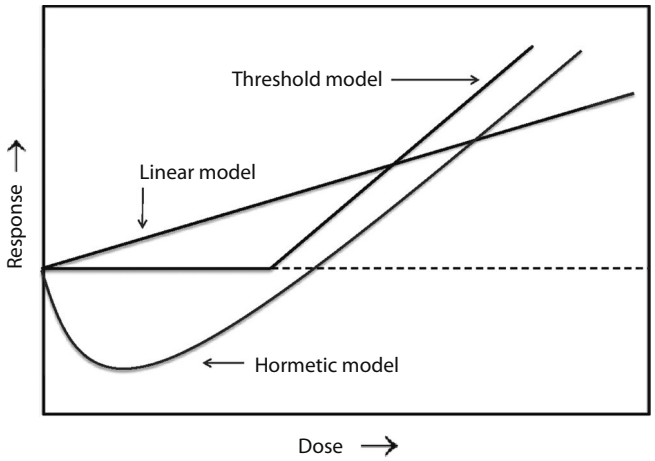

FIGURE 3.3 Most significant contemporary dose–response relationships.

modeling applying the law of mass action to the process of chemical disinfectants for bacteria was also undertaken in the first decade of the twentieth century [14–16]. Such modeling activities lead Chick and Martin [15] to derive concentration–response thresholds for the inhibitory actions of various disinfectants (e.g., mercuric chloride, silver nitrate, and phenol). Moreover, modeling-based threshold interpretations were based on experimental findings in the observable zone. Dose–response principles, practices, and applications were therefore developing in specific disciplines during the later decades of the nineteenth century.

The field of toxicology has tried to assert itself as a basic science just like physiology, pharmacology, genetics, microbiology, botany, and other biological disciplines have done. While toxicology has been largely successful in achieving this goal within the scientific and regulatory communities, its *basicness* is rooted not in a specific biological system (e.g., plant, microbe, animal), level of biological organization (e.g., in vitro, whole animal), or endpoint but in the dose–response concept, its mechanistic understandings, and applications. Ironically, the field of toxicology has inadequately grasped that its core is the dose–response and how this drives the hazard assessment, efficacy evaluation, and public health implications along with providing cellular mechanisms that account for its quantitative features. The dose–response concept not only provides the historical foundation of toxicology but also a fundamental context within which natural selection occurs at multiple levels of biological organization. When seen in a historical perspective, toxicology is a convergence of a broad range of biological disciplines in which dose–response evaluation and understandings are fundamental. That is, there are many differing biological disciplinary paths that require the use of the dose–response concept, modeling of dose–responses, and understanding of mechanisms that can account for the features of the dose–response and that lead to the discipline of toxicology. All are conducting toxicological assessments framed within a dose–response context.

This chapter will also explore the evolution of the dose–response concept for chemicals and radiation, underlying the development of the threshold and linear dose–response models, as well as additional models, such as the biphasic/hormetic (Figure 3.3) dose–response model and polyphasic (e.g., triphasic) models. Within this context, the dose–response concept in homogeneous experimental models will be compared with that of human epidemiology that is characterized by profoundly greater interindividual variation. The hazard assessment process will be assessed and how it was employed to validate the threshold dose–response concept that created it, resulting in a toxicological self-fulfilling yet failed prophecy.

The history of the dose–response is a rich one, with scientific, intellectual, and politicized disputes along with dynamic leaders that are far more than historical icons merely listed for completeness in historical assessments. This chapter will explore some of these key historical figures and describe how these intellectual leaders came to formulate the nature of what is called toxicology today, revealing their enormous contributions and perhaps their errors, conflicts, and successes, which have also come to mold and guide the evolution of this field.

DOSE–RESPONSE: HOW THE THRESHOLD DOSE–RESPONSE BECAME ACCEPTED

The widely acclaimed father of toxicology is Paracelsus. He is especially known for his insightful statement that "the dose determines not only the poison but also the remedy," a concept of dose and response that is extremely broad. This statement implies that the same agent could be both a toxicant

and one with therapeutic and/or health potential. According to Paracelsus, whether the agent was a toxicant or a tonic depended on the dose. While Paracelsus has long retained an esteemed status in the field of toxicology, his quote has often been truncated by recent generations of toxicologists to yield the following: "the dose determines the poison" [17]. This section explores the original Paracelsian insight into the realm of toxicology, addressing both toxicant and remedy as each is part of the dose–response continuum especially in light of the current experimental developments. It should be appreciated that the views of Paracelsus were not those of a toxicologist but one more closely tied to a philosophical perspective in which all things are interconnected and related. While there were many implications that emerged from this view, the previous idea of chemicals having both beneficial and harmful potential that was dependent on the dose was conceptual and intuitive. However, nearly four centuries would have to pass before these speculations could be experimentally tested.

While the concept of the dose–response may appear to be straightforward, it has had a highly controversial history. The first major debate of modern relevance over the dose–response was initiated by Samuel Hahnemann (Figure 3.4), the creator of the practice of homeopathy [18]. Hahnemann was a traditionally trained German physician who became strongly opposed to the medical treatment of his era, a period of the so-called heroic medicine. It was during this period when patients were treated by blood drawing and the administration of often intoxicating doses of heavy metals and other agents. He eventually came to the conclusion that such treatments were often worse than the disease. This led Hahnemann to seek alternative means of treatment that would be both healing and without toxicity. As a result of his intense moral conflict of harming patients while trying to cure them, Hahnemann quit his profession and began to support his family by becoming a professional translator of chemistry and related books. During the course of these translational activities, he developed an insight that was to change his life and lead him to create the medical practice of homeopathy. Based on information learned from reading of the medical literature, Hahnemann developed the belief that medicinal treatments could be obtained from plant extracts to relieve the symptoms of numerous diseases and to enhance healing and recovery from a broad spectrum of diseases and to do so without being toxic.

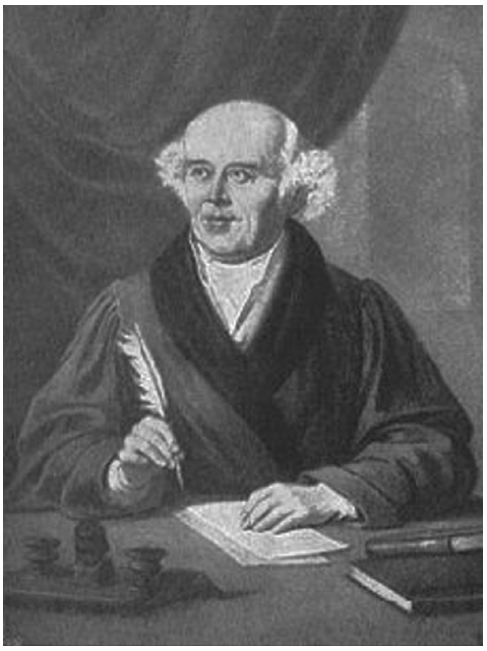

FIGURE 3.4 Samuel Hahnemann. (From commons.wikipedia.org/wiki/File:Hahnemann.jpg [PD-U.S.-not renewed].)

Hahnemann's drug discovery/medical treatment plan was based upon two principal components: The first was an effective drug needed to induce the same symptoms of the disease in healthy subjects that occurred in ill patients (i.e., the like cures like principle). Secondly, the medicinal treatment should be applied at a dose that was not toxic but sufficient to induce an adaptive response (i.e., called by Hahnemann a *vital force* that has been misrepresented to be an amorphous/undefined spiritual phenomenon). The general foundation of this dose–response concept is similar to that put forth centuries before by Paracelsus. However, over time, Hahnemann altered his dose–response concept, making it profoundly less acceptable to scientifically minded individuals by arguing that adaptive responses could be induced at infinitesimally lower doses. This high-dilution drug treatment position became untenable once Avogadro's number became quantifiably clarified in the early part of the twentieth century [19]. Using chemical molecule calculations based on Avogadro's number, it was shown that Hahnemann's extremism was indeed real, as many of his homeopathic preparations were estimated to have vanishing few, if any, molecules. Unease with this infinitesimal dosing/therapeutic practice had already been festering even during the life of Hahnemann who died in 1843. In fact, one of Hahnemann's former students was well known for challenging this infinitesimal dosing perspective [20]. Reflecting such disputes, it is clear that by the later decades of the nineteenth century, homeopathy, as a profession, had split into two main groups: the followers of Hahnemann (i.e., the high-dilution group) and those not accepting this view (i.e., the low-dilution group) but still accepting his first concept (i.e., law of similars). By the early 1880s, the high-dilution group represented only a small minority (<10%) of the homeopathy practitioners [18,21]. In practice however, many homeopaths actually tended to straddle the fence, being fiscally pragmatic, selling therapeutic preparations from both practitioner camps.

Despite this fracturing of the field of homeopathy, opponents of this practice, especially those within the field of traditional medicine, have invariably attempted to portray this entire profession as belonging to the Hahnemann *wing* of the movement. Why? Most likely, this was because the dose–response extremism of high-dilution homeopathy was an effective target. By lumping the entire practice

of homeopathy into the same dose extremism, it was easy to marginalize the entire field. The dose–response concept therefore that emerged from the work of homeopathy based on the early (i.e., prior to the adoption of infinitesimal dose scheme) writings of Hahnemann was that of a biphasic dose–response. The understanding of the dose–response during this time period was far more conceptual than experimentally based. In fact, this perspective was based on limited human exposure studies by Hahnemann and his school in a process called *proving*. Needless to say, by the time that Hahnemann died, the scientific basis of the dose–response was still fundamentally unexplored, lacking the technical capacity and resources for evaluation, especially on a reliably larger scale. However, this would change over the next 50 years with the onset of scientific advances in Europe, especially with the influence of the emerging fields of physiology, pharmacology, and toxicology.

While the well-known biologist Claude Bernard (Figure 3.5) introduced the concept of a threshold dose–response in the mid-1860s, relating to renal excretion [22], there was little serious scientific research concerning the nature of the dose–response until a report emerged from the laboratory of Hugo Schulz in the mid-1880s [23,24] (Figure 3.6). Schulz, a physician, as well as an expert in toxicology and pharmacology, became a highly polarizing figure when he reported that multiple chemical disinfectants not only inhibited yeast metabolism in a dose-dependent manner but also enhanced it at low concentrations, that is, below the toxicity threshold [25]. In his autobiography, Schulz ([26]; see Crump 2003 translation) noted that at first, he did not accept the low-dose stimulation response as real but probably the result of an experimental artifact. However, replication

FIGURE 3.5 Claude Bernard. (From commons.wikipedia.org/wiki/File:Claude_Bernard.jpg [PD-U.S.-not renewed].)

FIGURE 3.6 Hugo Schulz (1853–1932). (From http://dose-response.org/low-dose/scientists/schulz.htm.)

experiments revealed a reliable consistency of the biphasic dose–response:

> Since it could be foreseen that experiments on fermentation and putrescence in an institute of pathology would offer particularly good prospects for vigorous growth, I occupied myself as well as possible, in accordance with the state of our knowledge at the time, with this area. Sometimes, when working with substances that needed to be examined for their effectiveness in comparison to the inducers of yeast fermentation, initially working together with my assistant, Gottfried Hoffmann, I found in formic acid and also in other substances the marvelous occurrence that if I got below their indifference point, i.e., if, for example, I worked with less formic acid than was required in order to halt the appearance of its anti-fermentive property, that all at once the carbon dioxide production became distinctly higher than in the controls processed without the formic acid addition. I first thought, as is obvious, that there had been some kind of experimental or observation error. But the appearance of the overproduction continually repeated itself under the same conditions. First I did not know how to deal with it, and in any event at that time still did not realize that I had experimentally proved the first theorem of Arndt's fundamental law of biology [26].

While none of these observations should have been controversial, they became the object of intense scrutiny, debate, and ridicule. Why was this the case? Schulz made them so by claiming that he had discovered the underlying principle of homeopathy. Schulz set forth the premise that homeopathic remedies act by inducing adaptive responses that enhanced the capacity of biological systems to resist a broad range of environmental insults. The conclusion of Schulz was based on several lines of converging thoughts.

On the clinical side, there was a report in 1884 by Bloedau [27] that the homeopathic drug veratrine was successful in the treatment of gastroenteritis. Schulz [28] tried to verify that this botanically derived drug could actually kill the disease-causing agent. However, Schulz was unable to verify that this agent could kill the bacterial agent based upon considerable laboratory studies. Since the veratrine failed to kill the disease-causing agent in his laboratory but was apparently successful in the treatment of this disease in people, Schulz hypothesized that the drug acted not by killing the bacteria but by enhancing the adaptive capacity of the individual. While there could have been alternative explanatory possibilities proposed, Schulz then applied his biphasic dose–response observations with the yeast to account for the protective effect of the veratrine in people. It was by this linkage of the biphasic dose–response to homeopathy and its hostile relationship with traditional medicine that it (i.e., the biphasic dose–response) became the object of suspicion, hostility, and marginalization by the medical community starting in 1885 and continuing throughout the twentieth century [29–31].

Schulz [32] made this dose–response assertion in the midst of an intense rivalry between what is today called traditional medicine and the practice of homeopathy. In effect, he placed his newly observed biphasic dose–response into the homeopathic domain. His actions evoked a profound and prolonged series of professional criticisms and personal attacks as well as criticism of his dose–response model by leaders of traditional medicine as well as the medical faculty at his own institution [33,34]. This dispute about the nature of the dose–response could not have occurred at a worse time for the biological, biomedical, and toxicology communities. Experimental studies in these areas were just beginning, and various scientific concepts, including those related to the dose–response, were in the process of being formulated. The issue of the dose–response therefore was in its concept formation period. However, due to economic, philosophical, scientific, and personal conflicts with homeopathy, traditional medicine was *compelled* to attack all things homeopathic as if they were an enemy. Furthermore, the formation of the American Medical Association was explicitly based in part on the need to counter the growing societal influence of homeopathy. This is a generally underappreciated historical fact and one that had profound implications for the biphasic dose–response. As a result of being drawn into the homeopathy–medicine conflict, the biphasic dose–response concept would become collateral damage, leading to its marginalization and exclusion from medical, pharmacology, and toxicology textbooks, curricula, teachings, clinical practices, and government regulatory testing and evaluation procedures. The conflict with homeopathy and the involvement of the biphasic dose–response in the dispute would ironically play a significant role in the acceptance of the threshold dose–response model by the medical community. How could the rejection of Schluz's biphasic dose–response model have affected the acceptance of the threshold dose–response and, in fact, have even led to it?

FIGURE 3.7 Alfred J. Clark. (From Clark, D.H., *Alfred Joseph Clark (1885–1941): A Memoir*, C & J Clark Ltd., Glastonbury, England, 1985, 61pp.)

While Schulz had his many detractors, none were so visible and effective as Alfred J. Clark (Figure 3.7), a professor of pharmacology at the University of Edinburgh, a renowned researcher, scholar, an acclaimed textbook author, and a high-level government advisor. His influence upon the field was deep and pervasive and of long duration. One of Clark's goals was to discredit medical quackery. Unfortunately for Schulz, Clark labeled homeopathy as quackery and linked Schulz with the high-dilution wing of homeopathy, a charge that was demonstrably false [25]. Moreover, Clark vigorously attacked Schulz's biphasic dose–response hypothesis in his highly influential books [35,36] in an attempt to trivialize and marginalize Schulz and homeopathy. Clark also argued that research by Dannenberg [37] established that Schulz's findings could be explained by normal variation and that the low-dose stimulation findings were due to inadequate study design and chance and lack the capacity for replication. However, a reexamination of this critical paper by Dannenberg [37] revealed that it did not replicate the work of Schulz, using concentrations that were some 10–20-fold lower. In addition, the research did not collect data at multiple times, a necessary feature to evaluate Schulz's concentration–time response hypothesis. Thus, the paper was not relevant, yet the unfair criticism of Clark stuck because of his reputation and because Schulz, by temperament, would not usually defend himself and also that he was now several years into retirement. Furthermore, when Clark did have the opportunity to assess a paper that was specifically designed to replicate the experiments of Schulz, he never cited it. In this case, the research of Sarah Branham (Figure 3.8) [38] in the highly visible *Journal of Bacteriology* strongly confirmed the findings of Schulz.

FIGURE 3.8 Sara Branham (Matthews) (1888–1962). (Courtesy of Georgia Women of Achievement, Inc., LaGrange, GA, www.georgiawomen.org/_honorees/matthewss/index.html.)

FIGURE 3.9 Benjamin Minge Duggar. (From www.apsnet.org/about/history/pioneeringplantpathologists/Pages/DuggarBenjaminMinge.aspx [PD].)

Of further importance was that Clark's writings had a long reach, affecting several generations of pharmacologists and toxicologists. Such efforts were important in helping to ensure that the threshold model would be incorporated into research, teaching, and regulation, whereas the biphasic dose–response of Schulz would be excluded.

Over the ensuing next half century (~1885–1935), the so-called battle of the dose–responses was waged. However, it was a complex type of dispute. While many scientists were simply not part of the ideological battle between medicine and homeopathy, others, such as some very influential academic leaders of the British pharmacological community, were [29,39,40]. A review of the history of the dose–response reveals that traditional medicine was an organized, harsh, and powerful opponent for the biphasic dose–response. There is also little question that the lack of an *institutional* capacity for homeopathic activity resulted in researchers such as Schulz being isolated and without an institutional and professional support system.

A detailed accounting of the nonideological leading researchers documenting biphasic dose–responses revealed no tendency to organize around the biphasic dose–response concept or to promote it [8,9]. Most of these early leaders publishing in the area of biphasic dose–responses would transition professionally into other positions of leadership in science that, in effect, drew them away from this dose–response research domain [8,9]. In fact, many of these researchers who had demonstrated the occurrence of biphasic dose–responses were outstanding scientists with extensive reputations. For example, Benjamin Duggar (Figure 3.9) was involved in such research from the late 1890s throughout the first half of the twentieth century, becoming a major scientific feature in the United States and worldwide. Over time, he would become less involved with research and became more active with general academic scientific leadership. However, at the age of 72, following his retirement, he discovered the antibiotic Aureomycin in 1948 [41] and was nominated for the Nobel Prize. Yet he was not involved in the battle between homeopathy and traditional medicine.

The first biomathematical modeling of the hormetic–biphasic dose–response was published by the Cornell University microbiology professor Otto Rahn in 1932 [42]. The methods were based on the earlier modeling of the effects of disinfectants on bacterial survival by Chick [14] and others using the law of mass balance. Rahn [42] cited the extensive data supporting the findings of Schulz/Branham on the effects of metabolic poisons/chemical disinfectants on yeast metabolism. Rahn [42] also highlighted the earlier dissertation by Paul Hofmann [43] that paid very careful attention to the question of dosage and its optimization. In these experiments with 13 different agents, Hofmann reported not only that, at the lower concentrations, there were a larger number of colonies but that the colonies were enlarged.

Experimental proof for the biphasic dose–response was very demanding. First, a threshold response had to be estimated. Then both below- and above-threshold response areas need to be assessed when considering the possibility of a biphasic dose–response. While it may not have been appreciated in the early decades of the twentieth century, there was also a need for greater statistical power in the below-threshold doses. This added further difficulty in proving the reproducibility of the low-dose stimulation.

There was also the distinct possibility that there was a time component needed to demonstrate a biphasic dose–response. This was an initial finding of Schulz [23,24], Townsend, [44] and others, such as Branham [38] who carefully replicated the original findings of Schulz as noted previously. She clearly demonstrated that there was an initial dose-dependent toxicity/inhibition response, followed by a rebound or overcompensation response at low doses, resulting in the occurrence of the biphasic dose–response relationship (Figures 3.10 through 3.12). If the experiment included only one time measurement at the initial point of inhibition, then the stimulatory response would be missed but also the overcompensation response.

The low-dose stimulatory response was also invariably modest in magnitude, making it more difficult to observe and to replicate. Such a modest stimulatory response placed even more pressure on the need to replicate findings due to their greater inherent uncertainty as compared to high-dose toxicity effects. Thus, the dispute between followers of Schulz and those of traditional medicine about the dose–response was fully impacted by methodological and resource challenges that were needed in order to document the possible occurrence of biphasic dose–responses.

Despite these challenges to the study of biphasic dose–responses, there was a surprising and relatively copious

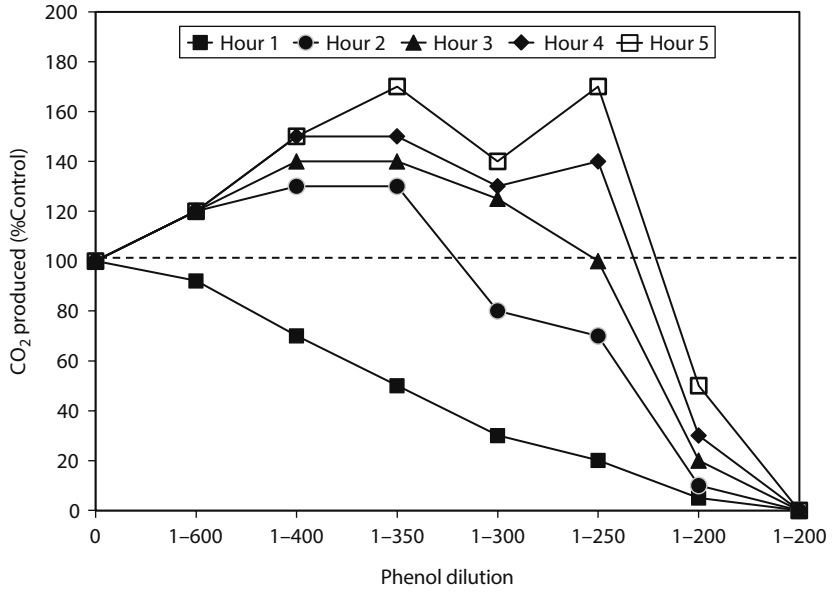

FIGURE 3.10 The effects of phenol on yeast metabolism. (Based on Branham, S.E., *J. Bacteriol*., 18, 247, 1929.)

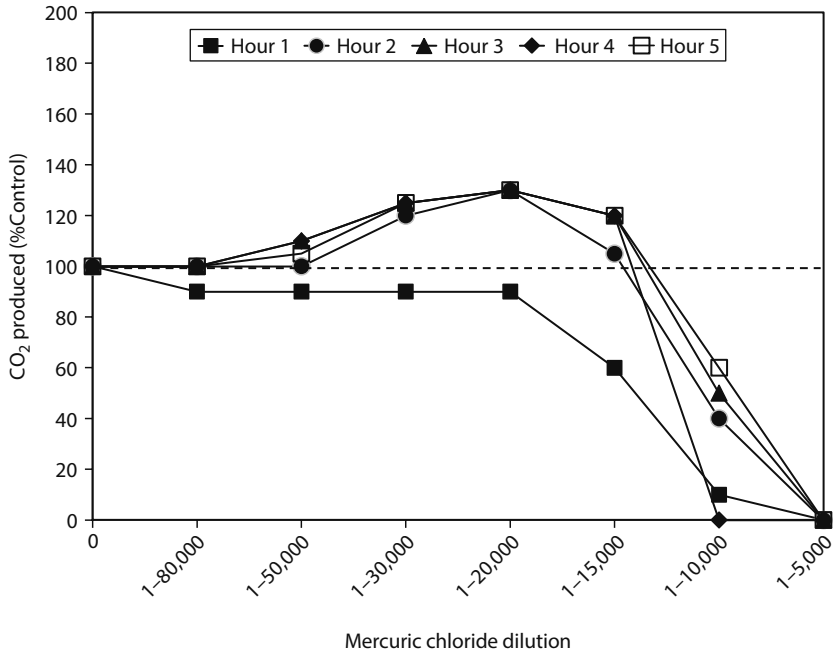

FIGURE 3.11 The effects of mercuric chloride on yeast metabolism. (Based on Branham, S.E., *J. Bacteriol*., 18, 247, 1929.)

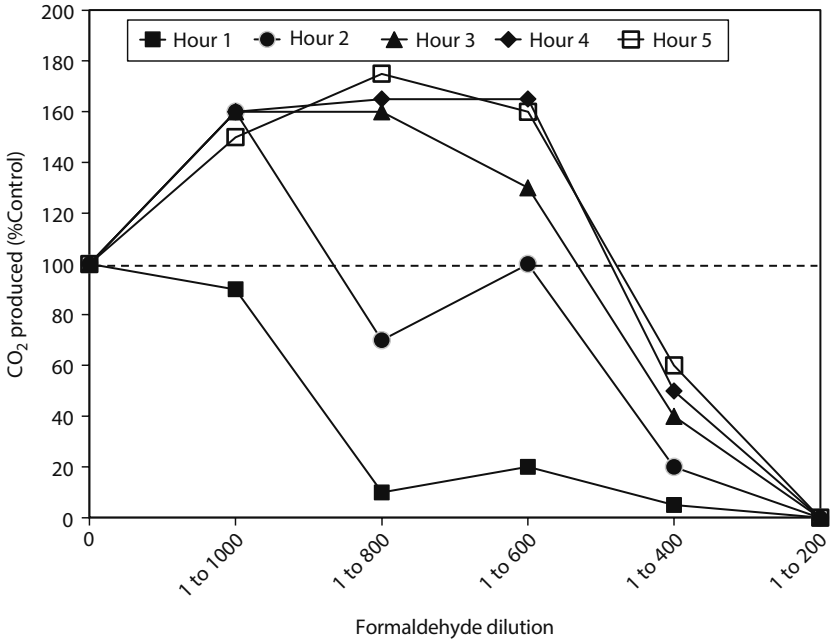

FIGURE 3.12 The effects of formaldehyde on yeast metabolism. (Based on Branham, S.E., *J. Bacteriol*., 18, 247, 1929.)

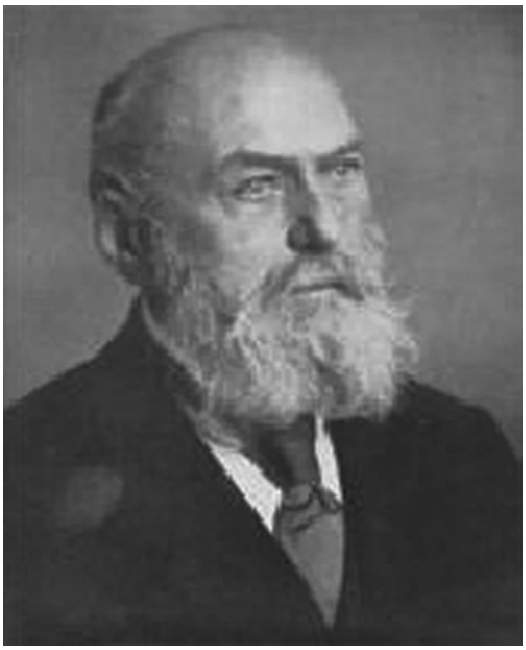

FIGURE 3.13 Fernando Hueppe. (From dose-repsonse.org/low-dose/scientists/hueppe.htm.)

literature documenting the biphasic dose–response (called the Arndt–Schulz law [32]/or Hueppe's [45] rule [Figure 3.13]) published in leading journals during the early decades of the twentieth century. This research was based principally on work with microorganisms, plants, and insect models. There was also a copious literature in medical journals related to the effects of x-rays on numerous medical conditions such as gas gangrene, arthritis, sinusitis, and otitis media. The publications were typically case reports [46,47], often extensive and supportive of the biphasic dose–response. The biphasic dose–response concept also became formally integrated into leading textbooks of the first half of the twentieth century for both botany [48] and microbiology [49–52]. However, it was not until 2000 when this historical literature on hormetic–biphasic dose–responses was finally comprehensibly summarized with respect to chemicals [3–7]. Luckey [53,54] had comprehensively addressed the area of radiation hormesis first some 20 years earlier.

While the biphasic dose–response was often observed in the early decades of the twentieth century, it was never the object of an integrative assessment by any of the various researchers of that era. There was no demonstrable understanding or appreciation of the quantitative features of the low-dose stimulation; due to the fact that this stimulation could be both directly induced by a compensatory response, there was no broad discussion concerning the design of experiments to study biphasic dose–responses. This lack of integrative assessment probably resulted from the fact that most researchers with experience in this area, as noted previously, moved on to other professional endeavors. A few researchers, such as Herbert Maule Richards [55,56] at Bernard College, directed a relatively large number of undergraduate students in the study of biphasic dose–responses but never provided a broadly integrative assessment. This was also the case for Charles-Edward Winslow (Figure 3.14), an internationally recognized bacteriologist at Yale University, who directed multiple PhD students in the area of biphasic dose–responses [57–59]. In the case of Winslow, he was to be redirected from dose–response research with bacteria to broader health issues, becoming the editor of the *American Journal of Public Health* and holding that position for about 20 years starting in the mid-1930s.

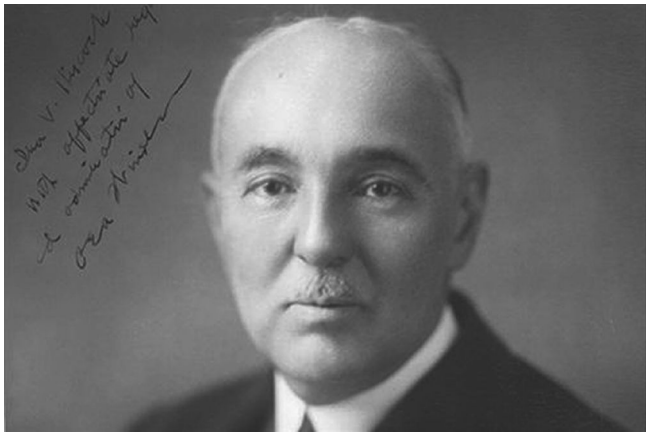

FIGURE 3.14 Charles-Edward Amory Winslow (1877–1957). (From http://www.google.com/imgres?imgurl=http://publichealth.yale.edu/Images/gallery202_74635History.)

The threshold dose–response model had a far easier time in this regard. It was markedly easier to evaluate, had profoundly less challenges with the replication of high-dose toxicity results, required far fewer doses, had smaller sample sizes, and had no complex time component. The threshold concept was also a model that resonated with personal experience. It also had leading researchers, including some in the area of biostatistics, which would advocate for its use and broad-scale applications. Thus, the road was much clearer for acceptance and understanding of the threshold dose–response than with the biphasic dose–response.

By the mid-1930s, the threshold dose–response model had made significant inroads of acceptance with the scientific [60–62] and regulatory communities, serving as the theoretical foundation for how hazard assessment would be conducted and the basis for the derivation of safety factors. While this concept would appear in the 1930s for chemical [63] and radiation safety factors [64], it was not until April 1953 that the safety factor concept became formally part of the FDA [65], the same month that Watson and Creek would publish their proposed structure of DNA. The recommendations of Lehman and Fitzhugh [65] would be broadly accepted affecting how environmental and food health exposure standards would be derived up to the present.

During this time period, the biphasic dose–response failed to become widely accepted, was poorly understood, and also continued to have a strong historical association with the practice of homeopathy, which itself had lost in the battle with traditional medicine in the treatment of patients. Furthermore, homeopathy also was the object of a blistering and profoundly influential attack by Abraham Flexner (Figure 3.15) [66] in 1910 that eventually lead to the closing of almost all the nearly two dozens of homeopathy medical schools in the United States by the early 1920s [67]. In effect, by the end of the 1930s, homeopathy was functionally decimated, and the biphasic dose–response of Schulz was fully marginalized, while the threshold dose–response model had become dominant along with traditional medicine. There were no other competitors.

FIGURE 3.15 Abraham Flexner. (From http://commons.wikimedia.org/wiki/File:Aflexner21.jpg [PD-U.S.-notrenewed].)

The threshold became the standard dose–response model for industrial chemicals, drugs, and radiation health effects evaluation. It received support from a variety of research perspectives including the occupational work experience for chemicals and ionizing radiation. Of particular importance was that the threshold dose–response model received mechanistic support based on the findings of researchers such as A.J. Clark who was able to demonstrate a relationship between receptor occupancy and the onset of biological effects [68]. He reported a threshold for acetylcholine that required approximately 20,000 molecules acting via receptors to produce an initial effect (i.e., isotonic contraction) on a heart cell. This was an impressive finding and one that Clark would exploit both in his extensive writings concerning the dose–response that would support a threshold model and in his criticism of the Arndt–Schulz law [35,36,69,70]. Therefore, the threshold dose–response appeared to have its foundation on secure scientific grounds. Clark also inspired an extensive series of statistical assessments of dose–response relationships that provided a solid foundation for application to wide-ranging subdisciplines within the biological sciences [71–80].

While the biphasic dose–response would come to have a scientific resurgence from the 1990s to the present, it simply appeared to be a dose–response concept in search of a cause or a proponent—which never seemed to materialize during the early 1900s, even when it was rediscovered and renamed as hormesis in the early 1940s by Chester Southam (Figure 3.16) and John Ehrlich [81] at the University of Idaho. Despite their rediscovery of the biphasic dose–response in research with fungi concerned with the rotting of wood, these two researchers, like so many other early researchers of the biphasic dose–response, soon redirected their focus from

FIGURE 3.16 Chester Southam. (From dose-response.org/low-dose/scientists/southam.htm.)

the dose–response. John Ehrlich moved to the University of Minnesota to work on methods to enhance the production of penicillin during World War II (WWII), later moving to the pharmaceutical company Parke-Davis in Detroit to participate in antibiotic/drug discovery activities (i.e., he became quite well known due to his role as a codiscoverer of chloramphenicol; in fact, one of the codiscoverers was nominated for a Nobel Prize). Chester Southam, on the other hand, moved to New York City, graduating medical school from Columbia University, later becoming an expert in the area of tumor antigenicity [8,9]. Thus, the early advocates of the biphasic dose–response model were to come and go, not having the focus and leadership of the medical community that A.J. Clark provided. Despite the dominance of the threshold dose–response and its generalizability, it nonetheless would be challenged during the twentieth century and into the twenty-first century.

HISTORY OF THE THRESHOLD DOSE–RESPONSE WITH PARTICULAR FOCUS ON OCCUPATIONAL RADIATION PROTECTION

In practical terms, the concept of a *threshold* dose had its origin in the domain of occupational health, with the focus on exposures to ionizing radiation from x-rays and gamma rays. In 1895, Roentgen discovered the x-ray and this ushered in a vast amount of research in a broad range of areas. It was soon learned that x-rays and gamma rays (which were discovered by Becquerel in 1898) could induce a wide range of harmful effects. Despite the early recognition of the hazard potential of ionizing radiation, it would take three decades for the regulatory community to begin to shape a response to this issue. That it might take considerable time to develop an organized response is not surprising given the nature of communications during that era as well as the occurrence of World War I (WWI) and its massive disruptions in Europe and the United States.

An organized assessment of the dose–response due to exposure to ionizing radiation was borne of the creation of the International Committee on X-Ray and Radium Protection by the Second International Congress of Radiology in 1928. The goal of this committee was to advise physicians on radiation safety measures, within a nonregulatory framework [9]. It had nothing to do with the issue of environmental contamination. In 1925, the first International Congress of Radiology met with the intention of renewing communication between opposing countries during the recently ended World War. The U.S. Bureau of Standards provided the liaison function for the United States, selecting Lauriston Taylor (Figure 3.17) as its representative. Taylor, an employee of the Bureau of Standards, would nonetheless act in a voluntary capacity to the international committee without an official U.S. government involvement in the activity. While the bureau would continue to provide the liaison function, the role of the bureau in this domain would become a political issue several decades later as President Eisenhower would create the Federal Radiation Council (FRC) to act as the official U.S. organization with direct accountability in the area.

In parallel to the actions of the international committee, Taylor created a U.S. national committee on radiation protection. This entity was initially called the American X-Ray and Radium Protection Committee. Almost two decades later (1946), after the conclusion of WWII, the name was changed to the National Committee for Radiation Protection

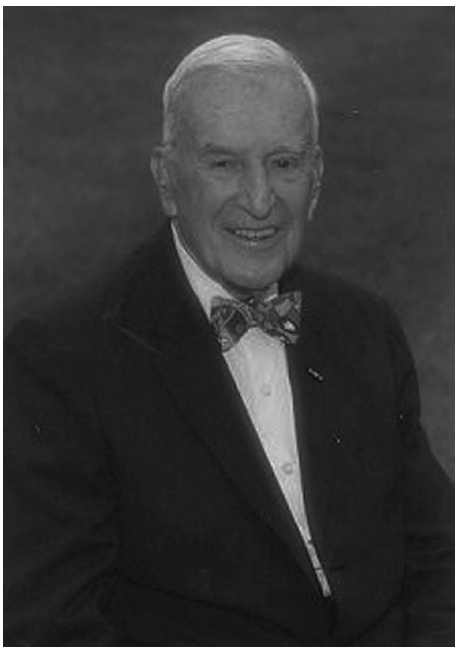

FIGURE 3.17 Lauriston Sale Taylor (1902–2004). (Courtesy of Health Physics Society, McLean, VA, http://hps.org/aboutthesociety/people/inmemoriam/LauristonTaylor.html.)

and Measurement (NCRPM). This committee was involved in the development of radiation protection standards and in related educational activities within the United States. The initial meeting of this organization was on September 18, 1929. This committee would come to publish documents about various safety procedures and exposure monitoring for workers involved with x-rays and gamma rays [64,82]. It was in the framework of evaluating radium that American X-Ray and Radium Protection Committee, as chaired by Taylor, concluded that the best indicators of ionizing radiation effects were skin changes seen with the fingertips. These areas displayed a reddening and shiny quality of the skin in the zone around the fingernails. An annual cumulative exposure of 600 R (i.e., 0.24 roentgens/day over 250 working days/year) was commonly viewed as the average erythema dose, that is, the exposure that affected a reddening of the skin. The value was obtained via a 1927 poll of radiotherapists. Their collective perspective yielded an average erythema dose of 550 R. The value was then rounded to 600 R in order to adjust for the presence of background scatter radiation [83].

The efforts of this committee of Taylor were preceded by the actions of Mutscheller who first proposed the concept of a tolerance dose for ionizing radiation in 1925, suggesting a value of 1/100 of the erythema dose averaged over 30 days. The approach of Mutscheller [84,85] was practically applied to estimate the quantity of lead shielding needed to protect workers. The concept of tolerance in this case was a type of equilibrium in which there would be a dose to which the workers could be exposed without noticeable harm. From this rather rudimentary start, the tolerance dose concept would proceed and provide the foundation for future radiation safety standards [64,82].

While the credit of first reporting on the concept of a radiation tolerance dose concept goes to Mutscheller, other radiation health experts were independently arriving at the same conclusion [86,87]. While each of these groups proposed a different time interval for the tolerance dose estimation, these values closely converged once similarly normalized. In these cases, the tolerance dose was dependent on the concept of a safety factor to reduce exposure using a fixed fraction of the estimated minimally affecting dose (erythema dose).

During the late 1920s, the International Committee for Radiation Protection (ICRP) offered their version of a tolerance dose for occupational radiation exposure, which was adopted in 1928. This ICRP standard was based on Mutscheller's safety factor of 1/100 of an erythema dose. In fact, it remained the principal basis for international and U.S. prewar radiation standards [83]. The ICRP standard was also principally employed for the calculating of the thickness of lead shielding in x-ray production.

This situation would change during the next decade as Professor Gioacchino Failla (Figure 3.18) of Columbia University Medical School would lead an effort to better understand effects of radium exposures on people and whether safety standards could be derived using skin and blood changes as biological markers. This research suggested that 0.6 R/month would be a safe dose, a value that

FIGURE 3.18 Gioacchino Failla. (From http://www.orcbs.msu.edu/radiation/resources_links/historical_figures/failla.htm.)

was 10-fold lower than the Mutscheller and ICRP recommendations. Despite the acceptance of the basic clinical findings of Failla and the safety factor concept, the committee agreed upon a higher tolerance dose using a lower safety factor. In this case, the tolerance dose was 0.1 R/day.

The tolerance dose and a threshold dose–response were not believed to be identical by the leaders of this era. The concept of tolerance assumed that there was probably some level of minor harm or damage. However, such assumed minor changes were not thought to be serious enough that they had to be avoided. Thus, the reddening of skin was clearly not a below-threshold response. The observation of reddening skin following radiation exposure was a definitive indication that a biological response threshold had been exceeded. The reddening of skin was used as a biomarker, as a type of warning signal for more serious concerns should the exposure exceed this level. Yet the issue of tolerance and threshold was often functionally confused. The use of a specific numerical limit was often interpreted to mean that there was a threshold of radiation exposure below which no harm would occur.

While the ICRP and other radiation advisory committees were debating the concept of a tolerance dose, thresholds, and skin and blood changes, their field would be turned upside down by a report from Hermann J. Muller (Figure 3.19) that x-rays could cause mutations in the male fruit fly sperm [88,89], which was extended to plants by Stadler [90] within a few months of the Muller report. Furthermore, the findings of mutations as induced by x-rays in fruit flies soon became widely reported by others working in the field [8].

These concept changes failed to be immediately implemented into regulatory guidance-based/exposure practices as committee activities were shut down during WWII. While

FIGURE 3.19 Hermann Joseph Muller (1890–1967). (From http://en.wikipedia.org/wiki/Hermann_Joseph_Muller [PD-U.S.-only].)

the genetic concerns were raised in the years leading up to WWII, such issues became dominant in the aftermath of the dropping of the atomic bombs on Japan and activities associated with aboveground testing of nuclear weapons. This set the stage for a long-term debate over the nature of the dose–response in the low-dose zone from ionizing radiation. This debate would be led by the genetics research community under the aegis of the NCRPM, ICRP, and National Academy of Sciences (NAS).

The debate over whether toxicologically based dose–response thresholds exist is a long one, approaching its hundredth year. It is difficult to think of other examples in the scientific literature where a major question still remains as broadly uncertain and contentious and as practically important as ever. On the surface, it would appear that this should not be a particularly difficult problem to resolve. However, this has not proven to be the case. As far as genotoxic carcinogens are concerned, regulatory agencies adopted a precautionary philosophy toward carcinogens based on fear of the cancer endpoint and the possibility that the process of carcinogenesis could be initiated by a single mutagen molecule or ionization in a single cell. This set of concerns gave the assessment and regulation of carcinogens a unique status within society and forced the regulatory agencies to deal with chemical carcinogens and ionizing radiation in a different manner than agents that were not classified as carcinogens. For noncarcinogens, there was believed to be a threshold below which no adverse effect(s) would occur. For genotoxic carcinogens, this was not the case. The regulatory agencies decided to treat them differently assuming there was no safe exposure. The next section will therefore address the theoretical foundations of why the scientific community has long adhered to a belief in a threshold dose–response model. These concepts have involved statistical, thermodynamic, and biological/toxicological domains. The subsequent section will provide a detailed assessment of scientific challenges to the threshold dose–response model, including that initiated by the radiation genetics community that ushered in the linearity at low-dose concept.

THRESHOLD DOSE–RESPONSE: THEORETICAL FOUNDATIONS

Introduction

While the threshold dose–response model was adopted without having been validated by the scientific, medical, or regulatory communities during the early decades of the twentieth century, this section of this chapter offers a perspective on what could be the toxicological foundations that would support a threshold dose–response. Five arguments are made with documentation that supports a threshold interpretation. These include the following: (1) there are observations that large numbers of atoms/molecules are needed to induce biological responses; (2) the second law of thermodynamics imposes an energy requirement for chemical reactions to occur, resulting in a threshold; (3) there are pharmacokinetic and pharmacodynamic processes; (4) the use of arithmetic/linear graphing can create a false impression of a linear dose–response, whereas logarithmic dose graphing using molecules/kg/day lead to the conclusion of a distinct threshold for chemical carcinogens; (5) since tumor latency is a function of dose, it is possible to obtain *practical* thresholds for carcinogens, even assuming a linear dose–response relationship.

Relationship between Biological Function and the Number of Atoms Inducing It

In 1943, George Evelyn Hutchinson [91] (1903–1991), Yale University professor and the father of limnology and modern ecology, explored the relationship of biological functions and the number of atoms required to induce such functions using the liver cell as his model. He provided a listing of various elements from the periodic table and estimated their presence in a human liver cell. Twenty-years later, he provided a considerably expanded view of the number of atoms needed to induce specific liver cell functions [92].

He concluded that it takes the presence of a relatively large number of atoms to affect a response even when there may be a highly evolved biochemical specificity for a reaction to take place. He hypothesized that even at its most sensitive level of activity, the liver cell, as well as probably most cell types, may require more than 10^4 atoms/molecules per cell to induce a response. Even in the case of nonnutritive agents, such as Cd and Pb, the number of atoms for each of these agents is 10^6–10^8/liver cell and was associated with no detectable effects.

Such findings were interpreted by Bertram Dinman [93], a prominent University of Michigan toxicologist, that the presence of specific atoms in a cell does not provide proof of biological activity nor does it offer any biological significance from a beneficial or harmful perspective. Using a complementary approach to Hutchinson [91,92], Dinman [93] concluded that nearly 400 in vitro studies of enzymatic inhibitors for 100 different enzymes [94] revealed that the lowest concentration at which most sulfhydryl enzyme inhibitors act was in the 10^4–10^5 molecules/cell range, a value similar to that proposed by Hutchinson [92]. Taken together, these different approaches lead Dinman [93] to suggest thresholds are the rule in biological systems. Dinman went on to conclude that

> To believe that such molecules cause an undesirable effect disregards the presence of multiplicity of interfering substances. Such thinking also does not take into account the fact that the dose of a foreign atom may be related to the probability of its interacting with an available active site, or that similar probability governs the answers to the question of whether interactions will occur at discrete topographical loci upon a structural or functional molecule (or on a possible precursor). While the construction of stochastically sound models is remote, the reasonableness of the hierarchy of cellular element concentrations as these relate to metabolic function suggests that a threshold for biological activity exists within a cell at 10^4 atoms.

A practical approach for assessing the threshold issue may be to consider the actual exposure that humans experience from environmental concentrations of well-known toxins. In the case of lead, the CDC has long used 10 μg/100 mL of whole blood as an action level (recently reduced to 5 μg/100 mL). At this concentration of Pb in blood (10 μg/100 mL), there are 3×10^{16} Pb atoms. If the exposure standard were to be reduced to 1 μg/100 mL, there would be 3×10^{15} Pb atoms per 100 mL, or 3×10^{13} Pb atoms/1 mL. It is difficult to consider that at 1 μg/100 mL, a value common today in apparently healthy humans, there are 30-trillion lead atoms for each of the 7500 mL of blood in the average U.S. adult. Such calculations support the existence of an individual and public health threshold for lead.

In a similar fashion, the U.S. EPA benzene drinking water standard of 5 μg/L has an estimated (1×10^{-5}) lifetime risk of developing leukemia based on linear nonthreshold (LNT) models. At the drinking water standard of 5 μg/L, there is an estimated 8×10^{15} benzene molecules/L. This cosmologically large number is further expanded when one considers that the EPA assumes that 2 L is consumed each day of a 70-year life. These two examples are not exceptions but illustrate the rule as far as the relationship between exposure to atoms/molecules and the onset of adverse health effects are seen in humans. In fact, regardless of whether the regulated agent is a carcinogen or not, the estimate of acceptable exposures is typically in the 10^{14}–10^{20} molecules/day for 70 years.

Second Law of Thermodynamics and Dose–Response Relationships

While individual thresholds are typically observed in toxicological and epidemiological studies, the issue of population-based thresholds has been less certain. This uncertainty is based on the fact that considerable variation exists between individuals with respect to their susceptibility to toxic/carcinogenic agents due to genetics, diet, developmental, and aging processes, among other factors. Thus, apparent thresholds can profoundly differ between individuals. The regulatory challenge therefore may not be that of an individual threshold estimation but a population threshold, thus transforming this question from a biological to a biostatistical one [95,96]. The question therefore is whether there is a population-based threshold for noncarcinogenic toxic substances and carcinogens.

A central fact that underlies the current debate over threshold or LNT for radiation-induced cancer is that each individual experiences cosmic numbers [97] of mutations/cell each day (e.g., depending on the cell type—millions to multiple billions) from normal metabolism and background radiation. If, in fact, a single hit were to be enough to cause cancer, as suggested by the LNT theory, it is difficult to explain why many, perhaps most individuals, would not quickly display various toxicities and/or cancer, unless reasonably efficient repair processes exist.

Despite the fact that vast numbers of mutations occur and are repaired on a daily basis, are there exposures that will not induce genetic damage? According to Schaeffer [96], the second law of thermodynamics provides a basis for such a threshold. This conclusion is founded on the premise that there is a minimum net free energy required for the reaction of an agent (in this case, a toxicant or carcinogen) with a target molecule [98–101]. If the threshold value is diminished, the net free energy that is related to the threshold value would also diminish. However, the net free energy cannot reach or become zero as this can only happen if both the activation energy and the net free energy are zero at all levels of exposure [99,102]. Since chemical bound energy is the "exclusive source of utilizable energy in biological systems, there is a minimum activation energy and a minimum net free energy above zero for all cellular reactions including the induction of cancer." Mutagen binding to DNA affects DNA alterations (i.e., mutagens) and can initiate the process of carcinogenesis. A thermodynamic analysis of this process has yielded a specific energy threshold that needs to be exceeded for the induction of chromosomal aberrations. Such an assessment provides a basis for why each interaction between molecules does not result in a chemical reaction and product formation that could lead to a biological effect. Each xenobiotic agent needs to provide the activation energy to react with the receptor molecule. Using the Maxwell–Boltzmann equation, Koch [103] estimates that the activation energy may vary between 16 and 42 kJ/mol for biochemical reactions in vivo. The minimum net energy required to induce a single strand break

was estimated to be at least 17 kJ/mol [101], supporting the existence of a threshold.

According to Schaeffer et al. [99], it may be appropriate to use the biological analogy of fertilization of an egg by a sperm when placing the process of chemical carcinogenesis in context. While there are billions of sperm that are present and actively attempting to fertilize the egg, only one does. Furthermore, the process of fertilization requires the presence of massive numbers of sperm. In a similar fashion, chemical reactions take place only where there are sufficient molecules, each displaying kinetic energy, eventually colliding with each other and eventually yielding a novel intermediate molecule that then transforms to the final product. The formation of the reactive intermediate represents a random event within an environment of many millions of molecules. The actions of a carcinogen must therefore be seen as a stochastic process involving massive numbers of targets and a cosmically large numbers of molecules.

Since chemical reactions are subject to entropy and free energy constraints, Schaeffer et al. [99] indicated that mutational effects and carcinogens likewise display activation and free energy requirements as well as entropic dependence. Since entropy represents a statistical estimate of system disorder, these reactions will show the mass requirements of chemical reactions. In fact, therefore, thermodynamic principles lead one to conclude that LNT is both chemical and biologically highly improbable. Pharmacokinetic reasons (absorption, tissue distribution, metabolism, storage, excretion) and the number of molecules that reach a target and thermodynamic parameters that affect molecule activation create the foundation for a physical/chemical/biological basis for biological/toxicological threshold.

This perspective reinforces the previous views of Dinman [93] that there is likely to be a minimal number of atoms/molecules needed to affect biological responses. In fact, the use of the number of Dinman [93] led Friedman [104] to estimate the minimally inducing number of molecules for various potent carcinogens (i.e., 8.6×10^{15} molecules/kg body weight or 10^8 molecules/kg). Using such analyses, Koch [103] estimated a *response threshold* level for various drinking water contaminants including chemical carcinogens. This modeling considered standard parameters such as average body weight, amount of water consumed/day, molecular conductivity as well as estimates of lipophilicity, and tissue distribution of biological activity. Koch [103] concluded that such outcomes provide not only a theoretical but also a practical means of translating toxicological findings into risk assessment practices. He also emphasized that this perspective illustrates that it is profoundly unrealistic to believe that a single molecule may affect the occurrence of cancer, thereby supporting a threshold dose–response perspective.

Despite their solid underlying foundations, the arguments of Hutchinson [92] and Dinman [93] and similar extensions by Claus [105] and Jukes [106] have not attracted significant formal attention in the literature. However, in 1980, Preussmann [107], a well-known experimental cancer researcher, attempted to apply such concepts to the issue of chemical carcinogen thresholds using the data of Mohr and Hilfrich [108]. In this study, kidney tumors were induced in rats via a subcutaneous (SC) injection of diethylnitrosamine (DENA) using eight different single-dose treatments ranging from 1.25 to 160 mg/kg. They reported 11/20 kidney tumors in the single dose of 100 mg/kg and 1/20 kidney tumors at 1.25 mg/kg. The 1.25 mg/kg dose was the equivalent of 0.3 mg/rat. The control group showed no kidney tumors. The 1.25 mg/kg level of exposure, which was presumably carcinogenic, corresponded to about 2×10^{18} molecules/rat or approximately 10^{16} molecules/kidney. Assuming the weight of the kidney was about 10^{-10} g, Preussmann [107] stated that there was about 10^6–10^7 molecules of DENA/kidney cell at this dose. The 10^6–10^7 molecule number range per cell was about 100–1000-fold greater than the postulated theoretical threshold of 10^4 molecules/cell.

DNA Adducts: A Method to Estimate Thresholds and/or Linearity?

As an alternative to the proposition of a theoretical molecule threshold that was postulated by Hutchinson [92], Dinman [93], Claus [105], and Preussmann [107] suggested a more mechanistic approach using DNA adduct biomarkers within susceptible tissue. This approach has demonstrated appeal in the subsequent decades with numerous researchers exploring the relationship of DNA adducts as biomarkers for chemically induced tumors. One of the most integrated of these attempts is seen in the publications of Williams et al. [109,110]. These authors have traced the biological processing of the chemical exposures from the portal of administration through the spectrum of pharmacokinetic processes to pharmacodynamic activity, inducing DNA adduct formation, location of the adducts, and efficacy of DNA repair, and to the route of neoplasmic cell transformations, including the number of mutations, development of tumor microvasculature, and tumor-suppressive elements. The documentation of this type of information was placed in a quantitative perspective, leading Williams et al. [111] to derive a new cell-based mechanism of action that could provide what they called a safe exposure level (SEL) for a DNA-reactive chemical carcinogen; the SEL was based on a DNA binding of less than 1 adduct/10^9 nucleotides for a single chemical agent. As suggested previously, the methodology used by Williams et al. [111] follows the administered agent(s) from the beginning to the end stage quantifying all changes that led to the final product and integrating the pharmacokinetic- and pharmacodynamic-mediated information along with tumor development activities.

Starting with an oral exposure to a stable procarcinogen, Williams et al. [111] noted that following ingestion, the agent will be subjected to the risk of degradation caused by chemical and biological processes including gut microflora. Following G.I. absorption, the agent will be transported via the portal blood flow to the liver and undergo the first-pass extraction. For that portion of the dose not taken up by the liver, it will enter systemic circulation, typically

in a highly diluted manner. For these *escaped* molecules, they become spread out to many organs and their cells, further diluting the dose.

As far as DNA-reactive carcinogens are concerned, the only cells at risk for initiation (and therefore cancer development) are reproducing stem (labile) cells. These cells typically represent <1% of the cell population in any tissue. Another factor affecting exposure to critical cellular sites is that cells display an active extrusion process via p-glycoprotein ATPase to pump hydrophobic cationic agents out of the cell and/or prevent their entry. Williams et al. then generalized that it may be reasonable to assume that perhaps 80% of the *carcinogenic* molecules may be detoxified in the liver. Of the remaining 20% of activated agent, a certain proportion would be *consumed* by encountering any of a wide range of macromolecular nucleophiles in the cytoplasm. In fact, the binding of bioactivated agents is typically much greater in the cytoplasm than nucleus, with a differential of about 100:1.

As each of the activated chemicals reaches the nucleus, it must still contend with the fact that the DNA is enshrouded with nucleophiles that further reduce the potential for mutagenicity. With respect to the cellular genome, it consists of about 3.2×10^9 base pairs. Within this framework, there are approximately 30,000 genes with an average size of 3,000 base pairs, with about 9×10^7 functional nucleotides. Up to about six genes would have to be mutated to effectively mediate the process of carcinogenesis [111].

In DNA adduct studies, at low levels of exposure, a relationship between dose and adducts has been shown as follows [111]: aflatoxin, 70 adducts/10^8 nucleotides; DMN, 360 or 5/10^9 for N^7 and O^6 methylguanine adducts; and 1/10^8 for meIQX. The question arises as to what level of DNA adduct displays biological/toxicological significance. Since spontaneous DNA damage based on labeling studies reveals about 1 lesion/10^6 nucleotides, it appears that background DNA adducts are about 10–100-fold greater than that reported in the previous experimental studies.

Based on such information, Williams et al. [111] hypothesized that an adduct formation rate of 1/10^{11} should be considered without biological significance. The occurrence of 1 adduct/10^9 nucleotides would represent about 3 adducts/cell, at most affecting only 3 of the 30,000 genes/cell. Even with 1 adduct/10^9 nucleotides, these authors concluded that there is little biological concern. Further, only a proportion of such adducts will be mutagenic, while others will have their mutagen damage repaired. In addition, not all mutations are of equal concern. Only mutations in certain locations of susceptible genes can alter gene function. Thus, many mutations can be without critical biological effect. For example, the vast majority of mutations of the p53 tumor suppressor gene did not significantly affect the amino acid structure of the protein [112]. Another consideration affecting the development of a tumor cellular mass is the need to circumvent host resistance and to form a neovasculature for expansion to a neoplasm. According to Williams et al. [111], the likelihood that a preneoplastic lesion will progress to a tumor is ≤1/1000. The capacity to progress is a function of dose, which can affect the replication potential of the affected cells and related promotional stimuli. At low doses, these promotional factors are far less evident.

Based on the previous mechanistic approach, Williams et al. [111] have addressed the issue of dose–response in general and the threshold concept in particular. Within this framework, these authors isolated specific stages of the process of carcinogenesis including cell proliferation and the induction of numerous preneoplastic and progressive hepatocellular lesions. For each of these steps, they report the occurrence of a threshold response. These collective factors led Williams et al. [111] to propose a change in the risk assessment of carcinogens—from that of an LNT model to the one that is both mechanism and threshold based using the frequency of DNA adducts as the key molecular endpoint to predict the risk of cancer.

The line of argument that has just been presented is the result of nearly 40 years of detailed toxicological investigations [113]. It confronts the long-held linearity perspective as stated in 1969 by Fahmy and Fahmy [114] that "there seems to be no such thing (i.e., thresholds) as far as mutations are concerned." Even more so, the toxicologist Bruce Ames [115] expanded on this area by stating

> It is worth emphasizing that one molecule of a mutagen is enough to cause a mutation and that if a large population is exposed to a 'weak' mutagen it may still be a hazard to the human germ line, since no repair system is completely effective, there may be no such thing as a completely safe dose of a mutagen.

While these historical cautionary statements of Fahmy and Ames still even now seem prudent, the cumulative findings as presented previously demonstrate that this type of conceptualization is fundamentally flawed stochastically, pharmacokinetically, pharmacodynamically, and thermodynamically.

Threshold Dose–Response: Dose-Dependent Transitions and Underlying Mechanisms

Within the past decade, there has been a strong consideration given to the topic of dose-dependent transitions in toxicity and how such descriptive changes in the dose–response could be mechanistically explained. The Health and Environmental Science Institute (HESI) conducted several workshops that addressed this issue [116,117]. These efforts were premised on the belief that a range of different mechanisms may occur for a toxic substance across a very broad dose–response range.

Such dose-dependent toxicities could also impact the risk assessment process and need greater clarification. The expert committee identified various processes that might contribute to the occurrence of dose-dependent toxicity transitions. These processes include gastrointestinal (G.I.) tract or respiratory absorption, tissue distribution as affected by protein binding and active transport systems, and chemical transformation, including bioactivation and detoxification,

TABLE 3.1
Mechanistic Basis for Dose-Dependent Transitions in Case Studies

Case Study	Mechanism
Acetaminophen	Metabolic activation and glutathione depletion
Butadiene	Metabolic activation
Ethylene glycol	Saturation of oxidation
Formaldehyde	Changes in cell kinetics
Manganese	Low-dose essentiality and high-dose toxicity
Methylene chloride	Metabolic activation
Peroxisome proliferator-activated receptor (PPAR)	Receptor activation
Progesterone/hydroxyflutamide	Receptor interaction
Propylene oxide	Altered homeostasis
Vinyl acetate	Altered homeostasis
Vinyl chloride	Adduct formation and DNA repair
VDC	Enzyme saturation and depletion
Zinc	Deficiency toxicity and high-dose toxicity

Source: Slikker, Jr. W. et al., *Toxicol. Appl. Pharmacol*., 201, 203, 2004.

receptor interactions, tissue and DNA repair processes, as well as altered homeostasis. Thirteen distinct examples of dose-dependent transitions were identified (Table 3.1) and discussed (Table 3.2). These examples were selected since they affect a broad spectrum of mechanisms such as metabolic activation, altered homeostasis, enzyme saturation and depletion, alteration in cell kinetics, adduct formation and DNA repair, and glutathione depletion.

An instructive example of a dose-dependent transition involved the toxicological assessment of acetaminophen, an agent whose toxicity has been closely associated with a depletion of reduced glutathione (GSH). This depletion begins to occur upon saturation of glucuronidation and sulfation pathways. This saturation process results in more acetaminophen becoming converted to the toxic electrophilic metabolite of acetaminophen called NAPQ1. Once formed, the NAPQ1 becomes quickly detoxified by GSH in the affected tissue, resulting in GSH depletion. At high metabolite doses, the GSH depletion becomes more extreme, leading to occurrence of NAPQ1-induced damage. Thus, the central factor

TABLE 3.2
Factors Affecting Dose-Dependent Transitions

Dose-Dependent Changes in Excretion

Considerable research has been conducted concerning the quantitative renal saturation behavior of drugs.

Two types of saturable active transport processes affect the urinary elimination of xenobiotics (tubular secretion and tubular resorption).

Saturation of tubular secretion tends to lower renal clearance at high doses; this results in yielding a larger internal body concentration at higher rates of external dosing.

Saturation of tubular resorption processes displays the opposite effect.

Dose-Dependent Alterations in Metabolism

Michaelis–Menten enzyme kinetics.

As activating/detoxifying enzymes have limited capacity, they will become saturated at some elevated dose.

At progressively higher doses, a larger fraction of the xenobiotic is metabolized and may be routed to other pathways.

Dose-Dependent Changes in Repair, Cell Killing, and Rate of Cell Replication

Tissue repair.

DNA repair.

Saturation of repair systems at elevated levels of exposure tends to result in longer biological half-lives of the induced lesions and a greater likelihood for the lesion to be incorporated into the genome following DNA replication. This type of process would enhance the incidence of mutations per incident of exposure.

Induction of repair process can also influence the degree of damage retained. It is generally unknown how the dose of mutagens affects the magnitude of the repair induction process and how this may vary between individuals. These factors are likely to affect dose-dependent transitions.

Source: Slikker, Jr. W. et al., *Toxicol. Appl. Pharmacol*., 201, 203, 2004.

in acetaminophen's induced hepatotoxicity involves both the formation of NAPQ1 and the tissue's ability to produce adequate amounts of GSH. The acetaminophen-induced hepatotoxicity will be affected by factors that influence that capacity for bioactivation as well as GSH production and regeneration. There is likely to be some degree of interindividual variation in the capacity of acetaminophen to transition into a toxicity mode. Another example of a saturable enzyme involves the metabolism of ethanol by alcohol dehydrogenase (ADH). Once this enzyme becomes saturated, the toxicity response becomes nonlinear. In contrast to the example with acetaminophen, the saturable enzyme is the one that led to detoxification.

Another example of an agent that is metabolized via cytochrome P450-dependent monooxygenases to a metabolite that binds to and depletes GSH is vinylidene chloride (VDC). According to Slikker et al. [116,117], the GSH depletion decreases in proportion to the concentration of the toxic metabolite generated. However, a dose-dependent toxic transition occurs when the GSH has been depleted by 60% that occurs at 50 mg/kg with no toxicity occurring at 25 mg/kg. It was speculated that the 60% GSH decrease may reflect the limits of reserve capacity below which the toxic mechanisms are able to alter plasma membranes leading to frank toxicities.

A particularly interesting example of a dose-dependent transition involved the mechanism by which propylene oxide induces nasal tumors in rats following exposure via the respiratory route [116,117]. Of particular interest was that the nasal tumor incidence reflected a clear threshold dose–response, whereas the occurrence of hemoglobin and DNA adducts followed a linear dose–response relationship and were thus not capable of accounting for the nasal tumor incidence. However, the cell proliferation induced in the nasal respiratory epithelium also followed a similar threshold pattern of the nasal tumors. These findings show a very strong association between cell proliferation and nasal tumor incidence (Figure 3.20).

The dose-dependent cell proliferation model has also been proposed in the assessment of formaldehyde-induced nasal squamous cell carcinoma in rodents. The doses associated with the cancerous responses were those that induced cell death while subsequently stimulating regenerative cellular proliferation (RCP). In a detailed cancer bioassay study with formaldehyde, Monticello et al. [118] employed five concentrations (0.7, 2.0, 6.0, 10.0, and 15.0 ppm) at which RCP was measured at multiple locations in the nasal tissue over days 1, 4, and 10 and at weeks 6, 13, 26, 52, and 78. Of particular interest was that the formaldehyde-induced dose-related changes in the rate of cell replication were J-shaped with below control responses seen with the two lowest concentrations. At low concentrations of formaldehyde exposure, the incidence of nasal tumors was less than in the control group. This finding was consistent with the hypothesis that the tumor incidence risk is well predicted by the cell replication rate as was the case with the propylene oxide. The decrease in the cell proliferation rate at low concentrations of formaldehyde was proposed to be caused by a reduction of DNA synthesis and cellular replication due to the occurrence of DNA cross-links that block cellular replication complex. The number of cross-links and their rate of removal were proposed to be rate limiting, therefore controlling the replicative synthesis. While this process was still ongoing at high concentrations, the capacity of formaldehyde to form the DNA cross-links was believed to be overwhelmed by its capacity for inducing cytotoxicity, leading to the increased tumor response at high concentrations of exposure.

Other examples of dose-dependent changes in the metabolism of xenobiotics as affected by enzyme saturations have been shown to affect dose-dependent toxicity transitions. For example, Belinsky et al. [119] assessed the implications of saturation of bioactivation of the carcinogen nicotine-derived nitrosamine ketone (NNK) on adduct formation. NNK forms O^6-methyl guanyl adducts eightfold more efficiently at low IP doses (0.3–1.0 mg/kg) than at 100 mg/kg. The difference in response between the high and low doses was hypothesized to be due to the saturation of a local enzyme that produced the methylating agent. A similar type of dose-dependent kinetic mediated response was reported for vinyl chloride. In this case, the tumor incidence response is linear at low dose but flattens out at high doses (1,000–10,000 ppm). Of interest is that testing at only high doses would have yielded an underestimation of tumor risks to the low-dose range with standard modeling procedures [116,117].

Plotting Data: A Key Element in the Threshold versus Linearity Debate

There has been considerable debate in the toxicological literature on the plotting/graphing of dose–response relationships. This debate has been led by William Waddell of the University of Louisville, who argues that dose–responses should be plotted via the use of logarithms rather than linear scales. Waddell has argued that current risk assessment methods typically assure that there is no safe exposure to carcinogens, only at a zero dose. He claims further that the selection of linear scales for plotting dose–response relationships has no scientific basis and, in effect, has concealed the existence of thresholds. The perspective of Waddell was stimulated by Rozman et al. [120], who argue that how one plots data can affect their perspective on the nature of the dose–response. These authors asserted that the case for low-dose linearity emerged from a conversion of the logarithm plotting of the dose–response as recommended by Gaddum in 1945 [121] to an arithmetic approach but a few years later. Based on data concerning the induction of CYP A1 by Tritscher et al. [122], who used nearly two dozens of doses of tetra-CCD, the data plots were compared with either an arithmetic or logarithmic approach. Arithmetic plotting created the distinct impression that the measurements were taken close to zero and that the Michaelis–Menten-based fit would be approaching zero. Rozman et al. [120] indicated that the arithmetic plotting creates a profound misrepresentation of the findings. They indicated that the dose–response

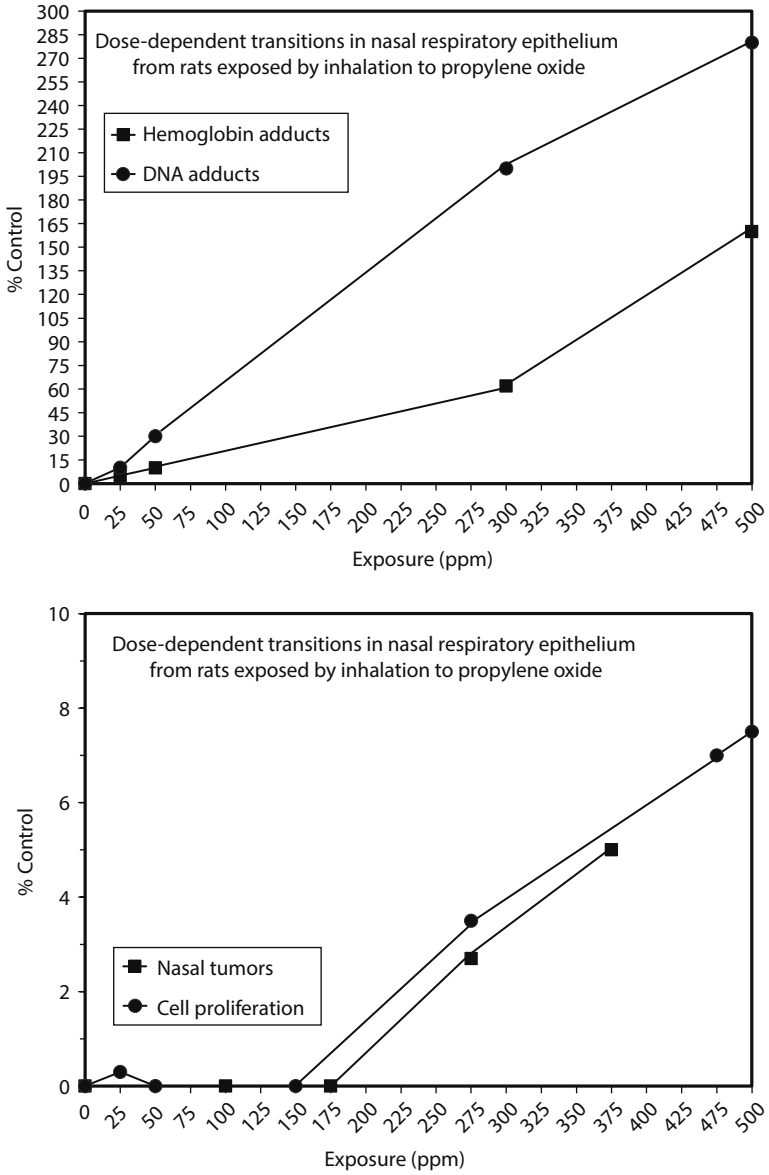

FIGURE 3.20 Dose-dependent transitions for nasal respiratory epithelium from rats exposed by inhalation to propylene oxide. (Based on Slikker, Jr. W. et al., *Toxicol. Appl. Pharmacol*., 201, 203, 2004; Slikker, Jr. W. et al., *Toxicol. Appl. Pharmacol*., 201, 226, 2004.)

should be represented logarithmically since one molecule is in close proximity to a zero point of reference. A limitation to using logarithmic is that the log of zero for the control is undefined. While this is an issue, it is commonly dealt with by giving the control group a very small positive value of no practical consequence.

It is generally accepted that quantitative risk assessment (QRA) for carcinogens was implemented to replace the apparent arbitrary judgment of extrapolating animal data to humans via the use of uncertainty factors. The underlying basis for this change was via the assumption that a single molecule can cause a mutation that can cause a molecular transformation with the result eventually leading to the development of cancer. Since there was an inadequate understanding of the process of carcinogenesis and great fear of this disease, it supported a precautionary perspective. Prior to the switch to QRA for carcinogens, there was the creation of the carcinogenicity bioassay. This assay had the simple goal of answering a qualitative question: was the agent to be tested a carcinogen or not? This being the case, a large number of doses were not deemed necessary. Typically there were only one or two doses that were administered. While this testing framework was adequate to answer the qualitative yes or no question, this design failed to address the quantitative demands for assessing the cancer dose–response [120]. A qualitative test system was used by the toxicology community for critical quantitative estimates of the dose–response in the low-dose range. To further distort the low-dose evaluation estimates, the qualitative design has incorporated the maximum tolerated dose (MTD) concept (see "Maximum Tolerated Dose/Dose–Response" section) and used it as a standard.

To illustrate the difference in plotting methodology, Waddell employed data from a 4000-rat cancer bioassay with nitrosodiethylamine. The dose–response data strongly fit a linear dose–response relationship when plotted on the linear scale. In contrast to the linear scale, Waddell introduced the logarithmic scale [120] that plots not via the mg/kg/day method but by using molecules/kg/day. On the abscissa, a logarithm represents dose as the number of molecules/kg/day from 1 to 10^{23}. The difference in the representation of the results is striking with the linear plotting giving the impression that the cancer risk approaches a background of zero exposure in a linear fashion. With the logarithm-scale molecule exposure model, the cancer incidence response does not approach zero molecules but approaches/collides with the abscissa at 10^{17} molecules/animal, thereby providing the distinct impression of a dose–response threshold.

Waddell demonstrated that this was the general pattern of dose–response relationships for numerous positive cancer bioassays [123]. The assessment of Waddell [123–134] attracted considerable attention of leading toxicologists and dose–response modelers, some of whom took issue with his methodology [135–140]. The interpretation of Waddell was disputed, dismissed as highly flawed by Crump and Clewell [135], and seen as a visual artifact based on the scale of the plot. Andersen et al. [136] stated there was no realistic representation of a threshold that could be demonstrated even in very large toxicity studies. Even though Andersen et al. were sympathetic to the likelihood of thresholds for chemical carcinogens, they argued that the only way to establish such a threshold was via a mechanistic understanding of the biological dose–response process. Lutz [137] offered further criticism of the Waddell perspective arguing that a major problem for this approach was that the logarithm transformation of the dose made it impossible to indicate a control group response since the log of 0 is undefined. He also criticized Waddell for creating this *problem* by simply subtracting the control group incidence from the treatment groups' incidence.

The series of papers of Waddell are important for a variety of reasons. The most substantial is that plotting of dose–response relationships using the number of molecules as the dose is highly instructive and provides an improved context for assessing toxicological data. Such plotting does not prevent the plotting of data via other approaches. In fact, such comparisons can lead to more insightful follow-up questions about the experimental findings. Waddell plotted tumor incidence dose–responses for 50 chemical carcinogens showing that the estimated zero response was in the 10^{17} molecule range/animal. This is an important observation that should not be dismissed.

An objective appraisal of this debate between Waddell and those who criticized his approach is that they came to the same practical conclusion. That is, the science reveals that there is no measureable enhanced risk at lifetime doses in which the number of molecules can reach 10^{17}/animal even at a de minimus level. The debate over whether there is linearity existing after an extrapolation over 17 orders of magnitude of dose is simply unresolvable in a technical/experimental manner. If advocates of an LNT position can retain a theoretical belief in LNT, then it should be accompanied with the recognition that there are, at the least, practical thresholds.

The Rozman plot has been widely publicized for the assessment of carcinogens. This concept is general and could readily be applied to noncarcinogens as well. As an exercise in this regard, the number of molecules in a liter of drinking water at U.S. EPA drinking water standards was estimated. These are exposure standards and are generally based on the reference dose (RfD)/no observed adverse effect level (NOAEL)/uncertainty factor process. That is, these exposure standards first select the most sensitive animal model, study, and endpoint, and then a threshold of toxicity is estimated. Uncertainty factors from 100 to 1000 are typically employed. At that point, a relative source contribution component is incorporated into the standard derivation process. Based on such a process, it may be expected that there should be a substantial level of confidence that an exposure of 2 L/day at the exposure standard should not be associated with any adverse health effects. That is, the exposure standards should therefore generally be considered as being of a highly precautionary nature. Yet, even with those *highly precautionary* daily exposures at the level of the drinking water standard, the number of molecules per liter is generally in the 10^{16}–10^{18} molecules/L. Once again at a rate of 2 L/day for 70 days, the number of *toxic* molecules that can be safely consumed in a lifetime from drinking water alone would be in the 10^{20}–10^{22} range. It should be noted that these exposure standards are based on the premise of protecting those at increased risk.

So what does this dose–response relationship based on molecules mean? The findings indicate that no demonstrable toxicological activity occurs until massive numbers of toxic molecules/day to noncarcinogens are administered. This is also the case for carcinogens as well.

CHALLENGES TO THE THRESHOLD MODEL

Introduction

The threshold dose–response has been the dominant dose–response model in pharmacology and toxicology throughout the twentieth century to the present. As discussed earlier, the biphasic dose–response model and the threshold dose–response models were advocated by homeopathy and traditional medicine, respectively, in the early decades of the twentieth century. By 1930, the biphasic dose–response, along with homeopathy, was significantly marginalized, whereas the threshold dose–response, along with traditional medicine, was transformingly dominant in the United States. In fact, the success of traditional medicine in the United States was due principally to major institutional, governmental, and performance reforms that were direct offshoots of the 1910 Flexner Report [66], transforming U.S. medicine education into a far more scientific and rigorous profession, with a strong research base [67]. This remaking of American medical education proved to be the key vehicle for

the transcendent dominance of American medicine in the twentieth century and as an offshoot, the dominance of the threshold model, and an offspring of traditional medicine. This critical association of the threshold model to the field of traditional medicine was the most significant factor affecting the acceptance and widespread use of this dose–response model in society. Despite its widespread acceptance and profound influence in the medical and scientific domains, the threshold dose–response model has also been the object of considerable criticism and challenged on multiple scientific and policy fronts. The following section presents and assesses the most significant challenges to the threshold dose–response model and their scientific foundations and places the threshold model and alternatives in a broad and integrative toxicological context.

Challenge #1: LNT and Ionizing Radiation

The radiation geneticist Hermann J. Muller [88], at the University of Texas at Austin, Texas, reported that high doses of x-rays induced mutations in the germ cells of male fruit flies. Based on limited experimental data, all of it at very high doses, and some of it contradictory to his hypothesis [9], Muller soon developed a strong belief that the mutation rate would be proportional to the dose of energy adsorbed. This implied that in the case of mutation, there would be no threshold. This issue would become progressively more contentious, especially with the expanding use of medical x-rays, the development of atomic weapons, dropping of the atomic bomb, and testing following their atmospheric explosions in the early cold war years.

During the 1920s to the early 1940s, the use of x-rays for therapeutic purposes for children and adults was extensive, treating arthritis, sinus infections, staphylococcal infections, otitis media, tuberculosis, pneumonia, and a myriad of other conditions besides cancers [46]. Seen within this perspective, one could sense the value of health concerns expressed by Muller, even if he might be incorrect with respect to the linearity hypothesis. It was only following the commercialization of penicillin after WWII and the rapid development of other antibiotics that the use of x-rays for such conditions was abandoned.

With respect to ionizing radiation, the idea of a threshold dose–response was first proposed in the mid-1920s based on occupational exposure concerns. The threshold concept was associated with the idea that workers could tolerate a certain amount of exposure without any significant health concern. That the threshold would need to be exceeded before harm would occur was generally accepted and especially accepted by the medical community. However, this view would become challenged by Muller following his discovery that x-rays induced mutations in fruit fly germ cells.

The first national impact of Muller's findings and advocacy occurred in 1935 within the American X-ray and Radium Protection Committee, later (1946) to be called the National Committee on Radiation Protection and Measurement (NCRPM). At this time, an unnamed committee member wrote to Taylor with the recommendation that the tolerance dose be reduced from 0.1 to 0.05 R/day based on the Muller findings that suggested a cumulative effect of x-rays on the genome over a prolonged period of time. Yet, the committee was at a loss as to how to extrapolate the findings in fruit flies to humans and to do so in a quantitative manner. Nonetheless, the data of Muller and others were kept at the forefront as the committee struggled with the issue of reproductive safety within the workplace and for patients. Over the next several years, the minutes of this committee reflect how the findings of Muller progressively came to challenge the view of a threshold. At the December 1938 meeting of the committee, a proposal was offered to amend the statement on the tolerance dose to the following: "The generally accepted tolerance dosage is taken as 10^{-5} r/sec for a 7 hr day. Geneticists on the committee pointed out that because of the cumulative effect of X-rays the tolerance dose should not exceed 10^{-6} r/sec ([82], see footnote 300)." By the December 1940 meeting, the committee explicitly stated that the rationale for lowering the tolerance dose was because of a concern with mutational effects ([82], see footnote 305). This recommendation did not sit well with the influential Failla who felt that a switch to a mutational endpoint would create considerable uncertainty since there may not be a threshold for a genetic effect. However, the data were still not viewed by the majority of committee members as adequate to prove that a threshold did not exist.

The committee tried to find a way through the confusing dilemma of the threshold versus linearity debate. While the committee believed that the term tolerance dose referred to a dose that could be tolerated or accommodated without any biologically significant damage, this was not the case with genetic damage that was thought to be irreversible, cumulative, and harmful. The dose–response change from threshold to linearity would lead to a new concept called *permissible exposure*. This was an important change because a permissible dose could still injure; it was not considered *safe*.

Permissible exposure was the means by which the *acceptable* risk concept became operational. Whether the general public understood or misunderstood the differences between a tolerance dose and a permissible dose is not known. Nonetheless, it would have important implications for the process of risk assessment and risk management. In offering a compromised position, the committee proposed not changing the exposure standard (i.e., a practical solution); this would placate the supporters of threshold; however, they would yield on the theory. The dose–response concept would change from a tolerance phenomenon based on the threshold concept to one without a threshold. The exposure would now be seen as a permissible dose, a concept built upon *acceptable risk*. The permissible dose terminology therefore represented a profound conceptual change, indicating that a threshold for x-ray-induced germ cell mutations was no longer assumed. However, despite the years of debate and the switch in terminology and concept, this critical policy change was never published (i.e., acted formally on); this failure to follow through on the agreed course of action would

lead to new controversy and debate in the years immediately following WWII.

The permissible dose concept was not lost during WWII despite the inactivity of the committee during that period, as committee members were reassigned to other activities for the duration of the war. However, following the end of the war and with the Nobel Prize recently received (i.e., December 1946), Muller was invited to join the NCRPM that was chaired by the more conservative Failla. In their 1947 draft report, the committee dropped the tolerance dose concept since ionizing radiation was now assumed to have no threshold and to display cumulative damage. Once again, organizational inertia and personal disputes hit and the 1948 recommended changes of the NCRPM would not be published for some 6 years! Based on these factors, it was hard to predict the behavior of the NCRPM committee. However, its actions were quickly overshadowed by a new NAS/NRC committee that was dominated by Muller and other radiation geneticists that were loyal to his perspective. The situation was also problematic since some scientists, such as Muller, with clear ideological perspectives, were serving on multiple national and international advisory committees, addressing the same dose–response policy topics [9].

In their groundbreaking publication of 1956, the NAS Biological Effects of Atomic Radiation (BEAR I) committee [141] formally asserted the concept of low-dose linearity for ionizing radiation-induced germ cell mutation. The geneticist community, as lead by Muller, saw the NAS committee as the vehicle to finally change the national risk assessment policy and to do so in a highly visible and far-reaching manner. That is, the 1956 report was widely distributed, was the subject of considerable media attention, and would have a profound impact on policy. The genetics section was intentionally written to be read by a general reader, further enhancing the spread of the linearity concept and fear of radiation in the media and general public.

The transition from threshold to linearity required about three decades, starting with Muller's seminal publication in 1927 [88]. During an oral history, the longtime chairman of the NCRPM Lauriston Taylor revealed that his committee was awaiting anxiously for the guidance of the prestigious NAS committee as they wanted to follow its lead. Within 1 year of the 1956 NAS report, the NCRPM generalized the concept of linearity for germ cell mutation to somatic cells, thereby affecting cancer risk assessment [9]. Soon other national and international committees would concur and a remarkable dose–response revolution had occurred, with significant long-term implications and consequences.

The generalization from the germ cell linearity response to somatic cell linearity was a key event, especially coming so soon after the NAS BEAR I report. It was also remarkable because even though the NAS BEAR I committee carried the greatest scientific credibility, the NCRPM committee, in effect, affected the major policy change. It is ironic that the NCRPM committee, which so intensely and anxiously awaited the guidance of the BEAR I committee, actually ignored guidance that emerged from the BEAR I committee activities on the question of somatic cell effects. More specifically, in a reading of the near-final draft report of the genetics panel of the BEAR I committee, Dr. Warren Weaver, chairman of that committee, stated on February 5, 1956 (see transcript of meeting), that "A little radiation, now and then, does not directly harm the person receiving it." Weaver then quoted from the next paragraph of the same report:

> From the above statements a very important conclusion results. Although there may be and probably is, a safe rate at which a person can receive radiation (say so much per week) if one is concerned only with direct damage to that person, the concept of safe rate of radiation simply does not make sense if one is concerned with genetic damage to future generations.

In the final report of the 1956 BEAR I committee, one can see a generally similar but slightly nuanced restatement of the earlier drafted statement as quoted by Chairman Weaver. The final report states:

> It has sometimes been thought that there may be a rate (say so much per week) at which a person can receive radiation with a reasonable safety as regards certain types of direct damage to his own person. But the concept of a safe rate of radiation simply does not make sense if one is concerned with genetic damage to future generations [141].

Both the near-final draft and final report statements are in agreement on the genetic effects to future generations. As for the somatic cell effects, the draft report is clear that a threshold effect is emphasized. The final report restates the somatic cell threshold interpretation but more subtly via a type of third-person comment rather than the more direct style of the draft report. Nonetheless, the NCRPM made the leap to linearity for somatic effects, not the genetics panel of the 1956 BEAR I NAS committee.

The generalizing of mutation linearity dose–response to somatic cells and its impact on cancer risk assessment was profoundly influenced by one of Muller's geneticist colleagues and future Nobel Prize winner, E.B. Lewis, and fueled by his widely acclaimed article in the journal *Science* [142] along with an accompanying and highly supportive editorial by the journal's editor in chief [143]. Muller likewise had obtained prepublication copy of the Lewis *Science* paper and distributed it to key groups such as the NAS BEAR genetics panel in December 1956, emphasizing the estimated cancer risk to the human population from radioactive fallout [82]. Thus, the first serious challenge to the threshold dose–response model was one of a developmental scientific perspective that was set within a highly charged international political context.

While the 1956 BEAR committee did not formally address the issue of somatic effects, the 1960 BEAR committee did. The conclusion of this committee on this issue is given in the following quote from its final report. The committee stated that it "does not consider it justifiable to predict human tumor incidence from small radiation doses based on extrapolation from the observed incidences following high dosage."

Furthermore, the 1960 BEAR committee acknowledged that the number of mutations in mouse sperm and oogonia was less when the x-rays were given at a lower dose rate than with an acute exposure in which the dose rate was greater but the total dose was identical under both approaches. This was a conclusion of the Caspari and Stern [144] study with *Drosophila* sperm that caused so much scientific confusion and subterfuge by Stern and Muller as they desperately tried to deflect the findings of the Caspari research [10,29,39,145]. Now some 12 years later, the findings were generalized to mice based on the work of William Russell at Oak Ridge. At his Nobel speech, Muller would argue that similar damage with the same cumulative dose, despite different dose rates, was de facto proof of the linearity model. Yet now, he would make no such statement in the context of the 1960 BEAR committee. Nor would the BEAR committee of 1960 and its genetic panel, still dominated by Muller, change their linearity recommendation for mutational effects in light of the challenging findings in mammalian cells to the linearity dose model.

These historical quotations are not simply the stuff of academic arguments but should be seen for how they came to impact major public policy in the dose–response and risk assessment domains [29]. The NCRPM gave the impression of deferring their position to that of the 1956 BEAR I committee; yet they developed a policy on somatic cells that was not formally addressed by this NAS/BEAR I committee in a direct policy sense but was actually in apparent conceptual disagreement with it. Furthermore, a position on somatic cell responses for cancer was explicitly addressed by the prestigious 1960 BEAR committee; it did not support the concept of extrapolating from high to low doses for cancer risk. This position was in direct conflict with the recommendations of the NCRPM. However, it was the 1956 report of the NAS BEAR I committee that received enormous publicity within the scientific community and the popular press, while the 1960 NAS BEAR committee's report was generally overlooked, lacking any notable impact. This point was explicitly addressed by a member of both committees, Dr. James Crow [146] and others (e.g., [147]), in a subsequent historical evaluation of the impact of such National Academy of Sciences efforts on radiation health policies in the United States.

While the 1956 NAS committee and other influential advisory committees were adopting the LNT perspective, an important independent action in the U.S. Congress was about to come to a similar conclusion, also with profound implications. That is, the Delaney *Amendment* to the Food Additives Amendment became law on April 26, 1958. It affirmed that no additive would be considered if it induced cancer in animal models or humans. The Delaney Clause was subsequently placed into the Color Additives Amendment of 1960 in response to the highly publicized cranberry crisis of 1959 [148].

The Delaney Amendment was premised on a belief in linearity at low dose for chemical carcinogens. Over time, the FDA would modify the Delaney Clause to address the issue of de minimus risks, that is, a risk that is both too low to be accurately measured and below practical concern. This modification enabled FDA to permit carcinogens to be added to food if the estimated risk had lower than a calculated risk (e.g., $\leq 10^6$/70 year lifetime) deemed to be de minimus, based on animal extrapolation and biostatistical modeling procedures. Despite their similarity in time, the development of the LNT cancer risk assessment model in the Delaney Clause developed independently of the NAS BEAR I-inspired LNT cancer risk assessment activities, although their implications converged [9].

The next serious challenge of the LNT model to the threshold dose–response model occurred two decades later in 1977 in the bursting forth of the environmental regulatory revolution in the United States when the NAS Safe Drinking Water Committee (SDWC) recommended that the EPA apply the guidance of the NAS BEIR II committee for ionizing radiation to the domain of chemical carcinogens [149]. From the late 1950s to the early 1970s, profound intellectual debate focused on the linearity versus threshold policy decision for ionizing radiation and cancer risk. As noted, the NCRPM first recommended the switch from threshold to linearity. In fact, the NCRPM orchestrated a very significant compromise between the opposing parties of this debate [8]. It got the linearity advocates to admit that the data supporting linearity at low dose were not convincing. Likewise, the same argument was used against the threshold perspective. As a result of this intellectual impasse/conundrum, the committee concluded that

> it was not possible to establish the exact character of the dose curve. Lacking sufficient unequivocal information, the committee believed it would be desirable to take a conservative position and to assume a non-threshold linearity dose response relationship [82].

While this position seemed to represent a compromise, it represented a major victory for the concept of the *precautionary principle*. This was similar to the type of compromise that was brokered in the 1940s over the issue of tolerable dose versus permissible dose. However, in that earlier case, the outcome was far different. The concept of permissible dose was accepted as scientifically more plausible, but the threshold concept would govern the regulation. Some 25 years later, the concept of linearity had lost scientific support, yet its underlying philosophical perspective (i.e., precautionary principle) was adopted by the NCRPM. Other leading advisory groups would also struggle with this problem with the NAS BEAR committee of 1960 rejecting linearity on scientific grounds, whereas the U.S. FRC (1960) accepted the linearity concept based on the precautionary principle. This type of back-and-forth struggle would continue between different committees and their varying compositions over the next decade. However, the precautionary perspective reemerged with the next NAS committee (now called BEIR I/II, 1972) [149], a perspective that would strongly affect the actions of the 1977 NAS SDWC [150]. This committee would also rely heavily upon a series of papers they claimed that supported the scientific foundations of low-dose linearity [151–154].

The EPA accepted this 1977 recommendation of the SDWC and, within 2 years, applied the LNT model to the risk assessment of trihalomethanes (THMs) and then soon to dozens of other chemical carcinogens up to the present. The theoretical foundation of this challenge to the threshold dose–response model for chemical carcinogens was built upon eight guiding principles as laid out in the NAS book *Drinking Water and Health* [150]. The eight principles are as follows:

1. Only one or two changes in a cell could transform it, and this could lead to cancer.
2. Human population heterogeneity was a factor, and some people may be at greater risk. Such heterogeneity leads to the conclusion that there was no population-based threshold.
3. A transformed cell will be irreversibly propagated.
4. If the mechanism involved mutation, there would be no threshold; in fact, if there were no information on mechanism and cancer occurred, mutation should be assumed.
5. It is necessary to assume that a single molecule or a few molecules can cause a mutation. Therefore, linearity at low dose can be assumed.
6. There is also the assumption that the response would be directly additive to background, if acting via the same mechanism. This would also support the linearity conclusion.
7. Available mutagenicity data with radiation indicated that it was linear at relatively *low* doses.
8. Since chemical carcinogens act like ionizing radiation, low-dose linearity should also be assumed to be the case for such chemicals.

It is also evident that the committee did not choose to document possible alternative opposing arguments to linearity at low dose or weaknesses in the so-called eight principles. Furthermore, it would have been of considerable value if the committee had ranked the principles in terms of the degree of scientific confidence that each had individually and its relativity to each other. The committee should have distinguished those principles that were guided by scientific data as compared to those more influenced by a protectionist philosophy rather than science. Furthermore, these key references used by the 1977 SDWC provided data and/or conclusions that directly contradicted the previous principles #1, #3, #4, and #5. For example, Nordling [154], Muller [152], and Iversen and Arley [153] argued that multiple successive mutations in the same cell (i.e., up to seven mutations) would be needed to cause human cancer, thereby challenging key principles of the 1977 committee that relied upon these papers.

While each of the eight guiding principles was important in order to provide the underlying support for the EPA to adopt low-dose linearity for carcinogen risk assessment, the intervening four decades have not provided confirmatory support for any of these *principles*. Rather, the findings that have emerged during this time period have, in fact, revealed that those views are now generally either scientifically untenable or practically impossible to study at a sufficient level of detection. For example, that a single alternation in DNA would be sufficient to lead to cancer or that the process of carcinogenesis, once initiated, is irreversible has been repeatedly demonstrated as not occurring [111]. Driver et al. [155] demonstrated a strong linear dose–response relationship from DMN-induced adducts and foci production in the kidney of male F344 rats. However, the linear dose–response pattern disappears displaying clear evidence of a threshold dose–response for this genotoxic carcinogen (Figure 3.21). Such a dose–time response strategy supported the concept of cancer being a multistage process and with repair-related processes occurring at lower doses, resulting in a regression or suppression of the carcinogenic process.

The idea that chemically induced cancer occurs via the same mechanism as similar as background cancer in the population has not been established. The level of mechanistic detail and biological complexity today reveals how superficial and implausible the *principle* was. In fact, in the domain of x-ray-induced germ cell mutation in fruit flies, it was shown that low dose of x-rays induced a DNA repair process that repairs background lesions while having no effect on those mutations induced by the x-rays [156]. The additivity to background concept would fail in this case as well. Thus, the concept of *reasonable* guiding principles became confused to mean something akin to scientific certainty. The confusion was not only passed on the general public but has affected the development of regulatory agencies' risk assessment actions for chemicals and ionizing radiation with widespread societal implications.

Challenge #2: Hormesis

Historical Foundations

The challenge to the threshold and LNT dose–response models from the hormesis dose–response is relatively recent, being little more than a quarter century old. While the concept of hormesis is about 130 years old, starting with the German physician and pharmacology/toxicology professor Hugo Schulz in the 1880s, that it might challenge current models or might inform the risk assessment process was probably first suggested by Donald Luckey et al. in 1975 [157] in the introduction of the book *Heavy Metal Toxicity, Safety and Hormology*:

> Agents which are found to cause stimulation when given in small quantities are colled [called] *hormetics* and the action is *hormesis*, taken from Southam et. al. [and Ehrlich] (1943). Unterstanding [Understanding] the extent of this phenomenon is essential before world wide committees and legislative bodies make recommendations which consider only toxic actions…

This publication was followed 5 years later by a detailed book by Luckey [53] on ionizing radiation and hormesis that raised the visibility of this concept within a new scientific regulatory context. While the issue of hormesis or biphasic

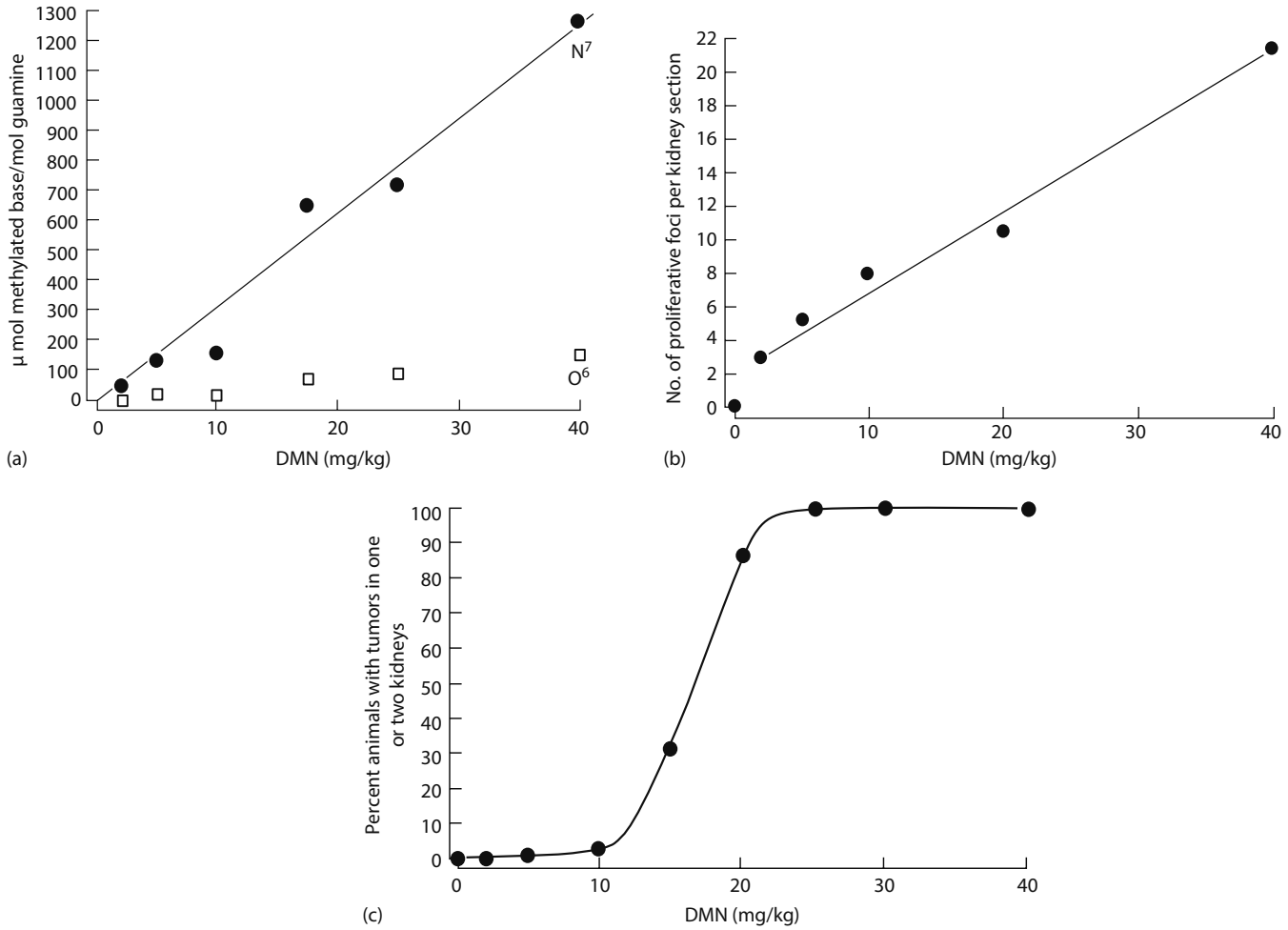

FIGURE 3.21 Dose–response for DMN: (a) renal adducts, (b) renal foci, (c) renal tumors. (From Driver, H.E. et al., *Br. J. Exp. Pathol.*, 68(2), 133, 1987.)

dose/responses seen in an earlier era in the confrontation between homeopathy and traditional medicine was in the attempted commercialization of treatments to enhance agricultural production [4], the goals of Luckey were different. He was far more academically based, with the ostensible goal to educate the regulatory community to the scientific foundations of the hormesis concept and its regulatory implications for setting exposure standards in all types of settings, be they occupational, environmental, food, consumer products, or within the home for concerns such as with radon gas. While these initial activities of Luckey created interest in the hormesis dose–response model, they were not persuasive to the regulatory communities and lead to frustration in dealing with agencies such as EPA in the regulation of carcinogens—especially for ionizing radiation as reflected in a number of his subsequent publications.

Despite the 1980 book by Luckey, the dose–response debate during the early 1980s was not focused upon hormesis versus LNT per se but threshold versus LNT. In fact, this still is the case, even today, all for practical reasons. The regulated industry in both the chemical and radiation areas supported the less conservative threshold dose–response model, whereas the EPA had established the LNT model for their estimates of risks from carcinogens. Using the LNT model in the risk assessment process and in guiding risk management decisions was proving to be very costly to the industry. A key goal of the regulated industry was to find a means to convince the EPA to switch back to the threshold dose–response model for risk assessment purposes. A practical problem for the industry was that the agency assessed each chemical in the chronic bioassay (i.e., the 2-year rodent bioassay) on its own merits, evaluating whether the data generated in the bioassay best fit a linear or threshold model. While this might seem to be an objective approach, there were too few doses to objectively differentiate which model best fit the data. The very few doses were also delivered at very high dose rates, that is, at the maximum tolerated dose (i.e., the highest dosage that would not cause frank toxicity and not reduce body weight by more than 10%) and one-half of that value. With so few doses and those that were used being delivered at such a high dose rate, there were high-dose toxicological concerns and biostatistical limitations that could not be overcome. For all the attempts to rationalize the adequacy of those standard types of bioassay studies, they were far from perfect and not adequate to provide the type of data needed for risk assessment purposes for low-level exposures.

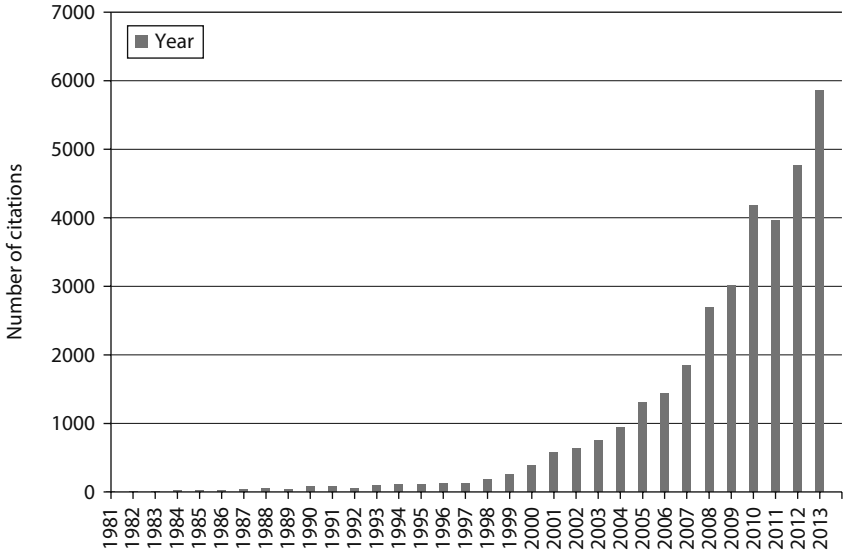

FIGURE 3.22 Citations of hormesis/hormetic in Web of Science database.

Typically both a linear model and a threshold model could similarly account for the observations that are produced in the chronic rodent bioassay. Since one biostatistical model is not particularly superior to the other for data generated in their experiments, the EPA, in such cases, invariably defaults to the most conservative or protective dose–response model. Thus, the linear model became the model selected, by default. There was no practical way around this risk assessment dilemma. The rules of the risk assessment process were very strongly biased toward using the LNT model. Thus, many in industry felt that the government's guidance rules were not scientific and were designed to always yield the same conclusion…. Thus, the *scientific* deck seemed to be stacked to yield a predetermined result.

Since the threshold model could not practically account for dose–response data better than the LNT model for the standard chronic bioassay data, some groups in the industrial sector became interested in the hormetic dose–response (HDR) as it might be easier to distinguish it (i.e., the HDR model) from the LNT than the threshold dose–response model. This might provide a better opportunity to avoid risk assessment estimates based on linearity. Secondly, the hormesis model had a threshold component and it was the establishment of a threshold that was the real goal. The fact that the hormesis model had the potential to estimate a possible public health benefit was politically problematic for industry since it would not be a wise strategy to claim that pollutants could induce beneficial effects at low doses, even if true. This can be readily seen in the range of policy concerns directed at the hormesis concept [158–161]. As stated previously, the goal of the industrial supporters of hormesis is for the threshold concept to gain regulatory traction, not for the concept of health benefit to be the focus.

The electrical power industry from the United States and Japan explored the area of hormesis by conducting a conference in August 1985 with the peer-reviewed proceedings published in the journal *Health Physics* in 1987. The proceedings were followed 2 years later by a debate on the issue of radiation hormesis in the journal *Science* by leaders of that initial conference [162,163]. While the issue of hormesis had resurfaced during the 1980s, it was a modest initial revival as reflected by citations in the Web of Science, which averaged only about 10–15/year (Figure 3.22).

Biphasic Dose–Responses: Multidisciplinary

Despite the rather slow unfolding of the scientific rediscovery of the hormesis concept during the 1980s, there were other developments that suggested an intellectual convergence on the topic of biphasic dose–responses. None of these newer developments were directly related to policies or specific actions of regulatory agencies. Reports from multiple scientific fields began to independently appear on the occurrence of hormetic-like biphasic dose–response relationships. For example, a significant development in the field of pharmacology occurred with the highly influential papers by Szabadi [164,165], which summarized the occurrence of biphasic dose–responses as far back as 1906, starting with research of the 1936 Noble Prize winner Henry Dale (1875–1968) [166] (see "Challenge #3: Pharmacology and the Biphasic Dose–Response" section). Of particular importance was that Szabadi [165] proposed a receptor-based mechanistic model by which such biphasic dose–responses could occur. The Szabadi [165] paper generated a spate of supportive commentaries and related papers [167–171], providing a strong foundation for further developments in this area. Likewise, the field of epidemiology published a number of studies showing U-shaped dose–responses for various types of medical and public health outcomes associated with environmental and occupational exposures [172]. A series of papers were published by Stebbing [173–175], providing a strong complement to the writings of Luckey concerning environmental toxicants and HDRs. During this period, researchers

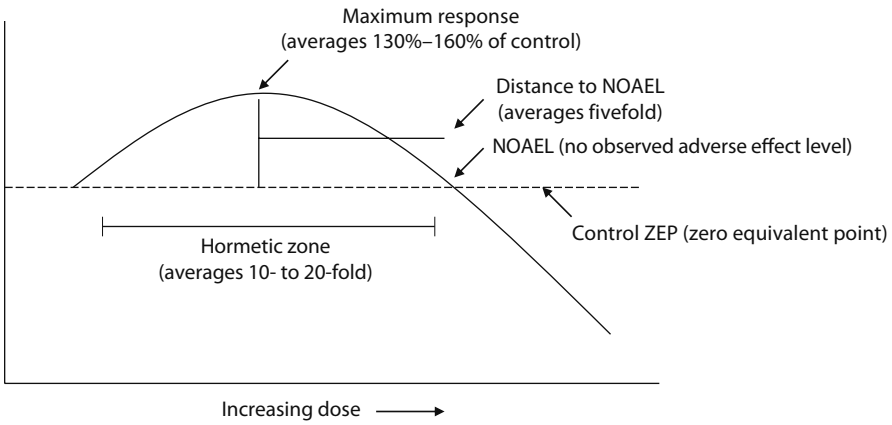

FIGURE 3.23 Dose–response curve depicting the quantitative feature of hormesis. (Based on Calabrese, E.J., *Am. J. Pharmacol. Toxicol*., 3(1), 56, 2008.)

in the area of genetic toxicology reported that low doses of chemical mutagens could induce DNA repair processes that would provide protection against subsequently more massive exposures to the same or different mutagenic agents [176]. By 1984, the concept of adaptive response to ionizing radiation had been reported by Shelly Wolff at the University of California at San Francisco [177]. In 1986, the concept of preconditioning in the biomedical sciences was published, which demonstrated that a short-term/low-dose prior hypoxic exposure would protect against a massive myocardial infarction-induced cardiac damage by about 70%–80% [178]. The findings from each of the previous cited areas of adaptive responses were soon replicated in extensive studies and then generalized to other cell types, organs, and biological models. The shape of the dose–response in each of these general areas was consistent with that of the HDR. In parallel with these emerging findings was the rapid development of in vitro methods and extensive economic and political pressure to employ fewer whole animals in biomedical and toxicological research. The switch to in vitro methods created the opportunity to assess more doses/concentrations per experiment. These changes have profoundly affected the conduct of toxicology, creating the opportunity to routinely evaluate from 8 to 10 doses per chemical screening. In fact, it was as a result of the newly acquired change to in vitro testing and its high-throughput dimension that profoundly expanded the number of reported cases of HDRs in the biomedical literature. The reliance upon test results with doses at the MTD and fractions of the MTD would not have the potential to explore the biological effects of low doses, including the hormesis hypothesis.

Hormesis Database

The 1980s therefore set the toxicological and biomedical stages for the evaluation of the HDR. The 1990s and the first decade of the new millennium witnessed a large increase in the reporting of HDRs in the toxicological and biomedical literature. This increase is especially evident in the second half of the 1990s. The hormetic response was found to be quite common, as well as very general, being independent of biological model, endpoint, and inducing agent. The HDR was also shown to display specific quantitative features, that is, the low-dose stimulatory response was generally quite modest with the maximum response being typically only about 30%–60% greater than a concurrent control group (Figure 3.23) [179–181]. This modest response would present challenges for the observation of hormetic responses, in attempts to distinguish it from normal background variation. Proving the occurrence of an HDR requires greater sample size/statistical power in the low-dose zone along with careful consideration of dose selection with more doses below the threshold along with a heightened need to replicate findings. Given the modest nature of the low-dose stimulation, it is essential that more detailed understanding of the historical variation in the control is known. Thus, false-positive low-dose stimulations could occur as a result of low-control group readings due to normal variation. This has also led to the need to conduct statistical simulations in order to estimate the frequency of false-positive responses. As a result of the recognition of such methodological challenges that are inherent in the assessment of HDRs, it has resulted in adding more dose–response insights, affecting the quality control, experimental design, and response criteria, thereby making conclusions concerning the likely presence or absence of HDRs more reliable. This is especially seen in the prospective testing of specific HDR hypotheses that are becoming more common in the biomedical and toxicological literature.

Defining Hormesis

As a result of the renewal of interest in the hormesis concept in the early 1980s, at least in part, via an industry initiative to avoid an LNT-driven regulatory-based cancer risk assessment, the low-dose stimulatory part of the HDR became viewed by some as beneficial. Without adequate evaluation, some in the environmental regulatory community became either suspicious or opposed to the HDR for philosophical/ideological reasons rather than scientific. However, the

low-dose stimulatory aspect of the HDR is neither inherently beneficial nor harmful [182–185]. One needs to assess not only the data from the experiment but also the biological context of the findings in order to make an informed judgment concerning whether the low-dose stimulation is potentially beneficial, harmful, or neither. When the low-dose stimulating results in enhancing longevity, greater disease resistance, improved memory, greater bone strength, and other comparable apparently desirable responses, it has generally been viewed as *beneficial*. When the responses reflect the enlargement of the prostate gland, the enhanced proliferation of tumor cells, or other undesirable responses, it has been viewed as potentially harmful [182]. In many cases, the clinical or public health implications of a low-dose stimulation may not be large enough to be practically significant. In such a case, the clinical or societal implications may be/are uncertain. Thus, the HDR is a scientific concept—not an ideological element.

Many in the endocrine disruption area describe such biphasic dose–responses as U-shaped nonmonotonic dose–response—not an HDR [184]. Yet, the quantitative features of the biphasic endocrine disruption dose–response follow similar quantitative features as that for the HDR. The underlying molecular mechanisms also follow similar receptor-based and signaling pathway-based processes. It is the opinion expressed here that the inverted U-shaped dose–responses reported within the context of endocrine disruptive agents are generally manifestations of the HDR model of hormetic response–endocrine disruption. The issue over terminology and language is important, especially in order to assist researchers, governmental officials, and others to better understand and apply the dose–response concept.

Hormesis Frequency

While the 1990s and early 2000s witnessed the publication of a large number of HDRs being reported in the biomedical and toxicological literature, there emerged the question of what was the frequency of hormesis in toxicology and pharmacology. Using rigorous entry and evaluative criteria, this question was assessed based on a review of approximately 21,000 papers from the toxicological, biomedical, and life sciences areas. It yielded a value approaching 40% for the frequency estimate of hormetic responses that satisfy rigorous a priori entry and evaluative criteria [186–190]. This observation was supported in follow-up large-scale evaluations [191–193].

Such results were striking since they represented a significant conceptual transition in the toxicological literature. That is, when the hormesis concept was beginning to be discussed in the 1980s, it was uncertain whether it was a real and reproducible phenomenon. If it was, then it was thought that it may be uncommon, rather infrequent, a type of paradoxical phenomenon. Yet, the deeper the investigations probed, the more general and frequent this dose–response was found to be.

Validation of Dose–Response Models

In the course of the previous evaluations of the HDR, the question was raised as to how the toxicology and pharmacology communities had validated the threshold dose–response model during the twentieth century. Since so many of environmental and occupational health regulations and FDA exposure standards were based on the use of the threshold dose–response model, it seemed logical to assume that these agencies as well as the scientific community must have validated the capacity of the threshold dose–response model to make accurate predications in the below-threshold zone. However, follow-up detailed searches of the literature did not yield any published attempts to test the validity of the threshold dose–response model predictions in the below-threshold zone. Likewise, interviews/discussions with senior toxicologists, both within and outside of government, also did not yield any published attempts that validated the low-dose prediction of the threshold dose–response. The below-threshold zone is critical since this is the area of the dose–response where humans principally live. While it is not possible to prove a negative, the results of multiple detailed searches of the literature to obtain articles that validated (or attempted to validate) the threshold dose–response model have been without success. This search failure has led to a tentative but confident conclusion that the toxicology, pharmacology, and regulatory communities failed to validate (or attempt to validate) the threshold dose–response model for accurate predictions in the below-threshold zone during the entire twentieth century.

When the concept of validation was then applied to the three most dominant dose–response models (i.e., threshold, LNT, and hormetic), using multiple, independent large data sets, the only one that was effective in accurately predicting responses in the low-dose zone was the HDR. The threshold model revealed that this model consistently failed to accurately predict responses across the entire spectrum of agents tested [193]. Inaccurate predictions were even more common for the LNT model. These observations were as challenging as they were surprising. These tests to validate the dose–response models upon which regulatory programs have been based were performed only long after these models had been adopted and applied by regulatory agencies in many countries. Thus, multiple decades after their establishment and acceptance, critical inadequacies were uncovered for key risk assessment dose–response models used for most risk-based regulations. Yet, enormous regulatory, administrative, and legal decisions have been made based on these dose–response models that were assumed by the public, elected officials, and probably the scientific community to have been vetted and validated by the scientific and regulatory communities.

The need to validate the capacity of dose–response models to make accurate response predictions, especially in the low-dose zone, is a surprisingly new development to the fields of toxicology, pharmacology, and risk assessment. It is a highly significant practical problem at present in these respective fields because the threshold dose–response model

was adopted nearly a century ago presumably on the assumption that it could make accurate estimates of responses below the threshold independent of biological model, endpoint, and chemical agent. The model estimates are supposed to be reliable and to provide confidence to the public that exposure standards are based on accurate dose–response estimates. The question must therefore be raised as to why the threshold dose–response model was accepted for regulatory use without having been evaluated. In fact, the model was accepted even in the absence of any documentation of what would be an acceptable model and how that decision would be made.

Beyond questions concerning what is an acceptable dose–response model for risk assessment purposes, the acceptance of a model has other important implications that may be overlooked. From a historical perspective, once the threshold dose–response model was adopted and integrated within governmental regulatory frameworks, a hazard assessment process was created based on the assumption that the threshold dose–response model provided accurate estimates of responses below the threshold. This hazard assessment testing protocol determined how chemicals would be tested and evaluated, including the animal models selected, the number and the spacing of doses, and the dose selection strategy. In fact, as a result of accepting the threshold dose–response as the model to be used in the estimation of responses in the low-dose zone, toxicology would become a discipline that would be characterized as using only a few very high doses in the chronic bioassay.

The hazard assessment concept was built upon several data requirements and goals: first, demonstrate toxicity at the highest dose while not exceeding the maximal tolerable dose; second, it is important that no statistically significant increase in toxicity occurs at the lowest dose so that a threshold dose might be more readily demonstrated. There was no interest in obtaining information on the entire dose–response continuum as it was assumed that only random bounce or noise would be observed for responses to doses below an estimated threshold. This type of hazard assessment methodology reinforced the assumption that the HDR did not exist. It was on this basis that past toxicological assessments were based.

There are multiple flaws in this hazard assessment process. If the high dose was too high, then the study would be dependent upon only a single dose. Since the second dose was still 50% of the high dose, this was also at risk for exceeding the MTD. Regardless of whether both doses provided valid data concern would still remain over what was the response at lower doses and whether the threshold was reasonably well characterized for the most sensitive endpoint. All the assumptions that were built into the bioassay and how the data that it produced would be interpreted were derived from assumptions about the threshold dose–response model. Nonetheless, the acceptance of the threshold dose–response model does not excuse the several decades of using only two doses before switching over to three. Even in the case of three doses, each separated by a factor of two is still far from satisfactory for estimating responses in the zone approaching a possible threshold as well as below the threshold.

The background tumor/disease incidence could affect the selection of the animal model. In the case of the HDR model, it would be necessary to test the predictive capacity of the model by ensuring that the control endpoint incidence for tumors and other disease endpoints would be such that one could detect a decrease in the incidence if hormesis were present. If the background incidence is negligible, then it would be impossible to detect the presence or absence of the HDR. The background disease incidence was not of theoretical concern in the selection of the threshold model.

The selection of the threshold model by the regulatory community therefore was never validated throughout the twentieth century in the United States and internationally as well. It was adopted for use by regulatory agencies such as EPA, OSHA, FDA, and all state regulatory agencies. It guided the testing of all chemicals and drugs for safety. The hazard assessment process also assures the testing of agents only at very high doses and with animal models that were given no consideration whether an alternative dose–response model might exist, such as the HDR model. In fact, it would have been a useful factor for this type of interpretational flexibility to have existed.

Attempt to Validate the LNT Model: The Megamouse Study

In contrast to the failure of governments to attempt to validate the threshold dose–response model, the U.S. FDA made an effort to validate the LNT dose–response model for chemical carcinogens. The strategy involved the selection of a well-studied chemical carcinogen (i.e., 2-acetylaminofluorene [2-AAF]) and evaluation of it in a massive experiment that used about 24,000 mice. The results of this massive study were extensively reported [194–196] and assessed in detail by a special expert panel of the U.S. Society of Toxicology (SOT) [197], highlighting the importance of the study. While the findings generated much discussion and debate, a major point of agreement was that the study was only able to assess tumor incidence to the level of 0.01. The report was therefore called the ED01 study. This was the most striking finding since it required about 24,000 mice and all that could be judged were risks to 10^{-2} when regulatory agencies need estimates to the 10^{-5} or 10^{-6} for de minimus risk estimates. It was not possible, therefore, to practically validate the LNT model for cancer risk assessment at the levels of risk that regulatory agencies need to estimate. As a result, no other rodent-based megamouse study estimate has been subsequently undertaken to validate risk in the low-dose range.

Despite these concerns and limitations, the SOT committee developed a dose-related time to tumor model evaluation. They found that the 2-AAF induced a J-shaped dose–response for bladder cancer, a finding that was consistent in all six rooms in which the large numbers of mice were maintained (Figure 3.24). The SOT expert committee unequivocally stated that there was both a threshold and a significant reduction in risks at doses below the threshold. Without using the term, the SOT panel concluded that the experimental findings demonstrated an HDR. This striking conclusion of

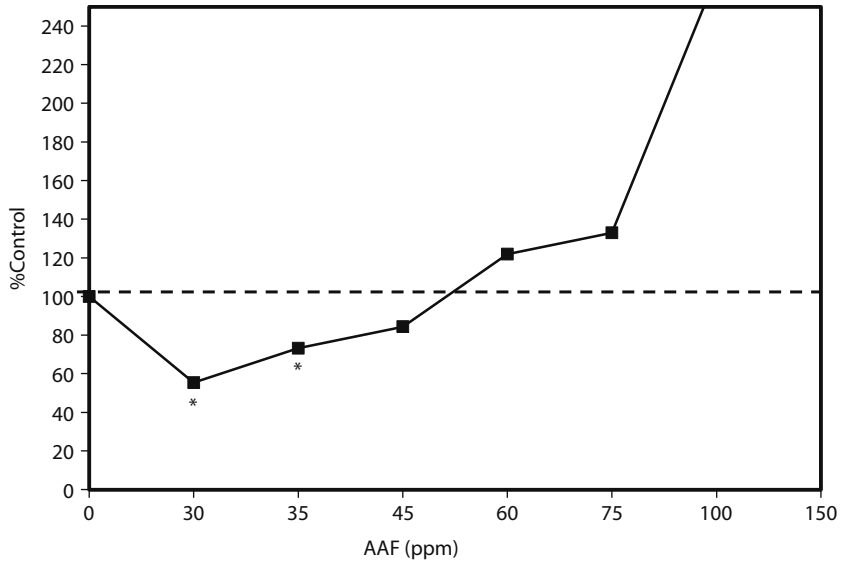

FIGURE 3.24 Bladder tumor incidence adjusted for time in ED01 megamouse study: HDR relationship. *Statistical significance. (Based on Bruce, R.D. et al., *Fundam. Appl. Toxicol*., 1, 67, 1981.)

the SOT panel was buried in the details of an analysis and was never widely cited. Yet, this is a principal finding of the largest rodent experiment, with a strong prospective study design to determine the nature of the dose–response in the low-dose zone. However, these results did not fit the preestablished regulatory agency LNT paradigm and were ignored by such agencies and the field of toxicology.

The validation of the LNT could have been undertaken by a far less expensive strategy and with the testing of more agents so that the issue of generalizability could be considered. For example, Japanese researchers assessed the dose–response for the liver carcinogen DDT using liver foci as the endpoint (Figure 3.25). The study was far less expensive and provided data using considerably less time; once again, the LNT model was not as supported as the hormetic model was.

Hormesis: Dose–Time Response (Overcompensation) Studies

Biphasic dose–responses are typically graphed as either an inverted U-shaped dose–response or J-shaped. The graphing of the inverted U- or the J-shaped seems to be a discipline-specific feature with those reporting disease incidence using J-shaped graphing and those disciplines reporting on growth or changes in longevity using the inverted U-shaped dose–response.

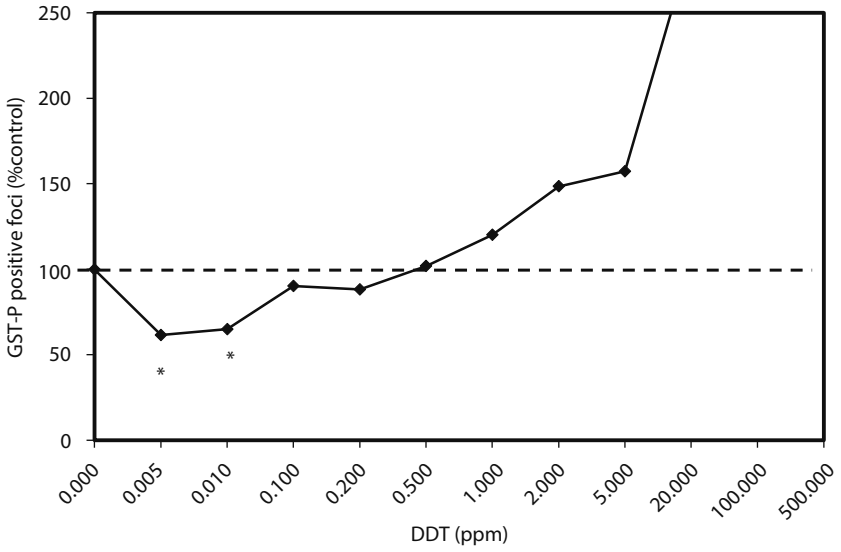

FIGURE 3.25 Effect of DDT on liver foci formation in male F344 rats: HDR relationship. *Statistical significance. (Based on Sukata, T. et al., *Int. J. Cancer*, 99, 112, 2002.)

The biphasic dose–response can also be not only dose-dependent but time-dependent as well. The biphasic dose–response can occur as a result of a direct stimulatory response at low dose followed by a high-dose inhibitory response (Figure 3.23). In contrast, the biphasic dose–response may also occur as a result of an initial dose-dependent decrease in response followed by an overcompensation response that over time yields the biphasic dose–response relationship [198,199]. At the lower doses, the compensatory response results in the response modestly exceeding that of the control group. However, at the higher doses, the compensatory response tends to fall short of a return to the control value. The resulting dose–response appears biphasic. Of particular interest is that the quantitative features for those dose–responses that are directly stimulatory and those that are compensatory are similar quantitatively, in fact, being distinguishable.

The compensatory stimulatory response has been widely observed in the pharmacological domain, with this type of response becoming very frequently cited since the early 1990s. The term *rebound effect* is widely used by researchers whose study designs typically include a dose and a time component. Dose–responses with the so-called rebound effect typically exhibit the same quantitative features of the HDR. In fact, in most cases, those dose–responses are examples of hormesis. For example, in the field of herbivory [200], it is commonly observed that following a low to modest removal of leaves, there is the occurrence of rebound effect in which there is an overcompensation of leaf growth. When excessive removal of leaves occurs, then the compensatory response is usually not adequate to return to control values. The quantitative features of the dose–response are strikingly similar to the hormetic–biphasic dose–response found in the toxicological literature. The term rebound effect is also commonly used in a wide range of biomedical disciplines as well. The use of multiple terms for similar phenomenon can make it difficult to gain a sense of the depth and breadth to which the HDR concept may have been studied.

Hormesis: Plasticity

Why would very large numbers of dose–responses using different biological models with different endpoints and inducing agents with or without a time component show biphasic doses with similar quantitative features, that is, similar magnitude and width of response? Such a highly diverse group of dose–response parameters showing similar quantitative dose–response patterns is not consistent with a random process. Given its general nature, it may have been highly conserved during the evolutionary process of natural selection. The consistency of this response across the plant and animal kingdoms is striking and indicative of an inherent adaptation consistent with providing an estimate of biological plasticity across biological systems, independent of endpoint and inducing agent and differential levels of biological organization.

The integration of the concept of biological plasticity into the interpretation of the dose–response is a novel one [201]. Plasticity has often been considered within several descriptive contexts. The adaptability of the brain with respect to repair following injury has been described as a manifestation of biological plasticity [202]. In a comparable manner, the variation in plant structure and form along a long and progressively steep mountain side would be another example of biological plasticity [203]. In the case of the plant example, plants experience numerous changes in the physical environment along the progressive mountain slopes. There would be changes in carbon dioxide and oxygen concentrations, temperature, soil types, access to nutrients, soil microbes, and many others. These changes along the slopes of the mountain affect the induction of the altered plant phenotypes displaying a range of plasticity changes within such a setting.

The examples of the plant alterations up a mountain side are changes that reflect the impact of numerous biological adaptations in response to alterations in environmental parameters (e.g., oxygen/CO_2), some of which conform to a dose–response gradient. While the changes in plant phenotype reflect biological plasticity, this example is one of the complexities with multiple variables. However, the field of toxicology can explore similar types of gradient changes with alterations in chemical or radiation dosage while keeping all other variables constant. In this manner, the change in treatment will affect the formation of new phenotypes that reflect biological plasticity [201]. The low-dose stimulation seen in the HDR is a manifestation of biological plasticity, a change in biological phenotype. It reveals how much gain or capacity there is for change. The HDR reveals that biological plasticity in response to a vast array of endogenous agents as well as exogenous chemicals is consistently very modest, being in the percentage range, that is, at maximum only about 30%–60% greater than background or control exposures (Figure 3.23). This observed biological plasticity capacity represents how the field of toxicology can interpret this concept within a dose–response context.

The limits of plasticity provide a biologically based resource management system. Large numbers of biological endpoints display HDRs. All have plasticity constrains in the low-dose region. Such responses are operational as part of normal living activities and under stressful conditions. In order to maintain stability in the management of limited biological resources, one strategy would be to regulate the allocation of such resources and to manage how they are handled. The low-dose stimulatory response displays how the *system* controls resources at multiple levels of biological organization and it does so by the use of the hormetic stimulation. The significance of this type of resource management strategy is seen across all types of life, from microorganisms to humans, representing a biological adaptation that is highly conserved. Thus, hormetic stimulation within the context of the dose–response plays a fundamental role in evolutionary biology. The HDR has the capacity to affect how evolutionary biologists consider dose–response processes.

The low-dose stimulation measures biological performance within the bounds of an overarching system of biological plasticity. The stimulation reflects an enhancement of integrated response endpoints, such as cell proliferation, wound healing, or memory. In each case of such integrated

endpoints, there is a limitation for its enhancement that is determined by the constraints of plasticity that is described by the quantitative features of the HDR.

At the time that the HDR was beginning its resurgence, another term was emerging in pharmacology called the *ceiling effect*. This term has generally been used to signify *the quantitative maxima* of a low-dose stimulatory response. The ceiling effect in pharmacology has been therefore used as another manifestation of the concept of biological performance within the framework of an HDR.

In 1907, Alexis Carrel stated that he hoped to find agents that would accelerate wound and other types of healing by 10–50-fold [204]. He hoped that broken bones could be healed in a few days rather than months. Yet, after more than a century, most reported attempts to accelerate healing show enhancements that are in the hormetic stimulatory range [205]. There is little doubt that rapid bone fracture healing (and other types of wound healing) would have important survival advantages. Thus, the degree of enhancement of repair is limited by the constraints of plasticity having a response *ceiling* in the percentage rather than fold zone, that is, being a manifestation of hormesis.

The concept of plasticity is also applied to biological regulation within the context of dose–response as these affect essentially all physiological systems and their control functions. Such low-dose regulatory-based responses represent a measure of biological performance, and such performance-related endpoints are in striking contrast to responses typically measured at the upper end of the dose–response spectrum.

The dose–response should not be interpreted as a simple continuum of agent-induced responses that increase or decrease in a monotonic manner. Substantial findings reveal a markedly different general dose–response pattern. That is, the effects that occur above the toxicological and pharmacological thresholds are often fundamentally different than those that occur below the threshold. The responses at high doses in the field of toxicology are typically, but not always, related to the induction of cellular and tissue damage, that is, true toxic responses. However, below the threshold, the response is not only adaptive but the means by which enhancements of responses occur and controlled and how these upregulated responses improve the chances of prolonged survival.

The dose–response therefore within a hormetic context is one of the low- and high-dose effects with very different biological response meanings. At the high end of exposure, one often encounters system toxicity; the high-end exposure can also reflect a system regulatory function in which the response may be slowed down via a receptor desensitization phenomenon or signaling pathway slow down, inactivation, or other alternative mechanisms. For example, low doses of anxiolytic drugs decrease anxiety in rodent models, while at higher doses, there is often an increase in anxiety. In rodent models, which prefer dark areas, low doses of anxiolytic drugs enhance the time spent in both open and lighted areas. This has been interpreted as a sign that the rodent overcomes some of its inherent anxiety of lighted zones. This suggests that there is an increase in traits such as curiosity and exploration. However, at higher doses, these behavioral patterns are reversed. From an evolutionary perspective, it would be an advantage to have the capacity to be curious and exploratory as well as cautious depending on the situation. Thus, the biphasic dose–response can be seen as displaying behavior patterns that are based in systems biology and of a regulatory nature.

Summary of Hormesis Challenges to the Threshold (and Linear) Dose–Responses

The HDR should assume a central role in the field of toxicology. Table 3.3 lists hormetic principles, while Table 3.4 summarizes scientific developments concerning HDRs. The amount of data indicating this biphasic dose–response is extensive and reproducible. There is also a substantial amount of mechanism understanding of HDRs that are based on receptor-mediated processes linked to signaling pathways. A key feature of the HDR model is that it is highly generalizable, being independent of biological model, endpoint, and inducing agents. The HDR model has illustrated biological and toxicological complexities that were either ignored or underappreciated for decades by the toxicological and pharmacological communities. For example, the low-dose

TABLE 3.3
Hormetic Principles

Low/modest stress induces prosurvival responses.
The quantitative features of the HDR are similar across species and individuals and independent of differential susceptibility and agent potency.
The magnitude of the stimulatory response is constrained by and defines the plasticity of the biological system.
Hormetic responses occur at multiple levels of biological organization, the cellular, organ, individual, and population.
Downstream processes integrate responses from multiple independent stressor agents/excitatory stimuli to yield an integrated dose–response (i.e., molecular vector) reflecting the HDR.
Hormetic responses reflect both a general response to environmentally induced stress/damage and some elements of chemical structure specificity for endpoint induction.

Source: Calabrese, E.J., *Am. J. Pharmacol. Toxicol.*, 3(1), 56, 2008.

TABLE 3.4
Major HDR Observations

Most commonly observed dose–response relationship.
Distinctive quantitative features, making it a unique biphasic dose–response relationship.
Most unique feature is the modest magnitude of the stimulatory response, usually less than twice control values.
The low-dose stimulation can occur via a direct stimulation or via an overcompensation to a disruption of homeostasis.
HDRs may be seen as an adaptive response that ensures tissue repair in an efficient manner and protects against damage from subsequent and more massive exposures.
HDRs are highly generalizable, being independent of biological model, endpoint measured, and chemical class.
Numerous specific mechanisms have been reported to account for HDRs.

Source: Calabrese, E.J., *Am. J. Pharmacol. Toxicol*., 3(1), 56, 2008.

TABLE 3.5
Implication of Hormesis for Toxicology/Risk Assessment and Clinical Practices/ Pharmaceutical Companies

Toxicology/Risk Assessment

Changes strategy for hazard assessment, altering animal model and endpoint selection, study design including number of doses, and dose range and number of subjects per dose
Alters biostatistical modeling to predict estimates of response below control background disease incidence
Differentiates dose optima (i.e., benefits) for normal and high-risk segments of the population
Creates evaluative framework to assess benefits or harm below traditional toxicological threshold
Creates new framework for quantitatively altering the magnitude of uncertainty factors in the risk assessment process

Clinical Practices/Pharmaceutical Companies

Drug performance expectation will be constrained by the quantitative features of the HDR.
Drugs that are designed to act at high doses may have hormetic effects at low doses with possible undesirable effects (e.g., tumor cell proliferation).
Modification of biological set points will be constrained by the quantitative features of the HDR.
Clinical trials need to recognize interindividual variation in the HDR.
Clinical trials need to be designed to take into account the quantitative features of the HDR.

Source: Calabrese, E.J., *Am. J. Pharmacol. Toxicol*., 3(1), 56, 2008.

stimulatory effect can be a beneficial or harmful effect. The effects may also be time-dependent. These factors indicate that the hazard assessment as used by regulatory agencies would have to be modified in order to take into account of the possibility of HDRs (Table 3.5); such changes would include alterations in the hazard assessment protocol, in study design (i.e., number of doses, dose spacing), sample size, and statistical power strategies and the need for replication. It may also affect the choice of the biological model with special consideration given to control background values and the variability of key endpoints. Table 3.6 briefly summarizes historical factors that impeded or prevented the concept of hormesis from being accepted by the toxicological community and by regulatory agencies within the risk assessment process.

Challenge #3: Pharmacology and the Biphasic Dose–Response

The historical foundations of the dose–response relationship in pharmacology were largely based on the classic research of Alfred J. Clark [35,36,68] and his contemporary and successor John Henry Gaddum [80,206]. Based on extensive research on the quantitative actions of drugs such as acetylcholine and atropine, these authors showed that combining of drugs with specific receptor groups could be mathematically similar to the function used in the Langmuir (1918) adsorption isotherm. The responses of a tissue were assumed to be directly proportional to its specific receptor groups as occupied by the drug, the receptor occupation followed the law of mass action, and drug molecules would have equal access to all receptor groups. This perspective became the dominant pharmacological dose–response paradigm for the remainder of the twentieth century.

Despite its general intellectual dominance within the field of pharmacology, this dose–response model of Clark and Gaddum needed further development, refinement, and correction over the subsequent years. In fact, not long after the theoretical formulation by Clark [35,36], it was found that adsorption equations besides that of Langmuir's could fit the dose–response data just as well and that a maximal response

TABLE 3.6
Factors Contributing to the Historical Demise of Hormesis

- Powerful conflicts with traditional medicine and homeopathy.
- Toxicology directly emerges from traditional medicine.
- Influential opponents to the hormesis concept controlling the vehicles of communication.
- Influential opponents (radiation) accepting data that supports hormesis and denying that it is hormesis.
- Biostatistical modeling being constrained to deny hormesis.
- Rejection of hormesis occurring during the period of toxicological concept consolidation.
- No intellectual counterforce at the time of concept consolidation (not yet addressed).
- Early supporters of the hormesis concept lack understanding of its dose–time response features.
- Hormesis being difficult to prove experimentally without proper study designs.
- Powerful regulatory agencies adopting rival dose–response models.
- Governmental hazard assessment protocols making it highly unlikely to observe hormetic effects.
- Being shunned from standard texts, society meetings, and academic teaching.
- Being excluded from research funding possibilities in major governmental grant programs.
- Biological/societal implications not appreciated nor anticipated.

Source: Calabrese, E.J. et al., *Toxicol. Appl. Pharmacol.*, 222, 122, 2007.

may not require maximal receptor occupation. However, despite such limitations in this pharmacological model of the dose–response, it became adopted along with the belief that the maximal response required the complete occupation of receptors by the agent [207].

While the previous conceptual framework of the pharmacological dose–response has had a long history of qualitative predictive utility, it frequently offered less than satisfactory quantitative agreement with experimentally derived data [208]. It was often reported that the observed dose–response did not follow the predicted form (some drugs [i.e., partial agonists] often stimulated tissues but did not produce the expected normal maximum response), nor could the model of Clark and Gaddum account for the antagonism shown by partial agonists to more powerful agonists, tissue desensitization following exposure to highly specific stimulants (acetylcholine, histamine), as well as responses when a vigorous excitation is quickly replaced by competitive antagonism [165].

These and other limitations lead to modifications in the receptor-based dose–response concept during the mid-decades of the twentieth century. Ariens [209] proposed that the drug–receptor union may have varying effectiveness, which he called *intrinsic activity*, while Stephenson [208] referred to this concept as *efficacy*. This efficacy or intrinsic activity concept was expressed quantitatively as the rate constant of the second step of a drug–receptor process that yields the active form of the drug. This concept was based on the studies on motor-endplate depolarizing activity [210–212].

Biphasic dose–response curves, especially those with a single active chemical group, offered a further significant challenge to the occupation receptor theory, proposing that such curves may be observed if a drug interacted with two-receptor systems [213]. In this context, Dale in 1906 [166] noted that adrenaline can act at both excitatory and inhibitory receptors in the vasculature; this observation lead Furchgott [214] to suggest that these receptors may occur on the same smooth muscle cell. By the 1960s, numerous, yet isolated, biphasic dose–responses were reported for drugs of pharmacological importance. These responses were accounted for by the presence of two opposing receptor populations. In his subsequent assessment of this literature, Szabadi [165] reported that most of the sympathomimetic amines activate opposite receptors in smooth muscle preparations with α-type receptors being stimulating, while β-receptors were negative. Likewise, by the 1970s, several researchers reported that histamine can activate both excitatory and inhibitory receptors [215,216] with H_1-type excitatory receptors being stimulating and H_2 receptors being inhibitory.

It also became recognized that two opposing receptor populations to the same agonist were often present on the same neuron consistent with the early suggestion of Furchgott [214]. This was the case of invertebrates for acetylcholine [217], dopamine [218], 5-HT [219,220], and histamine [221] on sympathetic ganglion cells along with numerous other examples.

As a result of these cumulative findings, Szabadi [164,165] proposed a theoretical model to explain how agonists and antagonists act in pharmacological systems containing opposite acting receptors. This model was derived from the combination of *double agonists* and functional antagonism as proposed by Ariens et al. [222]. Double agonism was generally defined as a phenomenon when a compound induces an effect by interaction with two different specific receptors. The Szabadi [165] model was a special case of double agonism when two receptors, which are activated by the agonist, mediate opposite effects. If the two types of receptors are assumed to be functionally independent, the model for functional antagonism is employed; the quantitative features of the model are described by the algebraic sum of the induced effects produced by the activities of both receptors.

The model of Szabadi [165] is very generalized, with the potential to account for a wide range of dose–response

relationships depending on assumed affinities and intrinsic activities. This model may also account for hormetic-like biphasic dose–response relationships. Since the publication of the Szabadi [165] model, the number of examples of biphasic dose–responses has markedly increased, including nearly 40 receptor families affecting a range of biological endpoints that govern essential physiological and behavioral functions [186–188].

The biphasic dose–response model of Szabadi [165] has been supported and extended by other researchers [168,169,223–225]. For example, based on research on α-lactalbumin production by rat mammary gland explants, Quirk et al. [226,227] were able to account for biphasic dose–responses even for highly specific glucocorticoids with binding to a single receptor. This response was accounted for with a model of a single *turn-on* nuclear receptor site for glucocorticoid receptors and multiple glucocorticoid regulatory refinements. This finding was consistent with those of Jarv that agonistic and antagonistic effects of ligands may be related to their binding in two separate sites of the same receptor molecule [228,229]. Evidence supporting this model was reported by Jarv et al. [230] for the muscarinic acetylcholine receptor.

While pharmacological evaluation of biphasic dose–responses has dominated the literature from the 1970s to present, there is both a convergence of concept and a reformulation of such perspectives in the area of toxicology and more broadly in systems biology. In 2004, Leuchenko et al. [231] presented a framework for biphasic regulation that is conceptually similar to the model of Szabadi [165] and the considerable research on the concept of hormesis. Leuchenko et al. [231] proposed four types of biphasic regulatory schemes, with the first two being similar to the one-receptor model [223,232] and the one-agonist two-receptor model [165]. However, the paper added a further dimension of progressive complexity.

In the third model (i.e., type III), the biphasic response results from signal transduction activities that are removed from the response element (ligand–receptor complex). In this situation, the biphasic nature of the dose–response derives from the properties of the signal-transducing components. The authors proposed a variety of ways in which the biphasic dose–response could occur depending on the type of properties of the RE, molecular inputs, and complexity of the biochemical pathways. An example of the type III biphasic regulation is seen with mesoderm induction in *Xenopus laevis* via the TGFB homologue that activates the genes *Xbra* and *goosecoid*. The activated receptor starts the signaling process by phosphorylating a signal transduction of the SMAD family that leads to gene transcription. Approximately 100 and 300 activin molecules need to bind the receptor to activate Xbra and goosecoid expressions, respectively. However, the expression of Xbra is suppressed by high goosecoid concentrations. Consequently, when activin activates about 100 receptors, Xbra expression is positively regulated, but if the activin concentration is increased by about 30-fold, goosecoid expression is enhanced, resulting in turning off Xbra expression.

The fourth model of biphasic regulation requires two inputs to affect the response rather than one as in the previous cases. This is seen in combinatorial inhibition when a molecule binds with two or more interacting molecules to form a single functional complex as is the case with scaffold proteins. A second type IV receptor involves two or more interactions between an activator and the activated molecules for full activation. Examples of both biphasic regulation with scaffold protein concentrations and distributive activator are seen in the MAPK cascades. Leuchenko et al. [231] speculated on the adaptive significance of biphasic regulatory strategies. They provide tunable filtering of the magnitude of the incoming signal and determine the sensitivity and strength of the response. The composite of these strategies may be seen as a framework of broad biological significance at multiple levels including global and local domains.

Challenge #4: Population-Heterogeneity-Based LNT

While it is often stated that each individual of the population may display a threshold response, the population itself may display no threshold due to normal heterogeneity within the highly outbred and socially and culturally diverse human population. While this is a conceptually logical statement, it is principally a theoretical one. The reason for this statement being theoretical is because the power of epidemiological studies to estimate low risks in the population is usually weak, due to limitations in assessing exposure, genetic variability, and numerous other disease and performance-modifying variables.

The principal controversy associated with the population-heterogeneity-based LNT hypothesis is the generalization of its applications to agents and endpoints, including those of a noncancer nature. It is also not clear how this concept would translate into what is permissible population-based risk [1]. However, as noted earlier, the 1977 SDWC [148] used this concept as one of their eight guiding principles to support the LNT model for carcinogens. Thus, this concept is not a new one—but one now reformulated to challenge the threshold dose–response for noncarcinogens. Would each adverse effect be treated in a similar manner, that is, with the same level of permissible risk, or would the permissible risk be related to the medical implications and cost of therapeutic interventions? This concept would appear to offer a profound challenge to regulatory agencies and would affect a reevaluation of environmental, food, and occupational chemical health standards.

The scientific foundations of a heterogeneity-based LNT model assume that there are multiple risk factors affecting susceptibility to all toxic substances and that these risk factors are randomly distributed within the population. This would be predicted to result in a rather broad distribution of susceptibilities with multiple interactive risk factors affecting a progressively smaller proportion of the population who would display a progressively greater level of risk and/or susceptibility. When taken to its extreme, those individuals with the greater number of significant risk factors will be the first to experience adverse effect and premature death.

The heterogeneity-based LNT concept also assumes that there is an additive to background adverse effect relationship providing further support for a linearity interpretation.

The underlying evidence to support the heterogeneity-based LNT is difficult to obtain since epidemiological methods have generally been shown to be hard to establish causality unless the risks exceed the control or reference population by a factor from two- to threefold. In the area of toxic torts, formal judicial guidance reflects this perspective, with judges being guided not to accept a causal relationship unless the related risk for the alleged exposure equals or exceeds a factor of 2 [233]. If, therefore, the population heterogeneity-based LNT dose–response model were to be accepted, it would come into conflict with the current standard for epidemiological guidance for tort-risk causality [234].

Despite the long-standing two- to threefold epidemiological causality rule of thumb as just discussed, this perspective has been challenged, especially with the area of fine particulates and community-based air pollution [235]. Numerous large-scale epidemiological studies in the United States and elsewhere have generally reported relative risks that have ranged from the slightly below 1 to about 1.2. Some investigators and regulatory agencies, especially in the state of California, have concluded that causality in such studies can be affirmatively made, even with population-based risk, below the twofold increased risk historical perspective. This has been a highly contentious matter since it is difficult to accurately identify known causes of deaths as well as underlying biological predispositions. Factors such as family characteristics, age, gender, stress, diet, prior disease, access to medical treatments, social factors, epigenetics, and other influences are likely factors affecting health outcomes. Given all the possible confounding variables and the extreme difficulty of their quantification or the general inability to control for such differences with surrogate parameters such as socioeconomic and other standard parameters, the capacity to use epidemiological methods to estimate risks to the less than the doubling rate is generally beyond the present capacity of this discipline. Thus, the population-based heterogeneity-based LNT model is limited by the inherent limitations of epidemiological methods. Even with marginal improvements in the sensitivity of epidemiological methods in the future, its capacity to address low-dose risks is principally theoretical and model dependent and has no capacity for validation. While this is a powerful argument against the acceptance and use of the population heterogeneity-based LNT, it would be similar to an argument against a toxicologically based LNT model based on experiments in which only a few very high doses were used within the standard chronic bioassay.

ISSUES

Dose–Response versus Dose–Time Response Relationships

The effects of the dose on biological systems may be expected to change over time. Biological systems are dynamic; they can be stimulated, stressed, or injured, and in response, such changes may display a wide range of adaptive or maladaptive processes. These responses to toxic substances may also affect the availability and utilization of various biological resources and the capacity of the system to respond to subsequent similar or different exposures. There is therefore value if experiments can assess such biological responses over time. In practical terms, investigators are typically confronted with a decision over whether to use a range of doses or to follow the response of a single dose over a range of time points. Combining these dose and time features would be the ideal research strategy as much is lost by the ignoring the other.

The fact that biological systems are dynamic and adaptive has long been known, first being explored in the 1890s [44]. Of particular importance to the history of dose–response was the work of Sarah Branham in 1929 [38], which was designed to provide a detailed and updated replication of the seminal findings of Hugo Schulz [23,24] on the effects of numerous chemical disinfectants on the metabolism of yeasts. Of particular interest was that Branham [38] assessed the effects of multiple chemicals over multiple concentrations each over multiple time periods. What Branham [38] demonstrated was that these agents initially induced a concentration decrease in metabolism. However, this was subsequently followed by a compensatory or rebound-type response that eventually led to a stimulation within the lower concentration range. Compensatory responses were also noted at the higher concentrations, but the degree of compensation was usually not enough to overcome the toxicity that was initially induced. The degree of stimulation was modest, being at maximum in the 30%–60% zone greater than the control values. Similar dose–time responses have been widely reported confirming the general nature of the dose–time overcompensation responses [199]. Such findings indicate the significance of study designs that include a broad range of doses along with multiple measures over time. Failure to incorporate both adequate dose and time point evaluations will lead to inadequate understandings of the dose–response. Unfortunately, the use of many doses and their evaluations over multiple time controls is resource intense. However, the value of such consideration is clearly represented in the work of Branham and others [38]. These findings reveal the dynamic nature of the biological affected systems when it is stressed and/or injured. The dose–response, therefore, is not static and the dynamics of cellular/system response are necessary to discern as it provides critical information of not only dose-induced damage but critical repair and recovery processes. In fact, it was the use of the time variable that provided the capacity to discover the phenomenon of preconditioning and postconditioning in the biomedical sciences and the adaptive response to radiation and chemical mutagens as seen in the following section.

Adaptive Response/Pre(Post)-Conditioning/Auto Protection

Another factor affecting the dose–response is the physiological state of the biological system. Prior exposure to an

agent can affect how the biological system will respond to subsequent and more massive exposures of the same or different agents. This phenomenon has long been recognized by different terms in the toxicological and biomedical sciences. A low dose of CCl_4, which caused a negligible toxic response to the liver based on the change in serum levels of AST and ALT, prevents rats from dying when they receive a subsequent LD_{95} dose of the same agent [236]. This phenomenon was called autoprotection, that is, a prior low dose of the chemical prevented adverse health effects caused by a subsequent and more massive exposure of the same agent. It was later shown that a prior dose of a similar but not identical agent could protect against a massive exposure of the similar toxic agent (i.e., heteroprotection). The mechanism of protection was widely thought to result from the possibility that the low prior dose may have reduced the cytochrome P450 activity, preventing the bioactivation of the CCl_4, thereby reducing the toxic potential of the subsequent massive exposure. This interpretation was challenged by Mehendale [237,238] who associated the enhanced survival due to the prior low dose with an accelerated process of hepatic tissue repair.

The concept of an autoprotection and heteroprotection for heavy metals was also reported during the same general time period. Yoshikawa [239] found that a pretreatment with low doses of various heavy metals could markedly reduce the acute toxicity of similar and other heavy metals in a mouse model. These findings were further evaluated over a broad dose range of the preconditioning/priming agent. A biphasic dose–response was reported for lead and mercury fully consistent with the quantitative features of the HDR.

That a low dose could protect against the effects of a subsequent more massive exposure was subsequently generalized to the field of chemical mutagenesis by Samson and Cairns [176] and for radiation-induced mutagens by Olivieri et al. [177]. These investigators referred to the phenomenon as adaptive response. Two years later, it was shown that a prior hypoxic stress to the heart of dogs reduced the toxic effects of a subsequent massive myocardial infarction, a phenomenon referred to as preconditioning [178]. Thus, the fields of chemical toxicology, chemical/radiation mutagenesis, and biomedical/pharmacology converged on the same low-dose–high-dose protection phenomenon and referred to this with differing names. These observations in each case were replicated and generalized, covering significant areas of research. Furthermore, in the aftermath of the research on preconditioning, investigators reported the phenomenon of postconditioning. That is, if the low dose was given after the more massive exposure, it affected a similar decrease in toxicity as the preconditioning phenomenon dose [240].

The autoprotection, adaptive response, and pre-/postconditioning phenomenon act via the use of an adaptive dose that induces mechanisms that reduce the toxicity of a larger dose. Of particular significance is that the magnitude of the protective response depends not only on the timing of the adapting dose but also on its dose level as well. That is, all adapting doses are not equal in their capacity to affect a protective response. Subsequent research revealed that the adaptive dose could be optimized. Detailed dose–response studies using a broad range of adapting doses were found to follow the hormetic–biphasic dose–response. This was also the case for postconditioning responses as well [240].

High-Risk Groups: Dose–Responses

The dose–response may be affected by a wide range of biological factors, including developmental processes, age, gender, dietary and nutritional status, genetic factors, preexisting disease conditions, and prior exposure history, among other factors. These conditions can be seen as either enhancing or reducing one's susceptibility to toxic substances, thereby altering the shape/slope of the dose–response. Since all individuals experience developmental and aging processes, there will be periods during the normal life cycle in which all people will be at enhanced risk to certain agents. In general, it is thought the young will be at enhanced risk to most, if not all, toxic substances. While this concept has been generally borne out in a wide range of toxicological studies, there are instances where the young are not more susceptible than a middle-aged population subjects. In fact, there are well-known situations (e.g., renal toxicity for fluoride and mercury) for which their risks are significantly lower [241]. These observations illustrate challenges that regulatory agencies confront in the assessment of risk.

The enhanced susceptibility within the high heterogeneous population is thought to be captured with a range of 10-fold starting from the median response of the normal population response [65]. This is a practical guidance value that has not been systematically assessed. For example, the effect of oxidative stressors on normal red blood cells can be assessed in a dose–response context. These cells could be compared with the response of red blood cells from individuals with various types of antioxidant enzymatic deficiencies (e.g., glucose-6-phosphate dehydrogenase [G-6-PD]), proceeding from moderate to more extreme [242]. Such experiments could provide information on differences in the magnitude of the cell damage at the same concentration as well as the differential threshold concentrations for each experimental subgroup. These population-based subgroups could be made even more complex by superimposing factors such as lower or higher exposure to dietary antioxidants, further affecting the threshold and magnitude of response.

How Physiological Status Affects the Dose–Response: The Anti-Inflammatory Phenotype

Low-level exposures to ionizing radiation can enhance several immune parameters while being inhibitory at higher concentrations [243]. These findings are in striking contrast to a rather large body of experimental data that demonstrates that low doses of ionizing radiation can have a depressive effect on immune functions if the biological system is in an inflamed condition [47,244–246]. In fact, the dose–response in this situation is U- or J-shaped. That is, at the lower doses, the ionizing radiation suppresses the inflammatory

condition, while at higher doses, it inflames it further. This phenomenon has been documented in five arthritic animal models with strong consistency [47,247–256]. Much follow-up work has been conducted on the underlying mechanisms contributing to the radiation-induced suppression of inflammation [257–265]. The key conceptual insight offered is that the dose–response relationship is dependent on the physiological state of the organism. At the same doses, the response could be opposite depending on whether the biological system was inflamed or not. Low doses of ionizing radiation can therefore induced two types of HDRs: one that enhances immune function at low doses and one that suppresses the immune-mediated inflammatory state, thereby creating an anti-inflammatory phenotype.

Triphasic Dose–Responses

While the principal focus on the assessment of dose–response relationships has been directed toward threshold, linear, and hormetic/biphasic dose–responses, triphasic toxic responses have also been reported. However, the frequency of such reports is quite limited. Nonetheless, the occurrence of triphasic dose–responses is possible based on biological theory. There are significant challenges in the study of such possibilities starting with requiring more dose/concentrations. A particularly interesting example of a triphasic dose–response relationship was presented by Hooker et al. [266] in an assessment on x-ray-induced mutations in the spleen of pKZ1 mice. At the high end of the exposure spectrum, the x-rays, as expected, enhanced the incidence of mutations. However, as the doses were lowered, the incidence decreased below that of the control group, demonstrating support for an HDR. As the dose was further diminished, the mutation rate returned toward the control value again, consistent with a hormetic interpretation. However, further reduction in dosing revealed a surprising and novel finding. The mutation incidence flared up again, significantly beyond that of the control group. The dose–response was triphasic, with an increase in the frequency of mutations, followed by a marked reduction below the control and then the increase at higher doses.

Radiation-induced triphasic dose–responses were also reported by Choi et al. [267] in zebra fish embryos, either one or two embryonic cells, irradiated by microbeam protons over dose range from 20 to 2000 protons. This study not only confirmed the occurrence of the triphasic dose–response but showed biological responsiveness down to the level of 20 protons.

Much data exist that could explain both the low-dose protection and high-dose enhancement of mutation. However, what could account for the increase in mutation rate at the lowest doses? While this remains an area of research, it may be hypothesized that at the very lowest doses tested, the x-rays/protons induced a low level of damage. However, in order for repair processes to be induced and the damage to be repaired, the damage signal must be first detected, thereby initiating the repair process. The question that is posed is how sensitive is the detection system in this particular biological model and to what type of damage. Thus, it has been proposed that the damage at low doses may reflect a level and type of molecular alteration that goes undetected and thus unrepaired, at least within the time period of the study observations.

These types of investigations raise a broad range of novel scientific questions concerning damage, detection, repair, the generalizability of this phenomenon to other tissues and biological models, and the adequacy of routine hazard assessment protocols to detect such effects. The issue of triphasic dose–responses and their toxicological implications is an intriguing area of study but is generally ignored by the regulatory and research communities. If these findings, as assessed in the mouse and zebra fish models, could be broadly generalized, it would have significant biomedical and risk assessment implications.

Maximum Tolerated Dose/Dose–Response

The information provided from the chronic bioassay based on the use of MTD-related doses has had a controversial history. The MTD is the highest dose that a treatment group can be exposed to over the 2-year period of the rodent chronic bioassay without experiencing toxicity and/or a loss of more than 10% body weight. However, it was not uncommon for some chemicals, especially at the highest dose, to cause cell damage in areas such as the liver. Lower doses were typically believed not to cause such cell damage or to do so with lesser frequency and intensity. As a result of the cell damage at the MTD dose, cell proliferation responses might be initiated in order to repair this damage, providing a tumor promotional response resulting in an enhanced tumor incidence at the highest/MTD dose. It was often the case that there was a significant tumor response at the MTD but not at the lower dose(s) [268]. Nonetheless, regulatory agencies would make a carcinogen designation for such agents. Based on cancer risk assessment methods, these data would be modeled via an LNT process. This area has been highly controversial as the cancer risk assessment is significantly affected by a toxicity/promotional effect at the MTD [268–270].

Shallow/Steep Dose–Responses

Descriptive considerations of the dose–response have revealed that some dose–responses are very steep, with the toxic responses occurring over a very narrow dose range. An example of this type of dose–response is seen with cyanide and its induced mortality. In contrast, some dose–responses are very shallow with toxicity occurring over a very wide dose range. An example of this type of dose–response would be petroleum. In general, a very steep dose–response is due to the fact that the risk factor is highly specific and not subject to a high degree of interindividual variation. In the case of cyanide, the reason for the steep slope is that this agent is not subject to considerable metabolism and its target for toxicity, that is, the key cytochrome oxidase is essential for energy/ATP generation, varies little between individuals.

Thus, the toxic effects of cyanide would be quick in most, if not all, animal models and humans. In the case of petroleum, it is a complex mixture, comprised of thousands of chemicals with differing metabolic patterns and with differing targets and mechanisms of toxicity. As a result, there are likely a wide range of risk factors broadly distributed within a highly heterogeneous population, resulting in a far shallower dose–response.

Cumulative Dose–Response versus Dose Rate Response

In the mid-1940s, the question was raised as to whether exposure to the same cumulative dose to ionizing radiation would result in the same degree of toxicity independent of the dose rate. This was a question raised during the Manhattan Project by the radiation geneticist Curt Stern at the University of Rochester since it was considered as a test for the linearity dose–response hypothesis. Initial studies assessed the effect of a broad range of x-ray doses administered to fruit flies (i.e., over seconds to minutes). The response gave evidence of a linear dose–response relationship. However, when the same cumulative exposure was given at a rate of only 1/15,000 of the acute dose, a threshold was reported. This was a seminal observation as it challenged support for the linear dose–response interpretation [144,271]. This challenge to linearity was believed to be so significant that Hermann Muller and Curt Stern, two of the most influential radiation geneticists, tried to prevent the acceptance of the findings of the Caspari research [10,29,39]. As noted earlier, research at Oak Ridge by William Russell with mice would support the Caspari findings and the belief that the genetic toxicity response to exposure was cumulative.

Carcinogens: Latency and Dose–Response

The principal way in which dose is used to evaluate carcinogenic responses is via tumor incidence. However, in 1943, Hermann Druckrey [272] published the first of a series of papers [272–275] establishing a relationship between dose and the time required to detect tumors, that is, the tumor latency period. This introduced a new concept with the potential to affect significantly how carcinogen risk might be estimated in a manner that was more responsive to human risk than the more limited dose–tumor incidence methodology. The key observation was an inverse relationship between dose and tumor latency. That is, the lower the dose, the longer the time required for tumor appearance. It was hypothesized that if the dose were reduced low enough, the latency period could theoretically be longer than the normal life span of the experimental species. In an analytical sense, the treated groups in such low-dose zones would have a tumor incidence indistinguishable from controls. An attractive aspect of this concept was that one could accept the linear dose–response model and yet still arrive at a *practical threshold* for carcinogens. The proposal of Druckrey represented a type of toxicologically based compromise between the LNT and threshold dose–response models.

The Druckrey dose–latency tumor incidence concept generated considerable interest worldwide. Several researchers were quick to validate the findings of Druckrey [276]. In the former Soviet Union, the concept of dose–latency was accepted in risk assessment practices by the late 1970s following its experimental validation by Yanysheva and Antomonov [277] for carcinogen benzo(a)pyrene. The authors stated that any carcinogenic effect of the benzo(a) pyrene would be observed only considerably after the normal life span of the species had been exceeded.

This idea also resonated with some leading researchers in the United States, including Hardin Jones at the University of California at Berkeley, an expert in human aging, especially with respect to the effects of ionizing radiation. He and his colleague Alexander Grendon published several papers that integrated the findings of Druckrey and others as well as new understandings of the process of carcinogenesis, deriving a predictive biomathematical model. In practical terms, Jones and Grendon [278] reported that if the dose were decreased by a factor of 1000 in the linear zone, the latency period would increase by a factor of 10. The Druckrey concept was also applied to the field of occupational cancer by Philip Enterline [279] at the University of Pittsburgh based on a meta-analysis of 11 major epidemiological studies of asbestos exposure and respiratory cancer.

Even though the findings of Druckrey were striking, substantially replicated and extended to a range of compounds, using animal models and epidemiological meta-analyses, it was rejected by U.S. regulatory agencies, with the concept failing to be integrated into and/or affecting hazard assessment and risk assessment processes. Why would this be the case? On the historical side, Occupational Safety and Health Administration (OSHA) conducted massive carcinogen policy hearings during 1978 [280]. The dose–latency concept of Druckrey and Jones was considered. However, Druckrey did not testify nor did any other researcher who had published supportive findings of the dose–latency argument. Jones was expected to offer testimony but unexpectedly died less than 2 months prior to the hearings. His relevant papers were entered into the record and commented upon, especially by those opposed to having this concept affect carcinogen risk assessment policy. Without his presence to explain and defend his position on this topic, there was little likelihood that dose–latency tumor position would even partially prevail as several notable opponents (e.g., David Hoel, Umberto Saffiotti, Richard Peto, and Marvin Schniderman) would counter his written statements without any real opposition. The most impressive of the criticisms was that by Hoel (based upon a paper by Guess and Hoel [281]). This paper argued that the findings of Druckrey of an inverse relationship between dose and latency not only were not unexpected but would be readily predicted via stochastic modeling assumptions. Thus, there was nothing new in this concept. As a result, the concept of dose–latency for carcinogens was dropped from the cancer risk assessment process and the hazard assessment testing scheme.

A counter argument not presented was that dose and time to tumor is an important concept and there was no reason to

restrict the assessment to only tumor incidence in the animal bioassay. A similar argument was made earlier by Albert and Altshuler [282,283] who argued for a novel approach in the extrapolation of laboratory studies in the derivation of environmental health standards. This approach incorporated the concept of age at the time of the appearance of an adverse health effect as well as its incidence, a position similar to that of Jones. Albert and Altshuler [282,283] stated that the usual approach for assessing carcinogenic risks such as providing the dose to incidence relationship could be extremely misleading and give an inappropriate degree of importance in the risk assessment/risk management process. The typical dose–incidence methodology used by regulatory agencies today fails to consider the age at which new tumors occur or the likelihood that additional carcinogen exposures could affect people who would have developed tumors from different causes. Supporting this perspective was a National Academy of Sciences [284] report, commenting upon the work of Albert and Altshuler [282], that it may be possible to establish standards for carcinogens that would limit the possibility of environmentally induced tumors to only very advanced age, with less than a 10% increase in the chance of cancer at 95 years of age. While 95 years of age may not seem as old today in the second decade of the twenty-first century than it did some four decades ago, the basic concept of the National Academy of Sciences was in agreement with the perspectives offered by Jones and Grendon [278] as well. Again, this OSHA hearing failed to bring forward representation of the National Academy of Sciences. Likewise, Albert and Altshuler, both prominent toxicologists, did not render testimony on this issue.

In the intervening years, several lines of research have supported the Druckrey/Jones perspective, revealing that it takes a higher dose of carcinogen to enhance tumor promotion than initiation. At higher doses, it is possible to turn on promoting processes that can markedly decrease the latency period. The reverse would also be the case. That is, at lower doses and with less promotion, the tumor latency would be extended. While the initiation process may well be stochastic in nature, this was not likely the case for the induction of promotion, which has been typically seen as demonstrating a threshold dose–response. Thus, the concept of dose affecting latency has a solid foundation, does not have to be associated with a stochastic process, and can provide a means to derive a practical threshold for carcinogens as originally intended by Jones. This area is likely to warrant considerable toxicological research as it has the capacity to profoundly affect the risk assessment process for carcinogens.

Epidemiology and the Dose–Response

There can be considerable variation in the dose–response that subjects display toward the same agent. If there is a wide range in susceptibility (i.e., interindividual variation) and if hormesis does occur within the various subgroups of the population, how may this affect the overall population-based dose–response? This will depend on the nature of each subgroup's dose–response relationship and each subgroup's proportion of the overall population. Consider the following subgroup-specific dose–response relationship and their impact on the overall dose–response relationship for the population. We are assuming that there are five population subgroups (G1–G5) and each has a defined dose–response relationship. Subgroups 1 and 5 show no evidence of an HDR, but subgroups G2, G3, and G4 display unique HDR patterns. For simplicity, assume that each subgroup comprises 20% of the population. The population average, as represented by the bolded line (Figure 3.26), displays a threshold-like response. If the population proportions for each subgroup were changed, then the composite (i.e., average) response

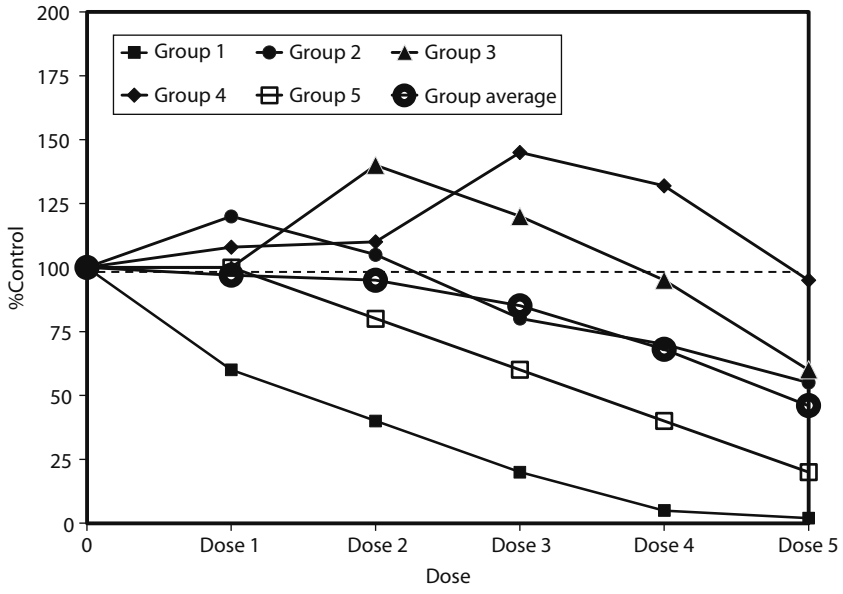

FIGURE 3.26 Population subgroup and population average dose–response relationship.

would also change. These findings demonstrate that a highly heterogeneous population has the potential to provide evidence that may or may not reveal a hormetic effect even when some of the subgroups display hormesis. This exercise demonstrates that the assessment of population-based data comparisons of substantial heterogeneity as may often be the case with humans may lead to findings that are not indicative of any underlying mechanism despite observed dose-dependent transitions. This is a situation that is not often appreciated, yet one with important biological and public health implications.

DOSE–RESPONSE IN PERSPECTIVE

One cannot underestimate the influence of biostatistical dose–response modeling in risk assessment since the mid-1970s when the EPA made the policy decision to apply linearity at low dose for carcinogen risk assessment. EPA selected what it believed to be a model with reasonable biological plausibility (i.e., the multistage model). The problem with this policy is that *assumed* plausibility does not equal validation. There was some toxicological support for a linear interpretation starting with the first systematic dose–response studies that were published during WWII, in Germany [272], and later extended by Druckrey and Küpfmüller [273] using butter yellow with five different daily doses ranging from 1 to 30 mg/rat. The administered total carcinogen doses were similar for all treated groups but that the median latency period (+) was inversely proportional to the daily dose. When plotted double logarithmically, the result was a linear dose–response relationship that followed a specific dose–time mathematical relationship in which the latency period for the time to the first appearance of a tumor was inversely related to dose. These findings were interpreted by Druckrey to mean that even very small daily doses of carcinogens could result in tumor development, that the doses were additive, and that the induction time is of considerable importance. Despite the fact that these findings clearly supported a linearity dose–rate relationship, they also indicated that latency was an important parameter that was affected by dose rate. Their data suggested that if the dose were decreased, low enough tumors would be predicted to occur long after the normal life span of the individual, in effect a practical population-based threshold. In a more statistical framework, the chemically induced tumor incidence would be indistinguishable from control group findings.

Another challenge to the linearity concept is that no LNT model, including the multistage model, could be validated in the low-dose zone. At the same time, numerous inconsistencies emerged that lead to an erosion of confidence in the estimated model predictions. For example, estimates of liver cancer in U.S. residents from aflatoxins, DDT, and chlordane were estimated via LNT modeling to be well over one million people/year [285]. Yet the number of liver cancers from all causes, including alcohol ingestion and virus infections, was orders of magnitude lower! These epidemiological validation challenges to LNT modeling predictions were complemented with the discoveries of a cascade of robust adaptive processes that further undercut the theoretical basis of the LNT model, while providing biological support for a threshold dose–response concept. Furthermore, during the late 1970s when the EPA was floating the idea for the LNT model, it also tried to support this position via two complementary approaches: one using the results of epidemiological studies in survivors of the atomic bomb blasts in Japan, while the second was via the use of mutagenicity data using the Ames data. In the case of the atomic bomb exposures, this support became quickly tentative given the nature of the exposures, the dose rates, and their relevance to low-dose exposures in society and the extreme difficulty in teasing out specific radiation exposures as its effects may have been affected by heat, shock, and other confounding factors and the apparent differential effects on different cancer types. The atomic bomb survivor study became more of a research vehicle rather than an environmental risk assessment instrument, forcing the agency to back away from using it as its central perspective to support a community-based linearity policy. A second policy probe put forward by the agency during this period was that of using evidence of linearity in dose–response with Ames mutagenicity strains. The EPA was linking the mechanism of genotoxicity to cancer with the Ames test data as the vehicle. This was also quickly withdrawn as the agency recognized the error of using DNA-deficient strains to support the LNT concept. Such tactics used by the EPA in the late 1970s were to be seen as scientifically amateurish, perhaps the action of a young agency. It was better to formulate a position based on a protectionist philosophy than to be embarrassed publicly on the science.

Much research and debate over the question of cancer risk assessment has focused on whether thresholds for mutagens might exist. Lost in this debate was the key paper of Caspari and Stern [144] that was marginalized by Stern and Muller (see Calabrese [10], Uphoff and Stern [286]). While the threshold debate initially focused on ionizing radiation [287–289], it was soon extended to chemicals [290–302]. During this period of the dose–response concept formulation for mutagens, the Environmental Mutagen Society proposed that mutagens be covered under the Delaney Clause and not be permitted to be added to food. They argued that there was no safe level of exposure since mutagens displayed a linear low-dose relationship. While this was a debate that the society lost, the debate over dose–response, especially for genotoxic agents and genotoxic carcinogens, has continued unabated to the present [112,303–321]. Regulatory agencies, such as EPA, have adhered to the LNT approach for carcinogen risk assessment for the next 40 years and continue to do so. Of interest in this regard was the striking statement of Kenny Crump, a key architect of the LNT multistage model used by the EPA in QRA in the mid-1970s to the present. In a major retrospective, Crump [322] tends to place this intellectual cacophony in the scientific world in perspective. He stated that "quantitative risk assessment, as currently practiced is broken. Despite a huge investment in 'risk research'

the effort has failed to resolve the shape of the dose response curve for any substance." Crump [322] further stated

> Science is not capable of determining the shape of the dose response at very low doses. Hypotheses regarding the existence or non-existence of thresholds are beyond the ability of science to resolve… Continuing to expend energy and time debating the irresoluble issue of thresholds, and base decision rules on it, are a detriment to fashioning a logical and workable comprehensive approach to risk assessment.

The argument of Crump is a statistical one. He notes that various research groups have reported on the occurrence of threshold and supported their conclusions by the use of a hockey stick dose–response model [316,323,324]. According to Crump [322], the best that can be said about the threshold dose–response in such cases is that it fits the data significantly better than a perfectly linear dose–response. Despite this factual conclusion, this assumption does not prove nor even imply that the true dose–response has a threshold. Crump [322] carried this argument further by assuming that the slope of the dose–response could, in fact, be quite shallow, yet still not a threshold. In this sense, he argued that this type of evaluation supports his conclusion that it is impossible to say which model is correct.

This statistical argument of Crump's has potential theoretical value when dealing with threshold dose–responses that also do not display a U-shaped or HDR. If an HDR were to be demonstrated, it would constitute proof that a threshold not only existed but had been reasonably demonstrated. This would be especially the case when the mechanism of the HDR was established.

OPEN LETTER

The concept of the dose–response has a long and controversial history in the field of toxicology and risk assessment. The controversy is not simply a modern one but one that emerged as soon as the first formal dose–response model was proposed in the 1880s. In fact, the controversies that swirl around the dose–response in the form of linearity, threshold, and hormesis can trace many of the issues back to the roots of the initial disputes. This chapter traced this history and its evolution. One of the further issues is that I have been at the center of the dose–response debate over the past two decades, leading the resurgence of the HDR in toxicology and the broader biomedical sciences. When one takes on both a leadership role and a scientific role in the development of a scientific concept, it can be challenging to be balanced in the assessment and in this case the writing of this chapter. The editor was quite clear to me that this chapter had to be about dose–response and not simply about hormesis. It was a challenge that I took seriously and looked forward to being successful with.

While I became very involved with the hormesis topic from 1990 to the present, I was first introduced to this concept as an undergraduate in a plant physiology class doing a laboratory exercise on the dose–response of a synthetic plant growth inhibitor with the peppermint plant. Unexpectedly, our research team observed that the growth inhibitor seemed to stimulate the plant growth. After the course ended, I followed up on this observation and worked with a professor to determine if the effects were reproducible. With multiple experiments and many hundreds of peppermint plants later, I was able to conclude that the findings were reproducible and showed an inverted U-shaped dose–response with a low-dose stimulation and a high-dose inhibition. The magnitude of the stimulation was always modest, being about 30%–60% greater than the controls. Since these initial findings were in soil, I did further work to see if the effects could be shown to occur in hydroponics. Similar reproducible findings occurred. The findings were published in the journal *Physiological Plantarum*. The dose–response was simply called a low-dose stimulation and high-dose inhibition as I had yet to hear of the term hormesis, even though I was to learn later that it had been created in the early 1940s.

I put aside the peppermint work and went to graduate school hoping to become an insecticide toxicologist. Over the next 20 years or so, I become very involved in the toxicology and risk assessment communities, serving on numerous major committees, including a series of NAS SDWCs, the Air Cabin Safety Committee that lead to the banding of smoking in airplanes, and an Institute of Medicine committee. I wrote nearly a dozen singly authored books, most of which dealt with the issue of dose–response. In rereading these books, my views were very much in line with the views of the federal agencies, supporting thresholds for noncarcinogens and linearity for carcinogens. In fact, this is the way that I taught my toxicology and risk assessment courses. My views on dose–response were completely *mainstream* and never challenging the status quo.

I was reintroduced to the topic of hormesis in 1985 when I received a brochure for a conference sponsored by the Electric Power Research Institute. I was invited to participate in the meeting by the late Leonard Sagan, with a paper addressing chemical examples of hormesis. While the paper was completed, it was presented by a graduate student, as I was unable to attend due to the birth of my second son. During the 1990s, my interest continued on the topic of hormesis, with an openness to discovering whether this phenomenon was very general and biologically significant. I was far from sure on this matter until the near to the end of the 1990s, when at last I had seen enough data within a sound evaluative framework that I finally concluded that there was something very significant going on with the hormesis concept. At that point, I threw myself fully into the study of hormesis, pretty much dropping all other previously funded areas. My scientific intuition told me that this could be very important to give it the highest priority. I have done this ever since and it was a good decision.

Despite this commitment to the study of hormesis, I did not give up my capacity to be objective, to see weaknesses in experiments, and to try to find ways to gain better scientific insights. Our work in this area is characterized by the publication of numerous articles in a broad range of high-level journals, with strong independent peer-reviewed activities. What I have learned is that it has been far harder to pass peer reviews on topics related to hormesis than all my previous areas of research. There is often an extra set of reviewers and a more skeptical associate editor that seem to raise the

publication bar far higher for things hormetic. While frustrating, this has been good in many ways as the finally accepted manuscripts are typically of higher quality. During the course of these years of hormesis focus, my work and I personally have been the object of much criticism and debate. Much of the criticism has been of excellent quality and has been helpful to improve future efforts. However, there are other criticisms that are clearly ideologically, based on a fear that hormesis may harm environmental regulations. Such criticisms often include personal attacks and unfounded accusations that hormesis is simply a ploy used by industry to weaken standards. We have shown from the early days of the hormesis revolution that the hormetic response could be beneficial, harmful, or neither. In fact, Baldwin and I were the first to publish this, a view that is now widely accepted. We have also reported cases in which the hormetic response was replaced by a triphasic dose–response, with damage occurring at the lowest levels of exposure. The point is that it is not hard to be objective, when one has been trained to be objective and where one has lived this way. This is the fun of science. The only way to gain solid insight is to follow the data. I have done this over 40 years since receiving my PhD, and I have tried to do this in this chapter on dose–response. Nonetheless, I have an informed perspective and I share it. It may not be fully correct and I suspect that my understandings and views will change with new data and assessments. Yet this chapter is my honest attempt to give the reader my best insight into the dose–response and how the toxicology community came to its understandings and whether they have been correct or wrong and why.

QUESTIONS

3.1 Could the inverted U-shaped dose–responses observed for some endocrine disrupting agents be examples of hormesis? Explain how and why you think that this might be the case or why not.

3.2 What are the risk assessment implications of the observation that latency of tumor development is inversely related to dose?

3.3 What are the toxicological advantages and disadvantages of having an animal model for the chronic bioassay to have a low or negligible background tumor incidence?

3.4 How does the concept of dose–response synergy differ depending upon whether the synergy occurs above the threshold or below the threshold in the hormetic zone?

3.5 In toxic tort litigation, epidemiological response less than twofold greater than the control group is not accepted as showing causality. Why is this the case and should there be exceptions to it, and what might they be and why?

3.6 Are their areas of toxicology and clinical medicine that do not show a dose–response? If so, what would they be? How would this phenomenon be explained?

KEYWORDS

Dose–response, Threshold, Linearity, Hormesis, Risk assessment, Biphasic, U-shaped, J-shaped

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4 Metabolism
A Determinant of Toxicity

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CONTENTS

INTRODUCTION

Understanding the metabolism, or biotransformation, of xenobiotics has come to be regarded as fundamental to appreciating the toxic mechanisms of chemicals, be they drugs, industrial chemicals, pesticides, or other molecule foreign to the body. This chapter discusses the major pathways of xenobiotic metabolism with an emphasis on the role that biotransformation plays as a determinant of toxicity. The material is organized in the traditional view of the functionalization of xenobiotics, that being biological oxidations, reductions, and hydrolytic conversions, frequently referred to as *phase I reactions*, and biochemical conjugation reactions, known as *phase II reactions.*

GENERAL FEATURES AND BASIC CONCEPTS OF XENOBIOTIC METABOLISM

The majority of organisms studied have biotransformation enzymes, although there is diversity in the occurrence, function, and rates of specific enzymes. Certain bacteria contain more primitive or less highly developed systems and may lack certain pathways altogether. Mammals demonstrate diversity in the activity or rates of specific systems, and as would be expected of genetically controlled functions, there are significant species and individual differences. This diversity extends to the organ level in multicellular organisms. Moreover, specific organs show different levels of activity, and specific cell types within organs demonstrate variation in biotransformation capability. There is even subcellular diversity, in that certain of these enzymes are compartmentalized whereas others are free in the cytoplasm.

The variety of chemicals to which organisms may be exposed requires that biotransformation enzymes have broad substrate specificity. This characteristic is not shared by the majority of enzymes involved in anabolic and catabolic metabolism. In addition, the types of reactions catalyzed are diverse, as shown in Table 4.1, including oxidation, reduction, epoxidation, deamination, hydroxylation, sulfoxidation, dehalogenation, and conjugation with endogenous compounds, to name a few. Although it is logical to initially focus on xenobiotic-metabolizing systems one at a time, in many cases, xenobiotic metabolism involves more than a single metabolic route. In addition, the eventual toxicity of a xenobiotic may be modified by a number of factors, including age, gender, physiological status, nutrition, diet, and the presence or absence of disease, among others.

Exposure of an animal to certain xenobiotics can result in the induction of specific enzymes associated with xenobiotic metabolism. When induced, their activity can dramatically increase, compared to their basal level. Induction is sometimes coordinated with more than one enzyme induced. Induction results in an increase in the ability of animals to metabolize xenobiotics, and in most cases, this reduces their susceptibility to its toxicity. Induction generally lasts only a few days. When exposure ceases, the enzymes return to their basal levels.

TABLE 4.1
Reaction Types and Enzymes That Participate in Xenobiotic Metabolism

Phase I Reactions

Oxidation
- Cytochromes P450
- FMOs
- Xanthine oxidase
- Amine oxidase
- MAO
- Semicarbazide-sensitive oxidases

Reduction
- Cytochromes P450
- NADP-quinone oxidoreductase
- Carbonyl reductases
- Glutathione peroxidases

Ester hydrolysis
- Carboxylesterases
- Amidases

Dehydrogenases
- ADHs
- ALDHs

Hydration
- Epoxide hydrolase

Miscellaneous
- Cysteine conjugate β-lyase
- Superoxide dismutase
- Catalase

Phase II Reactions

- UDPGTs
- SULTs
- GSTs
- Glucosyltransferase
- Thiotransferase
- Transacylases

Methylation
- O-methyltransferase
- N-methyltransferase
- S-methyltransferase

Acetylation
- NATs
- Acyltransferases

Miscellaneous
- Rhodanese

Because xenobiotic metabolism does not always result in detoxification, the term *biotransformation* has come into general use to denote the actions of xenobiotic-metabolizing enzymes, although it is still not semantically specific for xenobiotic metabolism. Biotransformation is divided into two distinct phases. Phase I reactions result in *functionalization*, the addition or the uncovering of specific functional groups that are required for subsequent metabolism by phase II enzymes. Phase II reactions are biosynthetic. These phase I and II reactions are often coordinated, with the product of phase I reactions becoming the substrate for phase II enzymes. A commonality of biotransformation reactions is the conversion of hydrophobic xenobiotics into more polar, more easily excreted compounds. Because the composition of cells is more lipophilic than their environment, nonpolar compounds tend to accumulate. This could lead to bioconcentration of chemicals within the cell to levels higher than that of the environment and increase the likelihood of a cytotoxic event; however, conversion of nonpolar chemicals to more polar metabolites allows them to be more easily excreted by the cell. Conjugation of a xenobiotic with an endogenous compound, a phase II reaction, increases water solubility, and in some cases, the added chemical group is recognized by specific carrier proteins or proteins involved in facilitated diffusion or active transport. This increases the ability of the cell to remove the xenobiotic.

Many diverse examples exist of xenobiotics whose toxicity is directly dependent on the activity of the biotransformation

enzymes. For most chemicals, increases in the activity of these enzymes result in decreases in toxicity, whereas decreases in activity result in increased toxicity; however, in some instances, the product of xenobiotic metabolism is more toxic than the parent compound. Conversion of a foreign compound to a more toxic metabolite is termed *metabolic activation*; for example, the majority of genotoxic and carcinogenic chemicals require metabolic activation to highly reactive species capable of interacting with DNA. The enzymes that protect the animal from the toxicity of certain compounds may be responsible for the toxicity of others. The susceptibility of an organism to the toxicity of a particular chemical is dependent, in many cases, on the delicate balance between detoxification and metabolic activation that exists during exposure to the xenobiotic. Due to the sensitivity of the enzymes of xenobiotic biotransformation to both endogenous and exogenous factors, this balance may differ among individuals and at different points in time.

BIOLOGICAL OXIDATION

Cytochrome P450-Dependent Monooxygenase System

The P450-dependent monooxygenase system is central to the metabolism of most xenobiotics. Not only is it the primary enzymatic system for metabolism of many xenobiotics, but it is also involved as the initial functionalization step in the further metabolism of many others. Consequently, P450 plays essential roles in several areas of research, including biochemistry, pharmacology, toxicology, physiology, and medicine. Several names for the P450 system exist in the literature. The names most commonly encountered include

- Mixed function oxidase
- P450 system
- P450-dependent monooxygenase system

Generally, these names either are related to a specific function or are descriptive of a biochemical mechanism. Currently, it generally is referred to in terms of a monooxygenase system to denote its ability to incorporate one atom of molecule oxygen into its substrates.

Components of the Cytochromes P450 System

The history of the discovery of P450 and elucidation of its functions and mechanisms of action is intriguing and has been reviewed by Estabrook.[1] P450 was first described independently in 1958 in microsomes isolated from rat and pig liver homogenates. The name P450 derived from the occurrence of a pigment that, when reduced and treated with carbon monoxide, yielded a spectrophotometric Soret band at 450 nm. The laboratory of Britton Chance at the Johnson Foundation was the first to observe the pigment. Six years later, Omura and Sato[2,3] published their pivotal papers describing P450 as a b-type hemocytochrome and demonstrated that the cytochrome was located in hepatic microsomes, which form from the endoplasmic reticulum upon cellular disruption. Upon isolation from the membrane, through the use of proteases, P450 is converted to an inactive form whose reduced carbon monoxide complex produces a spectrophotometric peak at 420 nm.

Before the discovery of P450, Julius Axelrod and his colleagues,[4] in the laboratory of Chemical Pharmacology at the National Heart Institute, were involved with studies on the metabolic disposition of drugs.[4] They found that the oxidative metabolism of amphetamine required the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) (reduced form) and the presence of oxygen. Estabrook et al.[5] established that P450 was the terminal oxidase involved with the C-21 hydroxylation of steroids in adrenal cortical microsomes, giving P450 a role in endobiotic metabolism. Many individuals and laboratories have since played major roles in the development of the current knowledge concerning P450.

It soon became obvious that although P450 played a major role in the activity of the monooxygenase, it did not act alone. In 1950, Horecher[6] isolated a flavoprotein from the liver, but no function was identified. This flavoprotein used reducing equivalents from NADPH and was termed *NADPH–cytochrome c reductase*. In 1955, La Du et al.[7] showed that cytochrome *c* could inhibit dealkylation of aminopyrine. This was followed by the studies of Gillette et al.[8] in 1957, which presented additional evidence that cytochrome *c* reductase was involved in xenobiotic metabolism. In the 1960s, it was reported that NADPH–cytochrome *c* reductase occurred in the endoplasmic reticulum of liver cells, that antibodies to the reductase inhibited xenobiotic metabolism, and that the reductase is required for monooxygenase activity when reconstituted from isolated components.[9–12]

Although the major components of the P450-dependent monooxygenase system appear to be P450 and P450 reductase, other components may also be involved with the metabolism of specific xenobiotics. Cytochrome b_5 reductase has been proposed to participate in monooxygenase activity through electron transport to cytochrome b_5 and, subsequently, to P450; however, several systems of electron transport in the endoplasmic reticulum and isolated microsomes, as well as other activities such as peroxidation, have greatly complicated the elucidation of the role of cytochrome b_5. In many cases, cytochrome b_5 has been found to enhance NADPH-dependent substrate oxidation by P450, although the effect of cytochrome b_5 is dependent on both the specific P450 isoform and the substrate under investigation.[13] Cytochrome b_5 may enhance electron transfer to P450 by at least four different mechanisms. These include faster provision of the second electron to P450, the rate-limiting step in catalysis; enhanced coupling between NADPH consumption and substrate oxidation; formation of a complex with P450 that is capable of accepting two electrons from NADPH–P450 reductase; and allosteric activation of P450.[13] An elucidation of the role of cytochrome b_5 must await further understanding of the complex electron transfer pathways that exist in the endoplasmic reticulum.

Although the catalytic activity of the monooxygenase system appears to require only two proteins, NADPH–P450

$$\text{R-H} + \mathbf{O_2} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{CYP}} \text{R-}\mathbf{O}\text{H} + \text{H}_2\mathbf{O} + \text{NAD}^+$$

FIGURE 4.1 Global reaction and stoichiometry of cytochrome P450.

reductase and P450, it is capable of carrying out a variety of different reactions on a large number of substrates. This ability is based on the occurrence of a variety of P450 isozymes, but it also is based on the basic reaction mechanism of the cytochromes and their overlapping substrate specificity. The nonspecificity of the monooxygenase provides important flexibility to xenobiotic metabolism, but this flexibility comes with a price. Generally, the enzymatic reactions of anabolism and catabolism are both extremely specific in substrate specificity and catalytically efficient, resulting in high activity and high substrate turnover number. The turnover number and efficiency of P450 are considerably lower than most enzymes. This is probably related to the inefficient electron transfer due to the presence of a water molecule at the active site. Some substrates are more efficiently oxidized because they exclude water from the active site.[14] Inefficiency of metabolism is more than made up for by the ability to metabolize a variety of chemical structures and the ability to catalyze a variety of reactions. An additional factor that compensates for the relatively low substrate turnover number is the high concentration of the system in organs important in xenobiotic metabolism.

Before discussing the various reactions catalyzed by P450, a discussion of the catalytic cycle is appropriate. Knowledge of the catalytic cycle will assist in understanding the various reactions catalyzed by the system and in predicting metabolic pathways for specific xenobiotics.

Catalytic Cycle of the P450-Dependent Monooxygenase System

The reaction catalyzed by the cytochrome P450-dependent monooxygenase system and its stoichiometry are illustrated in Figure 4.1. One molecule of substrate reacts with one molecule of molecular oxygen and NADPH to yield oxidized substrate containing one atom from molecular oxygen, water (containing the other oxygen atom), and $NADP^+$. The incorporation of one oxygen atom from molecular oxygen into the substrate is the source of the term *monooxygenase*. Oxidation of substrate and concomitant reduction of one atom of oxygen to water is the source of the name *mixed function oxidase*. Although the reaction stoichiometry appears simple, obtaining this stoichiometry in the laboratory is difficult.[15] The main difficulty is the number of oxidation–reduction reactions that occur simultaneously in the endoplasmic reticulum. These reactions use oxygen and NADPH and may yield water and $NADP^+$. When these diverse reactions have been accounted for, the predicted stoichiometry has been obtained.

It is recommended that the reader carefully follow the reaction sequence illustrated in Figure 4.2 during this discussion of the catalytic cycle. The initial step of the cycle is binding of the substrate (R–H) to P450 (Figure 4.2A).

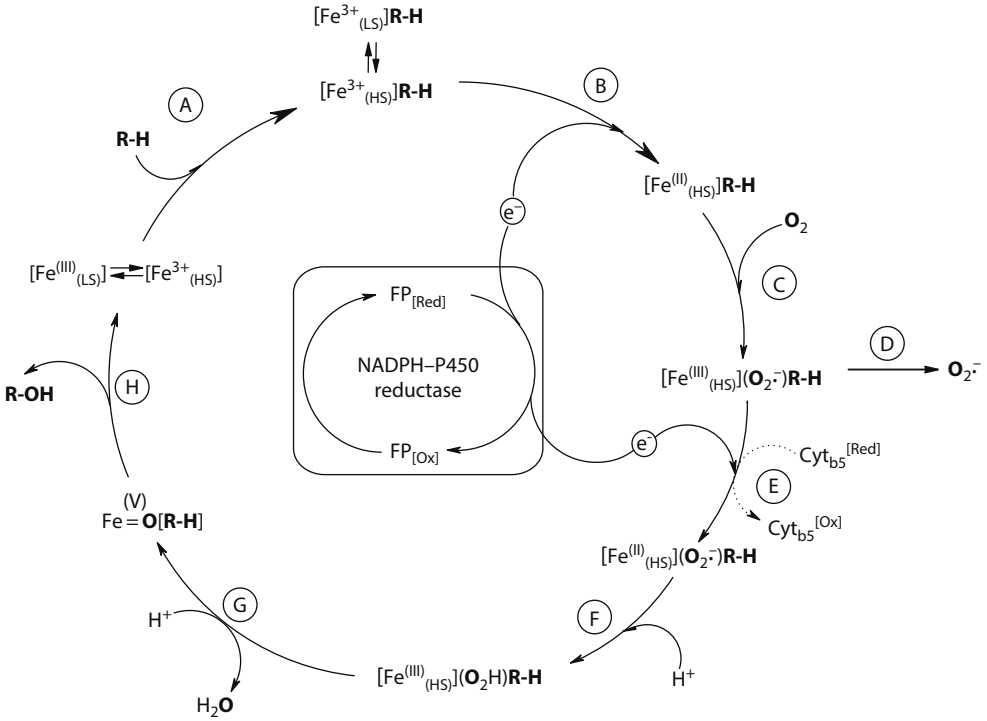


FIGURE 4.2 Catalytic cycle of cytochrome P450-dependent substrate oxidation. HS, high spin; LS, low spin; Cytb5, cytochrome B 5. See text for details. (Adapted from Testa, B., *The Metabolism of Drugs and Other Xenobiotics: Biochemistry of Redox Reactions*, Academic Press, London, U.K., 1995.)

As previously mentioned, P450 exists as a series of closely related isozymes, each of which demonstrates a degree of substrate specificity. This substrate specificity is not absolute, and overlapping is evident. At any one time, several isozymes of P450 exist in the endoplasmic reticulum. This is dependent on the specific genetic, environmental, and physiological conditions of the organism; therefore, binding of the substrate to the active site of P450 may represent binding to a single isozyme predominantly but not exclusively. The activity of the catalytic process (as well as the specific metabolites produced) is a function of the particular isozyme profile. Although our understanding of the structure of the active site of P450 is developing,[16,17] more remain to be learned. From the nature of the hemoprotein and its substrates, the active site contains the heme and a hydrophobic region. The substrate must have a specific orientation within the active site.

As occurs with many other enzymes, the binding of the substrate to the hemoprotein appears to produce conformational alterations in the enzyme that assist its catalytic activity; for example, substrate binding facilitates the reduction of P450 by NADPH–P450 reductase, in part, by lowering its redox potential. Binding of the substrate to the active site changes the absorption spectrum of the cytochrome. Because the oxidized heme iron is paramagnetic, electron paramagnetic resonance (EPR) can be applied to probe the environment of the iron in the heme. These studies have revealed alterations in the EPR signal that correlate with the blue shift in the Soret band from about 419 to 390 nm, observed when substrates bind the cytochrome. EPR and visible spectra changes result from the substrate binding in close proximity to the heme iron with a concomitant displacement of a water molecule from the iron. Substrate binding is energetically favorable and rapid, the heme is transformed from its low-spin form to the high-spin form, and the substrate is placed in close spatial proximity to the oxygen activation site on the heme. The relationships between the spin state of the cytochrome, interaction with the amino acids at the binding site, and substrate binding are more complex than described here. The reader is referred to discussions of changes in the spin state of P450 in Lewis,[16] Poulas and Raag,[14] Rein and Jung,[17] and Sligar and Murray.[18]

The next step in the catalytic cycle after substrate binding is the one-electron reduction of the substrate–P450 binary complex (Figure 4.2B). As mentioned, substrate binding and the concomitant alterations in P450 may facilitate this reduction step. The ferric (Fe^{3+}) hemocytochrome P450–substrate complex is reduced by a single electron to the ferrous (Fe^{2+}) hemocytochrome P450–substrate complex. This electron is provided by NADPH through NADPH–P450 reductase. This flavoprotein contains two flavins: flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). The flavoprotein appears to exist in its half-reduced (one-electron reduced) form and, upon reaction with NADPH, is fully reduced (two-electron reduction). The intramolecular electron flow appears to be from FAD to FMN. It is interesting that whereas the flavoprotein is a one-electron donor, its substrate, NADPH, provides two electrons. The mechanism for the two-electron shuttle by the one-electron donor flavoprotein is incompletely understood.[19]

NADPH–P450 reductase has at least two domains, one of which is imbedded in the endoplasmic reticulum membrane and the other above the plane of the membrane on the cytosolic side. The domain solubilized in the membrane consists mainly of hydrophobic amino acids. The actual interaction with NADPH and oxidation–reduction takes place outside the membrane. Another interesting aspect of P450 reductase is that the quantity of P450 is in large excess to the quantity of reductase (as much as 15- to 20-fold or more, depending on conditions). This means that each flavoprotein must reduce several P450 molecules, indicating that the interaction between the reductase and P450 is an important consideration, as discussed later.

Upon reduction of the ferric hemocytochrome P450–substrate binary complex to the ferrous state by the reductase, the enzyme binds oxygen (Figure 4.2C) to form a ternary complex. The oxygen binds at the free ligand of the heme iron and is believed to be oriented spatially with the substrate-binding portion of the active site. Uncoupling (interrupting the flow of electrons) the catalytic cycle at this point can produce the oxidized ferric P450 and a reduced form of oxygen, the superoxide radical (Figure 4.2D). Other reactive oxygen species can be generated by P450, including hydrogen peroxide and the hydroxyl radical. Generation of active oxygen species by P450 has been reviewed in Bernhardt.[19]

At this stage of the catalytic cycle, highly critical reactions take place that are still incompletely understood.[14] The major event is activation of the oxygen molecule. The ternary complex accepts a second electron required for the reaction (Figure 4.2E). The source of this electron can be either NADH or NADPH, depending on the mediator of electron transport. Because the purified, reconstituted system consisting of isolated P450, P450 reductase, and phospholipid requires only the presence of NADPH, NADPH–P450 reductase can mediate this step; however, as previously mentioned, in some systems, it appears that cytochrome b_5 can mediate the electron transfer employing reducing equivalents from NADH through NADH–cytochrome b_5 reductase. Whichever the source of the second electron, it results in the production of the peroxy P450–substrate complex, which has a net charge of −2 (Figure 4.2F). Of the variety of mechanisms proposed for oxygen activation and insertion into the substrate, two appear to be generally accepted. The first mechanism involves heterolytic cleavage of diatomic oxygen with the abstraction of hydrogen from the substrate and the insertion of oxygen into the substrate. The great majority of experimental evidence supports this mechanism,[20] particularly studies employing radical clock substrates.[21,22] Quite recently, direct spectroscopic evidence for the existence of the activated iron-oxo intermediate (compound I) has been obtained in CYP119 from *Sulfolobus acidocaldarius*, and its kinetic properties in oxygenating substrates have been defined.[23] The second more controversial mechanism involves homolytic cleavage, whereby two oxygen radicals are generated. Whatever the mechanism, one atom of this reactive oxygen is introduced into the substrate, whereas the other is reduced to water (Figure 4.2G). The oxidized substrate and water are released,

regenerating the oxidized ferric P450, which can again initiate the catalytic cycle (Figure 4.2H).

It must be emphasized that other pathways of electron transport in the endoplasmic reticulum can have significant impact on the catalytic activity of the monooxygenase by altering the availability of reducing equivalents. The interested reader is encouraged to consult other sources for a more comprehensive discussion of these pathways.[23,24]

This catalytic cycle is common to cytochrome P450-dependent monooxygenase activity associated with xenobiotic metabolism in a variety of organs and among different species; however, certain of these monooxygenases, especially the more specific forms associated with anabolic and catabolic metabolism, have different mediators of electron transport. For example, the adrenal cortex mitochondrial systems use a nonheme iron protein in addition to the P450 reductase in the electron transport chain, as does the monooxygenase system in certain microorganisms.[14,25]

The P450 system is not totally independent, and its activity is affected by a number of factors. One of these factors is the availability of reducing equivalents. The monooxygenase is primarily dependent on NADPH, as previously discussed, and possibly, to a lesser extent, on NADH. NADPH is generated from the pentose-phosphate shunt, isocitrate dehydrogenase, and the malic enzyme. Under most conditions, these pathways provide saturating levels of NADPH; however, certain conditions can compromise the ability of the cell to provide NADPH, and it may become rate limiting. Under conditions of high monooxygenase activity, starvation may reduce the activity toward certain substrates due to reduced levels of NADPH. It is generally believed that the decreased activity due to limiting NADH is an unlikely condition. A discussion of these and other factors that regulate monooxygenase activity can be found in Thurman.[26]

An additional factor that influences monooxygenase activity is the endoplasmic reticulum membrane. The asymmetric nature of the protein components of the system with respect to the membrane surface, coupled with the disproportionality of the concentrations of the components (i.e., a ratio of 1 to 15–20 between the flavoprotein and P450), indicates an interesting topology and interaction between the components. The membrane topology of the P450 system has been a topic of research for a number of years. The interaction between the protein components of the system and the interaction of these components with the lipid matrix of the membrane are important in the overall reactions of this system. P450 appears to be attached to the membrane of the endoplasmic reticulum by an anchor peptide at the NH_2-terminal end of the protein with the anchor peptide transversing the membrane. The active site, including the heme, is on the cytoplasmic side of the membrane. The active site portion is rich in alpha helix content, globular in nature, and not associated with membrane lipids. The area around the active site may be associated with the cytosolic surface of the membrane, providing a somewhat rigid character.

P450 appears to exist as multicomponent complexes of six P450 molecules clustered around a single P450 reductase. The NH_2-terminal regions on the opposite side of the membrane may interact to anchor this complex together. This allows for a catalytic advantage because of the close association of the components. This organization implies that each reductase would be capable of sequentially reducing several P450s. P450 may form a transient complex with the reductase that has an extremely short, non-rate-limiting half-life.[27,28]

Isozyme Heterogeneity and Substrate Specificity of Cytochrome P450

For many years, the apparent lack of substrate specificity of P450 intrigued investigators. It appeared that one of the major features of substrate specificity was lipid solubility. There appeared to be few other structural restraints for substrates. Intensive research on the nature of the hemoproteins has revealed that much of this apparent lack of substrate specificity results from the existence of multiple families and multiple subfamilies of P450 isozymes.

As the array of individual isozymes grew in number, nomenclature became increasingly problematic. It was sometimes difficult for investigators to know exactly which P450 they were working with because of inconsistencies in nomenclature. This led to attempts to develop a systematic nomenclature for the isozymes. P450 nomenclature has evolved from identifications based on spectral peaks to species-dependent nomenclature based on isolated and semipurified P450s to the current system, which is based on amino acid sequences that result from specific gene sequences.[29] P450 are now placed in families, which are further divided into subfamilies.

Names are based on the root CYP (derived from Cytochrome P450). The CYP is followed by a number identifying the gene family to which it belongs, such as CYP1, CYP2, and CYP3. The number for the gene family is followed by a letter denoting the subfamily to which the P450 belongs, such as CYP1A and CYP2A. The subfamilies are further defined by the addition of a number identifying the gene, such as CYP1A1 and CYP1A2; thus, P450 nomenclature is based on genetic relationships defined by protein and gene sequences. All P450s within a single family must exhibit a protein sequence similarity greater than 40%. P450s within the same subfamilies have sequence similarities greater than 55% within the same species. Subfamilies have sequence similarity that may be somewhat less than 55% when comparing species that are more distantly related. Members of subfamilies within a species appear to be located on a specific chromosome, and different subfamilies within a gene family may be clustered on the same chromosome.

As is generally found in biology, the classification system has exceptions resulting from P450s that do not fit the usual patterns. Although this nomenclature provides information on genetic and evolutionary relationships, it provides little information about substrate specificities and the reactions catalyzed by the different P450s. In fact, more is known

about the protein and gene sequences of many P450s than about their specific roles in metabolism. In the past decade, advances in molecular biology have driven an exponential increase in our ability to identify and sequence specific *CYP* genes, such that our knowledge of CYP primary structure has far outstripped our ability to define their specific roles in the metabolism of xenobiotics and endogenous compounds.

Different species may contain CYP genes or proteins that appear to be highly related; these are termed *orthologous genes* or *orthologs*. Orthologs are believed to have evolved from a single gene that existed before the two species diverged from a single species. Although these genes and their proteins may contain a high degree of sequence homology, it is not necessarily true that they share a catalytic similarity, or vice versa. A small change in an important amino acid sequence can result in a large change in the activity of a P450. Humans and rats have the CYP2 family and the CYP2D subfamily. The rat subfamily contains five genes, one of which is CYP2D1. This P450 has catalytic activity toward debrisoquine metabolism. The human P450 that has the highest catalytic activity toward debrisoquine is CYP2D6, which makes up less than 5% of the complement of P450 in the liver (Figure 4.3). Because these rat and human P450s have similar substrate specificity, it might be assumed that they have a high degree of sequence similarity, but this was found not to be the case. Therefore, even though these two isozymes have similar catalytic activity, they may have been derived from different ancestral genes. Sequence orthologs do not always predict similar catalytic activities. This is important for toxicologists who extrapolate toxicity from animal models to humans, as noted later.

The section that follows provides a brief description of the major P450 families involved in xenobiotic metabolism. Those families predominately involved in metabolism of endogenous substrates have been excluded. For a more complete discussion of the P450 families, the reader is referred to the excellent reviews in Ioannides,[30] Lewis,[31] and Smith et al.[32]

CYP1 Family

The CYP1 family contains two subfamilies of P450s: CYP1A and CYP1B. CYP1A has been much more extensively characterized than the CYP1B subfamily.

CYP1A Subfamily

The CYP1A subfamily contains CYP1A1 and CYP1A2, which appear to occur in all mammals. These two P450s may have been derived from a common ancestor approximately 120 million years ago. Both P450s are important in the metabolism of environmental xenobiotics. Historically, these two P450s were known collectively as cytochrome P448, due to their characteristic CO binding spectrum. Although these hemoproteins share a number of physiochemical characteristics, such as similar primary structures, they demonstrate different substrate specificities. For example, CY1A1 is highly active in the metabolism of planar polycyclic aromatic hydrocarbons (PAHs) such as benzo(*a*)pyrene (BP) (Figure 4.4), whereas CYP1A2 is active in the metabolism of acetanilide, caffeine, and other aromatic and heteroaromatic amines.[33,34]

Both CYP1A1 and CYP1A2 also play a role in catabolism of estrogens,[35,36] and CYP1A1 may be involved in heme catabolism.[31] Although low levels of CYP1A1 have been detected in liver, this isoform is constitutively expressed primarily in extrahepatic tissues, including lung, kidney, gastrointestinal tract, and breast. In contrast, expression of CYP1A2 is found primarily in liver, where it accounts for approximately 15% of total hepatic CYP (Figure 4.3). Both CYP1A isoforms are highly inducible by planar polycyclic aromatic compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene, and their induction is mediated by the aryl hydrocarbon (Ah) receptor. In addition, isosafrole

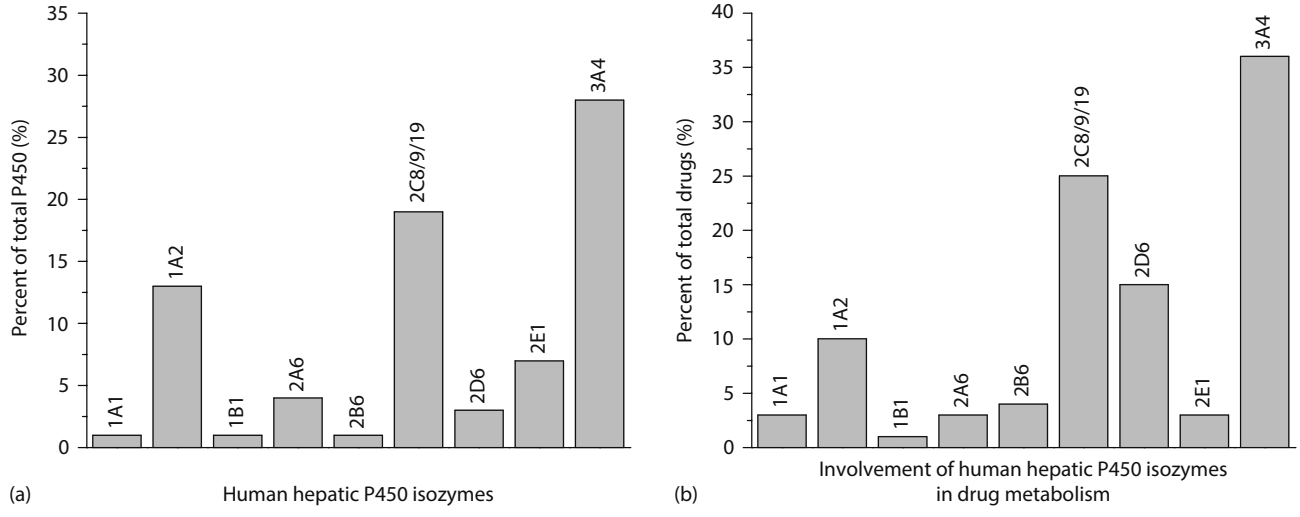


FIGURE 4.3 Cytochrome P450 isoforms and xenobiotic metabolism: (a) Hepatic distribution of P450 isoforms as a percentage of total hepatic P450. (b) Distribution of isozymes involved in drug metabolism as a percentage of the total number of drugs investigated. (Data from Jakoby, W.B., Glutathione S-transferases: Catalytic aspects, in Arias, I.M., Jakoby, W.B., eds. *Glutathione Metabolism and Function*, Raven Press, New York, 1976, pp. 189–211.)

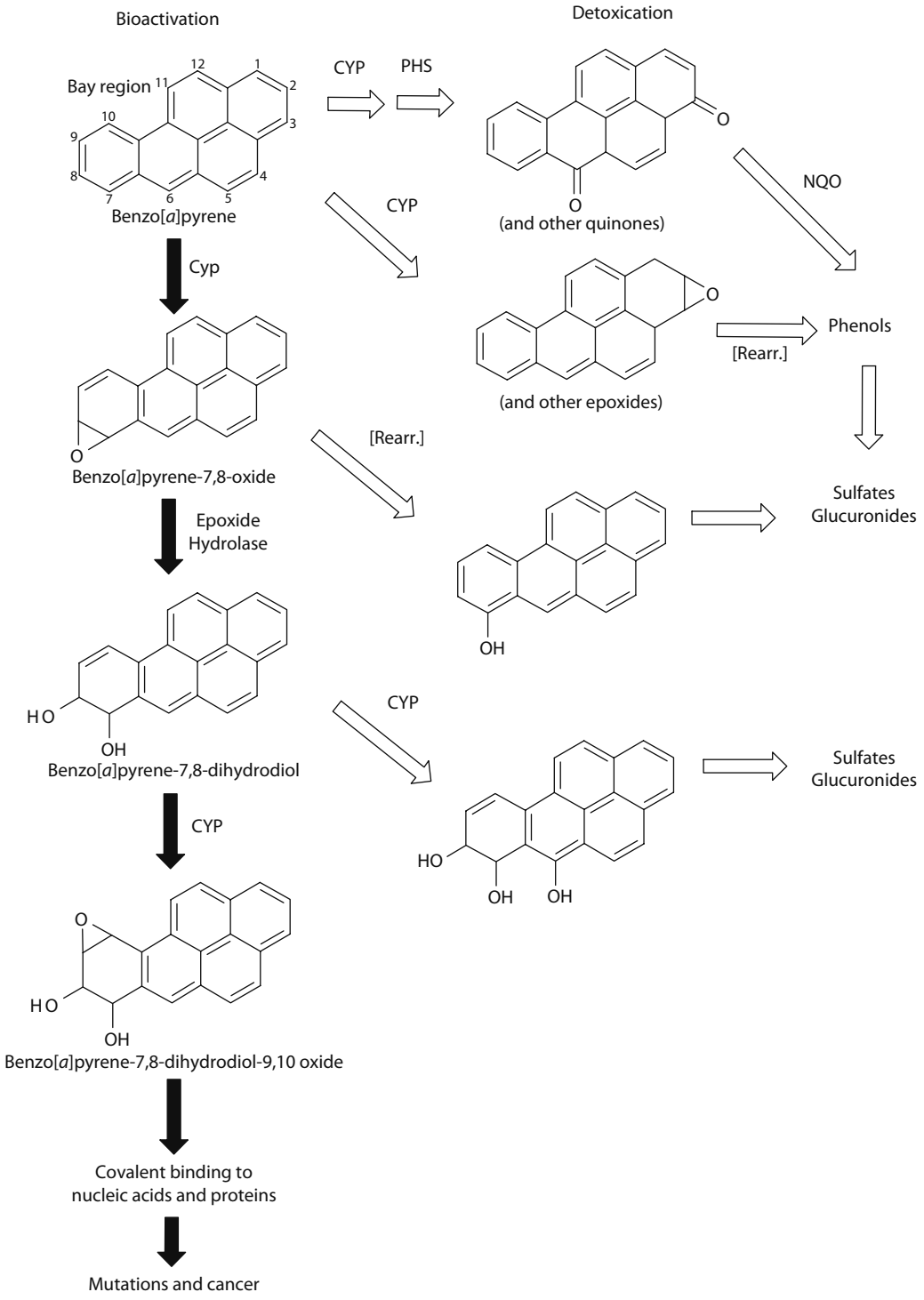


FIGURE 4.4 Bioactivation and detoxification of the carcinogen BP. Bioactivation pathways are indicated by filled arrows; detoxification pathways are indicated by open arrows. PHS, prostaglandin H synthase; NQO, NAD(P)H:quinine oxidoreductase.

is a specific inducer of CYP1A2. The O-dealkylation of ethoxyresorufin has been used as a functional marker for CYP1A1 activity in vitro, while ^{3}N-demethylation has been used as an in vivo marker for CYP1A2 activity. A variety of CYP1A1 inhibitors have been characterized, including furocoumarins,[37] flavonoids,[38] and acetylenic aromatic hydrocarbons.[39] However, the specificity of these inhibitors for CYP1A1 remains to be demonstrated. Furafylline has been used as a selective inhibitor of CYP1A2.[40] For a number of years, there has been an interest in the role of CYP1A1 in the metabolic activation of polycyclic hydrocarbons, such as BP.

Although many of the investigations on the role of CYP1A1 in the activation of PAHs have been done in animals with induced CYP1A1, these compounds may be

metabolized by other CYPs in uninduced animals. Because its concentration in the liver is low, CYP1A1 may be more important in the metabolic activation of polycyclics in extrahepatic tissues, such as the lung. In humans, both CYP1A1 and CYP1A2 demonstrate genetic polymorphism (discussed later), with at least 15 variants of CYP1A2 reported to date.[34] CYP1A2 is active in the metabolism of a wide variety of drugs and environmental contaminants in humans. Good substrates for CYP1A2 tend to be relatively planar molecules containing one or more hydrogen bond donor/acceptors, which play a significant role in the regioselectivity of substrate oxidation.[31] CYP1A2 has been shown to be associated with the mutagenic activation of aromatic and heterocyclic amines, such as 4-aminobiphenyl, 2-aminonaphthalene, and 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline (MeIQ). It also O-dealkylates phenacetin and 4-hydroxylates acetanilide. Although CYP1A2 does play a major role in the bioactivation of aromatic and heteroaromatic amines, other CYP isoforms, notably CYP1A1, CYP1B1, and CYP3A4, can also contribute to the mutagenic activity of this chemical class.[32]

CYP1B Subfamily

CYP1B1 is the only known member of the CYP1B subfamily, which was identified and characterized in the mid-1990s.[41,42] CYP1B1 has been identified in mouse, rat, and human tissues. Expression of CYP1B1 mRNA has been detected in a number of normal human tissues including liver, kidney, gastrointestinal tract, endometrium, breast epithelium, and brain[43]; however, expression of the functional protein in normal human tissues has been much more difficult to demonstrate.[43] Interestingly, CYP1B1 has been shown to be highly expressed in a number of human tumors,[44] suggesting potential roles as a tumor biomarker or a chemotherapeutic target.[45] CYP1B1 is regulated by both Ah-dependent and Ah-independent mechanisms (addressed later) and thus is inducible by TCDD[46] and PAHs, as well as by estradiol.[47] Recombinantly expressed human CYP1B1 is capable of activating a variety of PAHs, aromatic amines, and heterocyclic amines to mutagenic metabolites, as demonstrated by the *Salmonella* (Ames) mutagenesis assay.[48] In addition, CYP1B1 is active in the metabolism of estrogens, particularly 4-hydroxylation of estradiol, which is the most specific functional assay for this isoform demonstrated to date. 4-Hydroxyestradiol is carcinogenic in male hamsters and may play a role in estrogen-induced tumorigenesis. The potential of CYP1B1 as a chemotherapeutic target has fueled interest in the discovery and development of specific inhibitors. Although a number of highly potent inhibitors have been identified, realization of sufficient selectivity for CYP1B1 remains a challenge.[49] Recently, 2,3′,4,5′-tetramethoxystilbene has been used as a specific inhibitor of CYP1B1 for elucidation of the role of this isoform in salt-sensitive hypertension.[50]

CYP2 Family

The CYP2 family contains a number of subfamilies important in xenobiotic metabolism, including CYP2A, CYP2B, CYP2C, CYP2D, and CYP2E.

CYP2A Subfamily

The CYP2A subfamily contains at least 12 members that differ in their substrate specificity, tissue distribution, and response to inducers and inhibitors. CYP2A1, CYP2A2, and CYP2A3 are rat P450s; Cyp2a4, Cyp2a5, and CYP2a12 are found in mice; humans express CYP2A6 and CYP2A7. Rat CYP2A1, along with CYP2A2, hydroxylates testosterone, progesterone, and androstenedione. CYP2A1 and CYP2A2 can also metabolize aminopyrine, benzphetamine, ethylmorphine, aniline, acetanilide, and *N*-nitrosodimethylamine. CYP2A1 has low activity toward 3-hydroxylation of BP and 7-ethoxycoumarin O-deethylation and does not metabolize 7-ethoxyresorufin. CYP2A1 occurs in liver and testis, but not kidney and lung. In adult rats, it predominates in females and appears to be under endocrine control. CYP2A3 appears to be lung specific, and its substrate specificity has not been well characterized. Human CYP2A6 demonstrates coumarin 7-hydroxylase activity but no activity toward testosterone, in contrast to rat CYP2A1 and CYP2A2. Moreover, it has no activity toward probe substrates such as 7-ethoxyresorufin, 7-benzyloxyresorufin, ethylmorphine, and testosterone.

Typical substrates for CYP2A6 include both planar and nonplanar molecules, and the preferred site of oxidation is generally within six topological steps of a hydrogen bond acceptor. In addition to coumarin, other substrates for CYP2A6 include nicotine, cotinine, acetaminophen, and methoxyflurane.[31] Coumarin 7-hydroxylation is commonly used as a functional marker for CYP2A6 activity, and inhibitors include pilocarpine and 8-methoxypsoralin. In primary human hepatocytes, CYP2A6 can be induced by phenobarbital (PB) and rifampicin, and 7-hydroxylation of coumarin was found to be accelerated in patients taking anticonvulsant drugs.[51] CYP2A6 accounts for approximately 4% of hepatic P450 (Figure 4.3) and is also expressed in skin[52] and respiratory tract,[53] where it may play a role in activation of tobacco-specific carcinogens. Initially thought to be monomorphic, recent data indicate that CYP2A6 is a polymorphic enzyme and that polymorphism of this enzyme may be a determinant of lung cancer risk in smokers,[54] although other authors have not observed this association.[55] Interest in this potential association continues to be fueled by links between CYP2A polymorphism and susceptibility to tobacco-related lung tumors observed in animal models.[56] As mentioned previously, studies in human microsomes have indicated that this P450 may play a role in the metabolic activation of a number of nitrosamines and possibly in the activation of aflatoxin B_1 (AFB_1) and 1,3-butadiene to carcinogenic epoxides.

CYP2B Subfamily

This subfamily contains P450s such as rat CYP2B1 and rat CYP2B2 that are highly induced by PB. Hydroxylation of testosterone at the 16β position is used as a specific substrate probe for these isoforms in rats. Other substrates for this subfamily include benzyloxyresorufin, ethoxycoumarin, and pentoxyresorufin. In rats, CYP2B1 has been detected in the lung, adrenal gland, testis, and brain, whereas CYP2B2 occurs in liver and brain. Although its role in xenobiotic

toxicity has not been thoroughly investigated, the CYP2B subfamily can metabolically activate xenobiotics, such as bromobenzene, carbon tetrachloride, BP, AFB_1, and some nitrosamines in the rat. Although the rat P450s in the CYP2B subfamily have been studied for a number of years, members of the CYP2B subfamily in humans have received far less attention.

Estimates of constitutive expression of CYP2B6 in human liver are variable, ranging from as low as 0.2% of total hepatic P450[57] up to 6% of total microsomal CYP protein.[58] In addition to liver, CYP2B6 has been detected in a variety of extrahepatic tissues, including various regions of the respiratory tract,[53] skin,[52] kidney, brain,[59] placenta, and endometrium.[57] CYP2B6 catalyzes oxidation of a variety of drugs and other xenobiotics, including several coumarin derivatives, methoxychlor, bupropion, benzyloxyresorufin, and benzphetamine.[31,60] Although debenzylation of 7-benzyloxyresorufin has been used as a marker for CYP2B6 activity, the selectivity of this appears to be questionable[57]; rather, N-demethylation of *S*-mephenytoin and ring hydroxylation of the phosphodiesterase inhibitor RP 73401 appear to be more selective probes for CYP2B6. Orphenadrine and 9-ethynylphenathrene have been used as inhibitors, although the former appears to lack selectivity for this isoform. CYP2B6 is inducible by PB, as well as classic CYP3A inducers such as dexamethasone and rifampicin, but a selective inducer of CYP2B6 has not been identified. Although CYP2B6 contributes to the metabolism of approximately 10% of marketed drugs, there is little evidence for significant drug–drug interactions (DDIs), reflecting the considerable overlap in substrate specificity of this isoform with other CYPs.[60]

CYP2C Subfamily

At least eight members of the CYP2C subfamily have been identified in rats, and P450s in this subfamily are expressed in both hepatic and extrahepatic tissues. Expression of rat CYP2C isoforms is gender specific, in contrast to humans. Four functional CYP2C subfamily members have been identified in humans: CYP2C8, CYP2C9, CYP2C18, and CYP2C19. Together, isoforms of the CYP2C subfamily are estimated to account for metabolism of approximately 20% of prescribed drugs in humans. All four genes are expressed in liver and small intestine, and CYP2C8, CYP2C9, and CYP2C18 mRNA have been detected in a variety of extrahepatic tissues. To date, CYP2C18 protein has not been detected in any tissue examined. Marker activities for CYP2C8 include paclitaxel 6α-hydroxylation and N-dealkylation of amiodarone, and both of these compounds have been used as probe substrates for these isoforms.[61] Relatively selective competitive inhibitors of CYP2C8 include gemfibrozil and montelukast. Moreover, gemfibrozil coadministration results in DDIs with a number of drugs metabolized by CYP2C8, including rosiglitazone, piogitazone, repaglinide, loperamide, and cerivastatin.[62] CYP2C8 is inducible by the prototypical enzyme inducers rifampicin, PB, and dexamethasone and is one of the most inducible members of the CYP2C subfamily.[63]

Polymorphism of CYP2C8 was first reported in 2001,[64] and since this time, a variety of variant alleles have been described. One of these variants, CYP2C8*5, has been associated with the development of rhabdomyolysis following administration of cerivastatin. Although several CYP2C8 polymorphisms result in pharmacokinetic changes for a number of clinically relevant drugs, evidence for adverse consequences is lacking. CYP2C9 is quantitatively the most important member of the human CYP2C subfamily in liver, second only to CYP3A4.[65] CYP2C9 oxidizes a wide variety of clinically important drugs, many of which contain weakly acidic groups. Among these, warfarin and tolbutamide have been used as probe substrates for this P450. CYP2C9 also catalyzes S-oxidation of tienilic acid, resulting in bioactivation of this compound to a reactive electrophile. Benzbromarone derivatives and sulfaphenazole are used as high-affinity selective inhibitors of CYP2C9.

In common with other members of this subfamily, CYP2C9 is polymorphic, and it has been estimated that up to 40% of Caucasians carry at least one variant CYP2C9 allele.[66] Because of its prominent role in the clearance of several low therapeutic index drugs such as warfarin and phenytoin, polymorphisms of CYP2C9 represent a significant challenge in the development of drugs metabolized by this isoform.[67] CYP2C19 catalyzes oxidation of a number of different drug classes, including proton pump inhibitors, antidepressants, and benzodiazepines. *S*-Mephenytoin is a prototypical substrate for CYP2C19, and 4′-hydroxylation of this compound is frequently used as a selective marker activity. Both omeprazole and ticlopidine have been used as inhibitors of CYP2C19, although the selectivity of these compounds has been questioned. Recently, *N*-benzylnirvanol has been shown to be a potent and selective inhibitor of CYP2C19 suitable for diagnostic purposes.[68] Induction of CYP2C8, CYP2C9, and CYP2C19 has been observed following treatment with rifampicin, and the former two isoforms are also induced by PB. Like other members of the human CYP2C subfamily, CYP2C19 is polymorphic, and at least eight allylic variants have been described. Although adverse drug reactions have been observed following coadministration of CYP2C19 substrates and inhibitors, a recent extensive review failed to find convincing evidence of adverse drug reactions related to CYP2C19 polymorphisms.[69]

CYP2D Subfamily

Rats have six members in the CYP2D subfamily, whereas three have been identified in humans: CYP2D6, CYP2D7, and CYP2D8. Mice have five CYP2D members, and this subfamily has been identified in other mammals. Human CYP2D6 is the most important member of the subfamily for xenobiotic metabolism in humans and was the first human P450 shown to be polymorphic. CYP2D6 is expressed in liver, lung, small intestine, and skin.[52,53] CYP2D6 accounts for less than 5% of total hepatic P450 in humans (Figure 4.3) but is estimated to participate in metabolism of approximately 20% of clinical drugs.[70] Substrates for CYP2D6 are relatively lipophilic and contain a basic amine group, and hydroxylation

generally occurs within 5–7 Å of the basic nitrogen.[71] Typical substrates include debrisoquine, dextromethorphan, and tricyclic antidepressants. The O-demethylation of dextromethorphan has been used as a functional marker for CYP2D6 activity. Numerous alkaloids are potent ligands for CYP2D6, and quinidine has been used as a selective inhibitor of this activity. Reactions catalyzed by CYP2D6 range from aryl hydroxylation to N- and O-dealkylation.

CYP2E Subfamily

The CYP2E subfamily is one of particular interest to toxicologists, due to its involvement in the metabolism and bioactivation of a wide variety of industrial and environmental chemicals. Currently, CYP2E1 is the only member of this subfamily in rats, mice, and humans. CYP2E1 appears restricted to mammals and may have evolved more recently than certain other gene families. It is expressed in liver and kidney and occurs at low levels in a number of other tissues including lung, skin, esophagus, and small intestine.[52,53] Although it normally represents less than 10% of the total P450 in human liver, it is induced by a broad array of its substrates. Its hepatic concentration can vary up to 50% between different humans. CYP2E1 is highly conserved across species, and rodent and human forms of CYP2E1 share many similarities, including similar substrate specificities. It is known to metabolize more than 70 different chemicals with diverse structures.

Structural requirements for CYP2E1 substrates appear limited to small molecules with hydrophobic character.[31] CYP2E1 does not appear to be active in the metabolism of many drugs but does metabolize a wide array of alcohols, aldehydes, alkanes, aromatic hydrocarbons, ethers, fatty acids, halogenated hydrocarbons (including anesthetics), heterocyclics, and ketones. Aniline hydroxylation and *p*-nitrophenol hydroxylation have been used as marker activities for CYP2E1, but both substrates are oxidized by other isoforms of P450. More recently, 6-hydroxylation of the muscle relaxant chlorzoxazone has been used as a diagnostic activity for CYP2E1. Although the selectivity of CYP2E1 for this substrate is not absolute, this reaction has become the marker of choice for CYP2E1 activity.[72] Carbon-tetrachloride-dependent lipid peroxidation has also been used to follow CYP2E1 activity in vitro. CYP2E1 can be competitively inhibited by many of its substrates, and a number of sulfur-containing compounds, including disulfiram and diallyl sulfate, have been shown to be mechanism-based inhibitors (metabolism dependent).[73] As noted previously, CYP2E1 is induced by many of its substrates, including ethanol, and it has been suggested that chronic alcoholics may be more sensitive to chemicals that undergo CYP2E1-mediated bioactivation than nonalcoholics.

Interest in the role of CYP2E1 as a mediator of toxicity comes from two of its actions. First, it is known to be important in the metabolic activation/detoxification of a number of carcinogens and heptatoxicants. Second, it may have an important role in free-radical production and oxidative stress.[74] For example, CYP2E1 is believed to be involved in the metabolic activation associated with the carcinogenicity of benzene, butadiene, nitrosamines, and azoxymethane, as well as the hepatotoxicity of nitrosamines, acetaminophen, halothane, and enflurane.

With respect to free-radical production, CYP2E1 is involved in the formation of a reactive hydroxyethyl radical produced during its metabolism of ethanol to acetaldehyde. This hydroxy radical is believed to play a role in ethanol-related liver damage. It also appears to be involved in the production of a trichloroethyl radical produced by chlorine removal during the metabolism of carbon tetrachloride. This radical may initiate membrane lipid peroxidation associated with carbon-tetrachloride-induced hepatotoxicity. An additional mechanism by which CYP2E1 could produce reactive radicals is associated with its potential for futile cycling in the absence of substrate. CYP2E1 appears more loosely coupled than some of the other P450s. Oxygen activation during the catalytic cycle in the absence of substrate results in the production of highly reactive hydroxyl radicals, superoxide anions, and hydrogen peroxide. Indeed, cells that constitutively overexpress CYP2E1 exhibited a 40%–50% increase in generation of reactive oxygen species compared to wild-type cells in the absence of substrate.[74] If these reach concentrations that overcome cellular protection mechanisms, they may initiate oxidative stress leading to tissue damage.

P450s are not evenly expressed in the liver but occur in specific zones; for example, oxygen tension varies significantly across the hepatic lobule, ranging from ~13% in the periportal region to ~4% in the centrilobular region, and this gradient is thought to be an important determinant of CYP expression.[75] The highest concentration of P450 is generally found in a layer surrounding the terminal hepatic venules. This is especially true for induced CYP2E1. Enhanced CYP2E1 activity in the centrilobular region appears related to the centrilobular necrosis produced by hepatotoxicants, such as ethanol, carbon tetrachloride, benzene, nitrosamines, and acetaminophen. It may appear that CYP2E1 is predominately involved with metabolic activation; however, this is not necessarily true. As noted before, P450-mediated xenobiotic metabolism is generally associated with the production of less toxic metabolites, but in some cases, more toxic metabolites are produced. CYP2E1 is no exception to this rule and participates not only in metabolic activation but also in detoxification.

CYP2F Subfamily

Isozymes of the CYP2F subfamily have been identified in humans, nonhuman primates, rodents, and ruminants.[76] Unlike other members of the CYP2 family, only a single isoform of CYP2F has been identified in each of these species. CYP2F is expressed almost exclusively in lung with very little expression in liver. The human form, CYP2F1, has been shown to be involved in bioactivation of a number of environmental toxicants, including 3-methylindole, naphthylene, styrene, dichloroethylene, and benzene, and the cellular localization of CYP2F1 correlates with the site of injury of these toxicants.[77] Thus, this isoform may play a

significant role in the pneumotoxicity of inhaled xenobiotics. To date, no selective substrates for CYP2F1 have been identified; however, the pneumotoxicant 3-methylindole has been found to be a selective mechanism-based inactivator of this P450. No data on the inducibility of CYP2F isoforms have been published.

CYP3 Family

The CYP3 family of P450s includes CYP3A1, CYP3A2, CYP3A9, CYP3A18, and CYP3A62 in rats; CYP3a11, CYP3a13, CYP3a16, CYP3a25, and CYP3a44 in mice; and CYP3A3, CYP3A4, CYP3A5, and CYP3A7 in humans, along with others from rabbits, dogs, and other species. The CYP3 family contains P450s that are important in the metabolism of many xenobiotics, especially drugs.

CYP3A Subfamily

This subfamily contains at least four genes in humans, CYP3A3, CYP3A4, CYP3A5, and CYP3A7. Together, these isoforms constitute approximately 30% of total hepatic P450 and are estimated to mediate metabolism of around 50% of prescribed drugs, as well as a variety of environmental chemicals and other xenobiotics. CYP3A4 is the major form of P450 expressed in human liver. It is also the major P450 expressed in the human gastrointestinal tract, and intestinal metabolism of CYP3A4 substrates can contribute significantly to first-pass elimination of orally ingested xenobiotics. For example, the immunsuppressive agents cyclosporine and tacrolimus undergo significant metabolism by human intestinal microsomes, and significant species differences in intestinal metabolism of the compounds have been observed.[78] Small amounts are found in several other organs, such as the kidney and skin. X-ray crystallography studies have demonstrated that CYP3A4 has a cavernous active site, allowing it to oxidize very large substrates such as erythromycin (MW = 734) and cyclosporin A (MW = 1203).[79] In addition, the large active site allows for simultaneous binding of multiple ligands and is thought to account for homotropic (same ligand) and heterotropic (different ligands) cooperativity in substrate oxidation.[80] This cooperativity is thought to be responsible for the non-Michaelis–Menten (sigmoid) enzyme kinetics observed for some CYP3A4 substrates such as nifedipine.[78] As such, CYP3A isozymes do not demonstrate a high degree of structural selectivity with respect to their substrates, and the substrate selectivity of CYP3A4 has been difficult to generalize.

Prototypical substrates include erythromycin and midazolam, both of which have been used as probes for CYP3A4 activity. Of significance to toxicologists, CYP3A isoforms are also capable of metabolically activating carcinogens, such as AFB_1 and BP. CYP3A4 is also responsible for bioactivation of naturally occurring toxins sometime found in herbal medicine. An example is epoxidation of the hepatotoxic diterpenoids teucrin A and teuchamaedryn found in germander.[81] Moreover, polymorphisms in CYP3A isoforms may play a role in interindividual differences in toxicity of drugs with narrow therapeutic indices, such as tacrolimus and other immunosuppressive agents.[82] A number of selective mechanism-based inhibitors for CYP3A4 have been identified, and ketoconazole is frequently used for this purpose. Ritonavir is another inhibitor of CYP3A4 and is used as a pharmacoenhancer with antiretroviral agents to boost plasma concentrations at lower administered doses. CYP3A4 and other members of the CYP3A subfamily are induced by a number of drugs, including rifampicin, PB, and phenytoin. Paradoxically, ritonavir is also an inducer of CYP3A4 and other drug-metabolizing enzymes.[83] Because of the large number of drugs metabolized by CYP3A4, it frequently plays a role in a number of DDIs that may result in adverse effects, and this has become an important factor in the development of therapeutic agents.

An example of how dietary constituents can affect specific isozymes is provided by the interaction between the consumption of grapefruit juice and CYP3A4. Consumption of grapefruit juice can cause an increase in the oral availability of a number of drugs that are CYP3A4 substrates. Increased bioavailability is produced by inhibition of intestinal CYP3A4 activity by 6′,7′-dihydroxybergamottin, which is a component of grapefruit juice. This dietary compound is a mechanism-based inhibitor of CYP3A4 that results in the rapid partial loss of CYP3A4 activity.[84] Inhibition of metabolism of the CYP3A4 substrates during their intestinal absorption accounts for the higher than anticipated plasma concentrations of the drugs.

Other CYP3A Isoforms

CYP3A5 is the most extensively studied of the minor isoforms in this subfamily. This P450 may be polymorphically expressed in humans (discussed in the following). It does not appear to have the broad substrate specificity of CYP3A4 and has lower activity. CYP3A5 phenotype did have any significant effect on in vivo metabolism of a variety of CYP3A4 substrates, including midazolam, nifedipine, cyclosporin A, and docetaxel.[85] However, clearance of tacrolimus was more rapid in individuals expressing CYP3A5, suggesting that this isoform may be active in drug metabolism in humans. CYP3A7 is active in the metabolism of steroids and retinoids, but a role in xenobiotic metabolism has not been demonstrated.

Other P450s

A large number of P450 families and subfamilies have not been discussed here. Most of these are involved with the metabolism of endogenous substrates or occur in species that are beyond the scope of the topic of this chapter; see Ioannides[30] and Lewis[31] for a more complete discussion.

Role of the Cytochrome P450-Dependent Monooxygenase in Toxicity

The toxicity of any agent is dependent on its concentration at its target site. This is a function of many factors, including the route of exposure, the pharmacokinetics of the xenobiotic, the excretion of both the parent compound and its

metabolites, and the sensitivity of the target site. The ability of the organism to clear the xenobiotic through excretion will have a profound influence on the concentration at the target site. Directly associated with the ability to clear many xenobiotics is the ability to metabolize the xenobiotic to more water-soluble metabolites.

Without doubt, the P450-dependent monooxygenase plays a pivotal role in the metabolism of xenobiotics. It is the prime metabolic route for the majority of xenobiotics, acting either directly in detoxification or indirectly by priming the xenobiotic for further metabolism through functionalization, as illustrated in other sections of this chapter.

The original interest in the P450 system was associated with its ability to metabolize drugs and decrease both their toxicity and duration of action. It soon became evident that in certain cases, this enzyme system converted certain drugs from pharmacologically inactive forms to active forms. Examples of the metabolic activation of toxicants, such as the in vivo conversion of the inactive insecticide parathion to its active form, paraoxon, were soon encountered. It was also discovered that this enzyme system could activate stable molecules such as BP to highly reactive metabolites capable of damaging cellular macromolecules, as shown in Figure 4.4. Further studies have indicated that metabolic activation plays an important role in the toxicity of a number of xenobiotics. Recently, an extensive review of bioactivation has been conducted, and a compendium of bioactivation reactions has been published.[86]

Studies undertaken to understand the biochemistry of P450 played a large role in the development of the modern fields of biochemical and molecular toxicology. Currently, much effort is being placed on the determination of the balance between metabolite activation and detoxification and the detoxification of activated metabolites. This is providing new insight for toxicologists seeking to understand the toxicity of xenobiotics. Studies on the active sites of P450 and other xenobiotic metabolism enzymes and the factors that influence their activity and their expression are bringing toxicologists closer to being able to predict potential toxicity with more accuracy. These efforts are also aiding toxicologists in the difficult task of predicting human toxicity from studies done with cellular and animal models.

Reactions Catalyzed by the Cytochrome P450-Dependent Monooxygenase System

On first inspection, it appears that P450 can catalyze a bewildering number of reactions (Table 4.2); however, on closer inspection, a degree of commonality exists among these reactions. The first area of commonality is that most of the reactions represent oxidations. Second, the reactions convert lipophilic substrates to more hydrophilic products. Third, many of the reactions can be understood as hydroxylations. For a detailed review of P450 reactions, see Testa[90] and Guengerich.[88] Representative examples of the various reactions catalyzed by P450s are illustrated in Figure 4.5.

Aliphatic Hydroxylation

Aliphatic hydroxylation may be thought of as a special case of the oxidation of an sp^3-hybridized carbon atom, and examination of aliphatic hydroxylation reactions is illustrative of several important aspects of monooxygenase activity. Hydroxylation of aliphatic carbon atoms represents one of the most common reactions in phase I metabolism of xenobiotics. The reaction mechanism, which may be common to several other types of monooxygenase metabolism, appears to occur by a hydrogen (or electron) abstraction mechanism (Figure 4.6). Oxygen activation produces a $[FeO]^{3+}$ at the heme of P450. Hydrogen abstraction from the substrate results in production of the carbon-centered radical. This radical interacts with activated oxygen (through

TABLE 4.2
Distribution of Reaction Types Catalyzed by Major Human P450s Involved in Xenobiotic Metabolism

Reactions	1A1	1A2	2C9	2C19	2D6	2E1	3A4
N-dealkylation	19	24.4	19.8	23.6	23.8	15.2	26.5
O-dealkylation	9.8	10	8.8	11.8	22.7	8.3	9.6
S-oxidation	3.4	3.4	3.9	3.9	6.5	2.3	5.8
Aromatic hydroxylation	25.9	25.2	23.7	21.4	24.5	21.2	12.1
Aliphatic hydroxylation	13.7	13.2	24	27.9	11.9	24.9	24.6
N-oxidation	6.8	7.2	4.9	2.6	5.4	6.0	6.5
Nitro reduction	0.5	1.1	0.4	0.9	0	0.5	1
Peroxidation	14.1	9.7	7.1	4.8	0.7	14.7	3.4
Hydroxycarbonyl oxidation	2.9	2.3	4.2	1.3	2.5	4.6	4.1
Desaturation	2.4	2.9	3.2	1.3	1.4	1.4	5.6
Aldehyde oxidation	1.5	0.6	0	0.4	0.4	0.9	0.9
Total reactions (%)	9.6	16.3	13.2	10.7	12.9	10.1	27.3

Source: Adapted from Lewis, D.F.V., *Pharmacogenomics*, 5(3), 305, 2004.

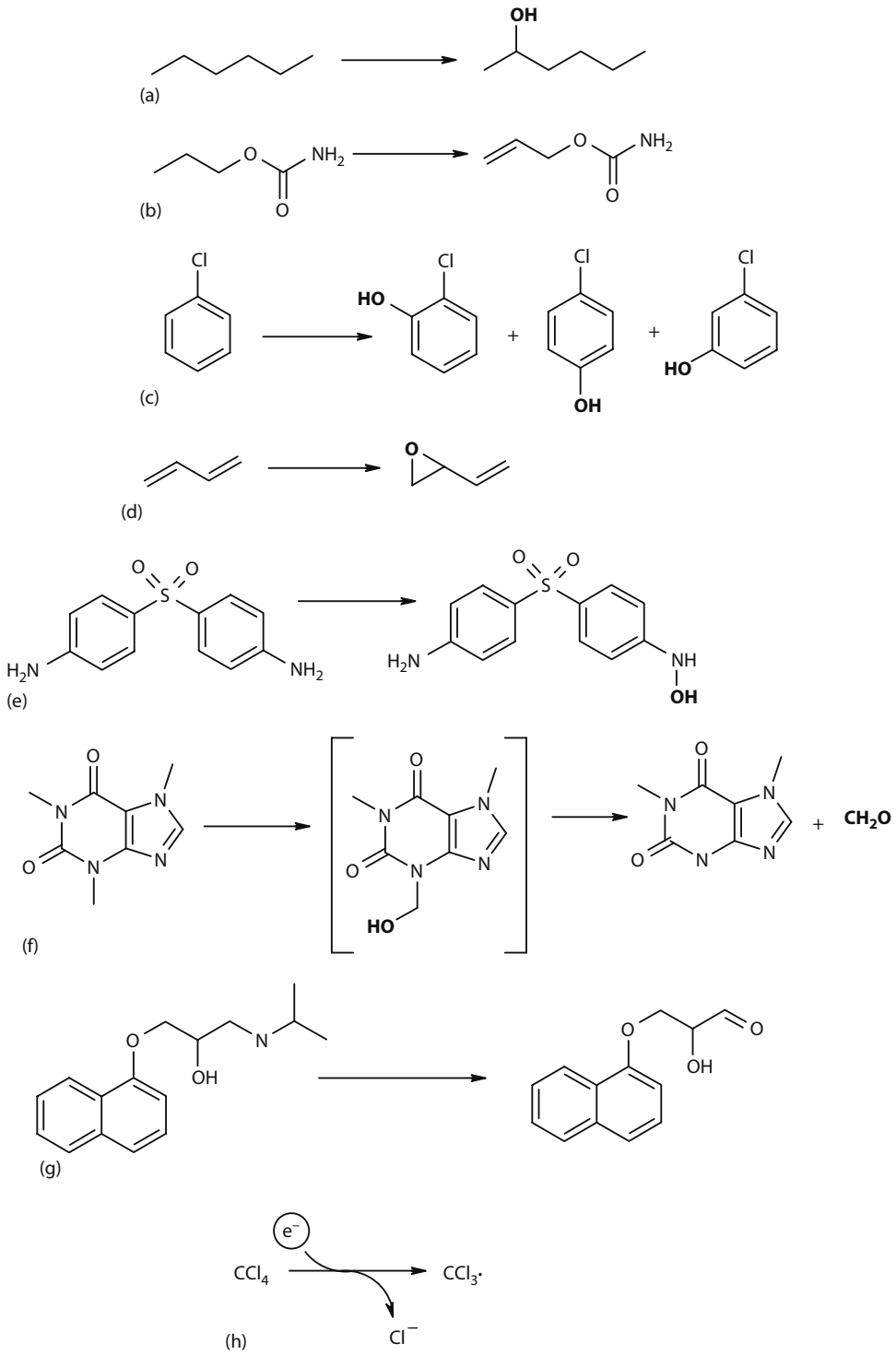


FIGURE 4.5 Examples of reaction types catalyzed by P450: (a) aliphatic hydroxylation, (b) desaturation, (c) aromatic oxidation (hydroxylation), (d) epoxidation of alkenes, (e) N-hydroxylation, (f) heteroatom dealkylation (N-dealkylation), (g) oxidative deamination, (h) reductive dehalogenation.

oxygen rebound) to yield hydroxylation. Other reactions, such as O-dealkylation of ethers and carboxylic acid esters, may proceed through this mechanism with decomposition of unstable hydroxylation products. Hydrogen abstraction is site selective, resulting in a nonrandom hydroxylation. The specific hydroxylation site is determined by structure and the specific spatial orientation of the substrate at the active site. Different isozymes of P450 show different degrees of site selectivity. For example, *n*-hexane hydroxylation can occur at C_1, C_2, C_3, or C_4. P450 isozymes induced by PB metabolized *n*-hexane to yield a four- to fivefold increase in the 2-, 3-, and 4-hydroxylated metabolites and only a slight increase

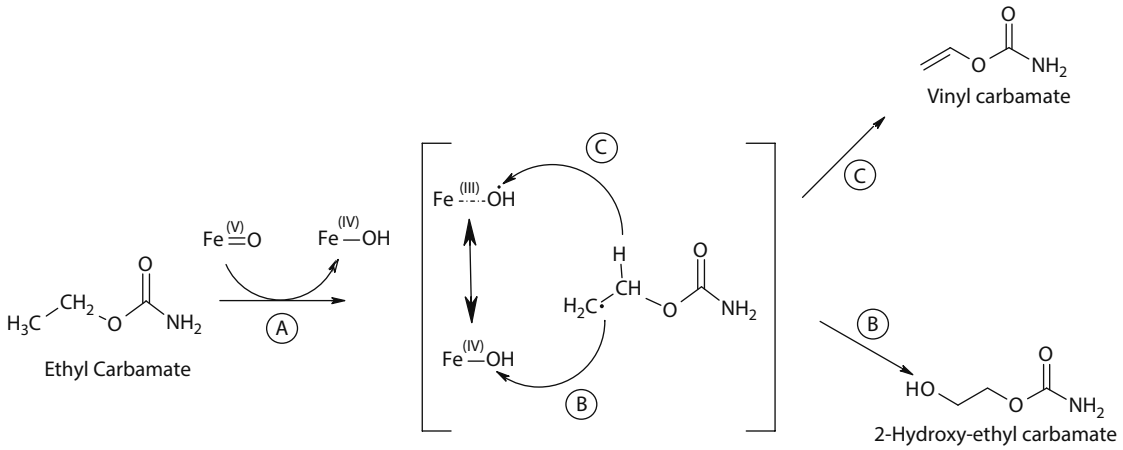


FIGURE 4.6 Mechanism of P450-mediated aliphatic hydroxylation and desaturation. Oxidation of aliphatic carbon begins with abstraction of a hydrogen atom by the electrophilic oxene form of P450 (A). The intermediate may collapse to the hydroxylated metabolite via the oxygen rebound mechanism (B) or may abstract a second hydrogen atom from the substrate resulting in desaturation (C). These two pathways compete kinetically.

at the 1 position. On the other hand, BP-induced isozymes result in decreased yields of the 1- and 2-hydroxylated products but increased yields of the 3- and 4-hydroxylated products.[89] Hydroxylation of aliphatic compounds is generally considered detoxification because of the greater water solubility of the products, but one must be cautioned against overgeneralization, as products that are more toxic could be produced by subsequent metabolism.

Aliphatic Desaturation

Aliphatic desaturation is another special case of the oxidation of an sp^3-hybridized carbon atom. Mechanistically, the first step of the reaction is abstraction of a hydrogen atom, similar to aliphatic hydroxylation; however, instead of oxygen rebound leading to insertion of oxygen, the second step in the mechanism results in abstraction of a second hydrogen atom and formation of a double bond. Compounds of toxicological significance that undergo CYP-mediated desaturation include the carcinogen ethyl carbamate and valproic acid.

Aromatic Oxidation

The mechanisms of aromatic hydroxylation are not completely understood but probably involve several alternative pathways. The exact mechanism for a given hydroxylation may be based on a number of factors, such as the steric features of the substrate and configuration of the active site of the specific P450. Potential mechanisms include direct oxygen insertion into the C–H bond to form an epoxide through radicaloid reactions or through intermediates bonded to $[FeO]^{3+}$. Evidence for intermediacy of an epoxide in the mechanism of aromatic hydroxylation comes from the so-called NIH shift, in which deuterium or halogen substituents are observed to migrate around the aromatic ring in a characteristic pattern during arene oxidation[90]; however, the necessity of an epoxide intermediate in the NIH shift has been challenged.[91] The production of arene oxides has been widely studied because of their importance in the formation of epoxide ultimate carcinogens.

Oxidation of Alkenes and Alkynes

In addition to aromatic compounds, both alkenes (aliphatic double bond) and alkynes (aliphatic triple bond) are subject to epoxidation by P450. The mechanism of these reactions involves abstraction of an electron to form the first C–O bond and a carbon-centered radical, followed by oxygen rebound giving rise to the epoxide. Epoxidation generally, though not always, occurs with retention of stereochemistry. With some terminal alkenes and alkynes, the carbon-centered radical may also react with one of the porphyrin nitrogens in the P450 heme, resulting in covalent binding and inactivation of the enzyme. In the case of polyhalogenated alkenes, migration of a halogen atom may occur with rearrangement of the product to the corresponding aldehyde, as seen in the metabolism of 1,1,2-trichloroethylene to 1,1,1-trichloroacetaldehyde. Other important examples of toxicological significance include oxidation of the industrial monomer 1,3-butadiene and the mycotoxin AFB_1. It is generally agreed that these reactive epoxides are the ultimate carcinogenic metabolites of the parent molecules.

Heteroatom Oxidation

P450 not only oxidizes carbon atoms but also nitrogen, sulfur, phosphorus, and halogen atoms. A number of nitrogen-containing compounds can be oxidized to stable N-oxides. Another hepatic enzyme, flavin-containing monooxygenase (FMO), can also catalyze this reaction, though the substrate specificities of these two enzymes are different (discussed later). P450 and FMO may form N-oxides from the same xenobiotic; however, FMO generally prefers substrates with an electron-deficient nitrogen, whereas P450 prefers an electron-rich nitrogen. P450-mediated N-oxidation is possible with primary and secondary aromatic amines to produce

hydroxylamines. This reaction is the first step in the bioactivation of this class of compounds to mutagens and is therefore thought to play a central role in the induction of cancer by many aromatic amines. S-Oxidation of secondary sulfides can also be catalyzed by P450 and FMO, leading to formation of sulfoxides, which can be further oxidized to sulfones. Free thiols can be oxidized to sulfinic and sulfenic acids, which may be electrophilic, reacting with protein thiol and glutathione (GSH) to produce mixed disulfides. The mechanism associated with all these reactions is believed to be electron abstraction from the heteroatom by $(FeO)^{3+}$, followed by oxygen rebound.

Heteroatom Dealkylation

P450-dependent heteroatom dealkylation begins like heteroatom oxidation with electron abstraction from N, S, O, or Si. This is followed by abstraction of H^+ from the carbon attached to the heteroatom (α-carbon). Alternatively, direct oxidation (hydrogen atom abstraction) of the α-carbon may also occur. In either case, the α-carbon is hydroxylated by oxygen rebound to form a carbinol intermediate. Carbinolamines and related intermediates are generally unstable and undergo carbon–heteroatom bond cleavage, followed by rearrangement to the corresponding aldehyde or ketone. Regardless of the identity of the heteroatom, the products of this reaction are the hydrogenated heteroatom compound and an aldehyde or ketone. Sulfur and silicon atoms generally are not as readily dealkylated as nitrogen and oxygen atoms. Dealkylations unmask more polar functional groups, facilitating conjugation and excretion; however, dealkylation can also facilitate bioactivation, as in the case of N-dealkylation of secondary aromatic amines.

Oxidative Deamination, Desulfuration, and Dehalogenation

Primary amines can be deaminated with the elimination of ammonia and the formation of an aldehyde or ketone. In a similar manner, P450 can catalyze desulfuration and dehalogenation, with the heteroatom being replaced with oxygen. Mechanistically, this reaction is identical to heteroatom dealkylation, discussed earlier.

Reduction Reactions

Reduction reactions are an interesting series of reactions in which P450 may participate under special conditions. These appear to involve transfer of electrons from Fe^{2+} to the substrate. Examples of such reactions are nitro reduction, azo reduction, arene oxide reduction, and reductive dehalogenation. These reactions generally are studied in vitro under anaerobic conditions in the presence of isolated microsomes and NADPH. Because these reactions require low oxygen tension to progress, their in vivo role (if any) is not well understood. Whether or not these reactions represent simply a curious phenomenon associated with P450 or a viable metabolic pathway is not known. It may be possible that under certain cellular conditions of low oxygen tension, these reactions could proceed in vivo.

Induction and Inhibition of Cytochromes P450

Induction

When animals are exposed to certain xenobiotics, their ability to metabolize a variety of xenobiotics is increased. This phenomenon is termed induction. Induction produces a transitory resistance to the toxicity of many compounds; however, this may not be the case with compounds that require metabolic activation because their toxicity may increase. The exact toxicological outcome of this increased metabolism will be dependent on the specific xenobiotic and its metabolic pathway. Because the toxicological outcome of a xenobiotic exposure can depend on the balance between those reactions that represent detoxification and those that represent activation, increases in metabolic capacity may, at times, produce unpredictable results. Induction of P450 has been reviewed in Bresnick,[92] Wang and LeCluyse,[78] and Waxman.[93] Approaches to understand induction potential of drug candidates in the discovery phase have recently been reviewed by Fahmi and Ripp.[94]

One of the initial reports of increased metabolic capacity associated with xenobiotic exposure suggests how induction may provide a survival advantage. In 1954, Brown et al.[95] were studying the metabolism of methylated aminoazo dyes and found that xenobiotics in the animal diets enhanced the P450-dependent demethylation of these compounds. Free-living animals consume a variety of feeds that may contain toxic constituents. If the animal can respond rapidly to these toxic compounds by developing resistance, it can continue to use the feed source and obtain a survival advantage. One mechanism of rapidly developing such resistance is through increased detoxification resulting from stimulation of xenobiotic-metabolizing enzyme activity. Conney[96] published a pivotal review in 1967 that indicated that more than 200 chemicals could induce P450-dependent metabolism, and most of these chemicals were monooxygenase substrates.

The classical definition of enzyme induction requires transcriptional activation at the level of DNA and increased production of mRNA, followed by an increase in the synthesis of the enzyme. The term has taken on a broader definition when used in respect to xenobiotic metabolism. This broader definition includes mechanisms such as mRNA and enzyme stabilization, all of which are associated with xenobiotic induction. The classes of P450 inducers are listed in Table 4.3; however, the concept of inducer classes has become less meaningful in light of recent advances in our understanding of CYP induction.

The PAH class of inducers includes 3-methylcholanthrene, BP, and TCDD, and their mechanism of induction in animals has been extensively investigated. These inducers induce CYP1A1, CYP1A2, and CYP2B1, which are expressed in the liver or extrahepatic tissues of rodents and humans. The low constitutive hepatic concentrations of CYP1A1 result from suppression of transcription by a nuclear repressor protein. Within the cytoplasm of the hepatocyte exists a receptor protein termed the Ah receptor (AhR), which is complexed with heat-shock protein (Hsp 90). When a

TABLE 4.3
Inducers of Cytochrome P450

Structural Class	Primary Examples	Other Examples	Receptor	CYPs Induced
Polycyclic hydrocarbon type	3-Methylcholanthrene	TCDD, BP, β-naphthoflavone, chlorpromazine, isosafrole, ketoconazole	AhR	1A1, 1A2, 1B1
PB type	PB	Phenytoin, griseofulvin, chlorpromazine, ketoconazole, dieldrin, BHT	CAR–RXR	2A1, 2B1, 2B2, 2B6, 2C6, 3A4
Glucocorticoid type	Rifampicin[a]	Dexamethasone, pregnenolone 16a-carbonitrile, spironolactone, prednisolone, methylprednisolone	PXR–RXR (GR)	3A
Ethanol type	Ethanol	Acetone, heptane, pyrazole	None	2E1
Clofibrate type	Clofibrate	Phorbol esters, WY-14,634	PPAR–RXR	4A

[a] Marked species differences exist in inducer selectivity. Rifampicin is the prototypical glucocorticoid-type inducer in humans; dexamethasone is a prototypical inducer in rodents.

polycyclic-hydrocarbon-type inducer enters the hepatocyte, it binds and activates the Ah receptor, resulting in the release of Hsp90. The Ah receptor is phosphorylated and subsequently binds to the AhR nuclear translocator (Arnt) protein, which is also activated by phosphorylation. This complex then moves to the nucleus of the hepatocyte. In the nucleus, this complex binds to a DNA regulatory sequence termed the xenobiotic response element (XRE). A DNA segment similar to rat XRE has been found in mouse and human cells. The XRE has also been found in genes of other xenobiotic metabolism enzymes, such as glutathione S-transferase (GST), aldehyde dehydrogenase (ALDH), and uridine diphosphate (UDP) glucuronosyltransferase (UGT), where it may be involved in regulation of their expression. Binding of the ligand-bound Ah–Arnt complex to XRE enhances transcription of the CYP1A1 gene, resulting in increased quantities of CYP1A1 mRNA followed by an increase in the hepatic concentration of CYP1A1.

CYP2A isoforms are induced by a structurally diverse array of xenobiotics from several different inducer classes[97]; for example, human CYP2A6 is induced by PB, dexamethasone, and rifampicin in primary hepatocyte cultures, and CYP2A3 was induced in rat lung by 3-methylcholanthrene. In addition, several metal salts induce CYP2A5 in mice. Induction of CYP2A is poorly understood and appears to occur by transcriptional activation, RNA stabilization, and protein stabilization. Further, the mechanisms of induction vary with the isoforms, species, and tissues examined. Roles in CYP2A induction have been proposed for several of the orphan nuclear receptors (discussed in the following), as well as for a variety of RNA binding proteins, which appear to bind in the 3′-untranslated region of CYP2A mRNA in response to pyrazole treatment.

PB and other compounds of diverse structure induce expression of CYP2B1 and CYP2B2, as well as, to a lesser extent, CYP2A1, CYP2C6, CYP3A1, and CYP3A2. PB also produces a general pleiotropic response in the liver, resulting in proliferation of the smooth endoplasmic reticulum in hepatocytes, increases in total microsomal protein, and increases in NADPH–cytochrome P450 reductase, as well as other xenobiotic-metabolizing enzymes, such as UDP-glucuronosyltransferase (UDPGT) and epoxide hydrolase. Induction of CYPs and other enzymes by PB occurs by transcriptional activation, but until relatively recently, no cytoplasmic receptor had been identified for the PB-type inducers. Over the last 10 years, however, significant gains have been made with respect to mechanisms of CYP induction by the PB-like inducers. Convincing evidence now suggests that PB-mediated CYP induction is associated with a cytoplasmic receptor known as the constitutively active receptor (CAR), so named because early studies in HepG2 cells indicated that this transcription factor was active even in the absence of ligand.[78] It has since been demonstrated that certain androgens, including androstanol and androstenol, bind to CAR and inhibit transcriptional activation of CYP2B and other genes[98]; hence, CAR is also known as the constitutive androstane receptor. CAR is one of the so-called orphan nuclear receptors, whose endogenous ligands are unknown. Other orphan receptors include the pregnane X receptor (PXR) (discussed in the following), the retinoid X receptor (RXR), and the peroxisome proliferator-activated receptor (PPAR) (discussed in the following).

The details of CAR-mediated induction of CYP are unclear. As is the case with several other orphan receptors, CAR forms a heterodimer with RXR, and the heterodimer forms a DNA-binding complex with the steroid receptor coactivator SRC-1. CYP induction in physiological systems is strictly dependent on the presence of PB or other PB-like inducers, but actual binding of the inducer does not appear to be required. It is thought that PB somehow causes dissociation of the androstanes from CAR, resulting in depression of transcriptional activation.[78] Compounds in the PB inducer class, such as terpenes, organochlorine pesticides, and polychlorinated biphenyls, may act through a common pathway of induction.[99]

CYP2E1 induction has been studied in detail in the rat and represents an interesting situation where induction is controlled at the transcription, mRNA stabilization, translation, and enzyme stabilization levels. CYP2E1 is induced by the ethanol-type inducers. Although not true for all P450s, the

CYP2E1 inducers generally are substrates for the isozyme. In many cases, the regulation of expression of CYP2E1 is controlled by stabilization of CYP2E1 mRNA and stabilization of the enzyme apoprotein, along with possible increased efficiency of translation. Cycloheximide, which blocks translation, blocked the increase in CYP2E1 apoprotein when mRNA was unchanged, indicating that the increase in apoprotein was related to increased translation. Actinomycin D, which blocks transcription, did not block the apoprotein increase, indicating that it was not transcription related. Many of the CYP2E1 inducers act by posttranslational stabilization, including acetone, low ethanol doses, pyridine, and pyrazole. With these inducers, CYP2E1 concentration increases, whereas no change in mRNA occurs. This indicates that CYP2E1 degradation decreases while synthesis remains constant, with the net result being increased CYP2E1.

Nutritional factors and disease conditions can also result in increased activity of CYP2E1. High-fat diets and starvation produce an induction of CYP2E1, as does insulin-dependent diabetes and obesity. One common factor in all of these conditions is increased plasma ketone body concentrations. Whether or not this induction is produced by increased ketone bodies, including acetone, or by other factors is currently under investigation.

The glucocorticoid-type inducers, such as dexamethasone and pregnenolone-16α-carbonitrile, induce CYP3A1 and CYP3A2 in rodents but not in human hepatocytes. In contrast, rifampicin is an effective inducer of CYP3A isoforms in humans and rabbits but not in rodents. Regulation of CYP3 induction is now known to be under the control of PXR, another of the orphan nuclear receptors. In humans, PXR is found primarily in the liver and gastrointestinal tract and in lower amounts in kidney and lung; thus, the tissue distribution of PXR tracks with the tissue distribution of CYP3A4 in humans. As with CAR and other orphan nuclear receptors, PXR consists of a DNA-binding domain, which is highly conserved across species, and a ligand-binding domain, with lower sequence homology across species. Differences in the selectivity of CYP3A inducers are due to structural differences in the ligand-binding domain of PXR, and site-directed mutagenesis has been used to convert the rodent PXR ligand-binding spectrum to a humanlike ligand selectivity that no longer responds to rodent CYP3A inducers.[100] Like other orphan receptors, PXR forms a functional heterodimer with RXR. Evidence suggests that the glucocorticoid receptor also mediates CYP3A induction, although the role played by this receptor is unclear. One hypothesis is that binding to the glucocorticoid receptor upregulates PXR, leading to induction of CYP3A and other PXR-dependent genes; however, some studies demonstrate glucocorticoid induction of CYP3A by a PXR-independent mechanism. Thus, the exact role of the glucocorticoid receptor in induction of CYP3A genes remains to be resolved. Because of the large number of drugs metabolized by CYP3A4 and the wide array of natural and synthetic PXR ligands, DDIs due to CYP3A4 induction are common, and PXR reporter assays have become popular in the pharmaceutical industry as a screen for CYP3A4 induction.

Clofibrate-type inducers induce the CYP4A subfamily that is, in the most part, associated with metabolism of endogenous compounds. The clofibrate-type inducers are structurally diverse but in general are highly lipophilic and possess a carboxylic acid functional group. They also cause hepatocyte peroxisome proliferation in rodents.

Induction of CYP4A and enzymes related to peroxisomal fatty acid β-oxidation is mediated by another member of the orphan nuclear receptor superfamily, PPAR. Three members of the PPAR family have been identified and designated as PPARα, PPARβ, and PPARγ. PPARα is responsible for induction of CYP4A in rodents and rabbits. Upon ligand binding, PPAR forms an activated heterodimer with RXR; the heterodimer binds to the peroxisome proliferator response element upstream from CYP4A and related genes, activating transcription. Many compounds that cause peroxisome proliferation in rodent liver are also hepatocarcinogens; however, no CYP4A induction has been observed in humans by PPARα ligands, and humans are resistant to peroxisome proliferation, suggesting that this phenomenon may have little relevance for humans. The low concentrations of CYP4A in human liver and its limited number of xenobiotic substrates reduce its role in DDIs in humans.

Although CAR and PXR were originally identified as mediators of CYP2B and CYP3A enzymes, respectively, there is significant overlap in the selectivity profiles of inducers of these two subfamilies. As such, many PB-type inducers are capable of inducing CYP3A genes, and several glucocorticoid-type inducers are effective inducers of CYP2B genes. This overlap may be related in part to the ability of CAR and PXR to recognize and bind to each other's response elements, a phenomenon referred to as cross talk. Another mechanism for cross talk may be the overlapping ligand-binding selectivity of CAR and PXR; for example, PB is a ligand for both CAR and PXR in humans.

Inhibition

Just as induction of xenobiotic metabolism can have important toxicological ramifications, inhibition of the ability to metabolize a xenobiotic can result in profound changes in its toxicity. Inhibition of the metabolism of a compound can result in a higher plasma concentration than predicted and unexpected toxicity. During treatment with multiple drugs, unexpected adverse effects can be produced through DDIs where one drug inhibits the metabolism of another, resulting in higher than expected plasma concentrations.

Four mechanisms are generally associated with inhibition of P450-mediated detoxification. First, two xenobiotics may be substrates for the same P450 isozyme and will compete for the active site of the enzyme, a phenomenon known as competitive inhibition. An example of this type of mechanism is the inhibition of bioactivation of the rodent carcinogen ethyl carbamate by ethanol, both of which are found in fermented beverages.[101] A second mechanism of competitive inhibition is the binding of a xenobiotic to the active site of a P450, although it is not a substrate for that P450. The presence of the nonsubstrate at the active site blocks the binding

of the true substrate, inhibiting its metabolism. Examples of this mechanism of CYP inhibition are rare. A third mechanism of inhibition involves the metabolism of a xenobiotic to a product that has a higher affinity for the active site than the parent compound, forming a so-called metabolite–inhibitor (MI) complex. The active site is then occupied and additional substrate cannot bind. This essentially makes the enzyme inactive and is an example of noncompetitive inhibition. Compounds such as erythromycin, nicardipine, and diltiazem reversibly inhibit human CYP3A4 by this mechanism.[102] The fourth mechanism is another example of noncompetitive inhibition resulting from the production of a highly reactive metabolite that binds (often covalently) to the heme or apoprotein of P450, destroying its activity. This type of inhibitor is termed a suicide substrate. As mentioned previously, inhibition of CYPs by trichloroethylene is an example of suicide inhibition. Both MI complex formation and suicide inhibition are examples of mechanism-based inhibition.

Other less common mechanisms can result in inhibition of P450-mediated xenobiotic metabolism, including compounds that may modify protein or heme synthesis or degradation, those that may uncouple electron transport to P450, those that may interfere with cofactor availability, and those that may directly inhibit NADPH–P450 reductase activity. Just as some substrates may demonstrate a higher affinity for specific P450s and others may not, inhibitors may show a narrow or broad range of affinity for a specific P450. Inhibitors have been useful tools in determining mechanisms associated with xenobiotic metabolism and in attempts to predict specific DDIs. Induction and inhibition of human cytochromes P450 have been recently reviewed in Pelkonen et al.[103]

Pharmacogenetics, Human Polymorphism of P450 Isozymes, and Their Toxicological Significance

Pharmacogenetics is the study of the hereditary basis of the observed differences in response (both therapeutic and adverse) to drugs by individuals and populations. The term can be expanded to include not only drugs but also dietary and environmental chemicals. This field has seen a large expansion over the last decade, as the understanding of the genetics, genetic regulation, and interindividual variations in P450 and other xenobiotic metabolism enzymes has increased. Maturation of methodologies from molecular biology and refinement of other methodologies to study genetic differences in individuals and populations have spurred interest in the pharmacogenetics of xenobiotic metabolism.

Many studies of the adverse effects of chemicals in animals and humans have indicated that highly significant differences exist between animal strains, among individual animals, and especially in individual humans and different human population groups. These genetic polymorphisms can result in unexpected drug and environmental toxicities and complicate safety assessments and the extrapolation of data from animal studies to humans. It has been estimated that almost 50% of drugs for which adverse reactions have been reported are metabolized by significantly polymorphic P450s.[104] As discussed later, this has led to recommendations that the specific family and subfamily of P450 that metabolizes a specific drug candidate be determined during early drug discovery efforts. This could avoid unexpected interactions and suggest potential adverse effects before additional developmental efforts with a drug or other chemical product are undertaken.

Several alleles in the CYP2D6 family, for example, are known to contain specific nucleotide deletions that result in inactive genes and a lack of production of the CYP2D6 protein.[105] Individuals homozygous for these gene variations will be *poor metabolizers* (PMs) of CYP2D6 substrates. In contrast, some individuals have multiple copies of the CYP2D6 gene, possibly due to gene duplication.[106] These individuals have enhanced capability to metabolize CYP2D6 substrates and are *ultrarapid metabolizers* (UMs).[107] A chemical whose detoxification depends on CYP2D6 would be more toxic than expected in PM, but less toxic than expected in UM individuals. In contrast, a chemical that is metabolically activated would be less toxic in PMs and more toxic in UMs. To predict toxicity, it is obvious that not only must the role of metabolism in the toxicity of a compound be known but also the potential genotype of exposed individuals.

Several human P450s have been shown to exhibit significant polymorphic expression, including CYP1A1, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP3A5, and CYP2E1. Many other CYP polymorphisms are known, encompassing most of the known human CYP-mediating xenobiotic metabolism, but the functional significance of many of these polymorphisms is unclear. The most up-to-date information on CYP polymorphisms can be found on the homepage of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles). The chromosomal locations of these P450 genes have been identified, and the genetic basis for the altered P450 activity is becoming understood. From a toxicological perspective, the most important P450 polymorphisms are those of the CYP2 and to a lesser degree the CYP3 subfamilies. These polymorphisms have been the subject of several recent reviews[103,108,109] and are summarized here.

CYP2A6

CYP2A6 is the major isoform responsible for C-oxidation of nicotine. Several significant allelic variants of this isoform have been identified, including CYP2A6*2, which is inactive due to a Leu–His substitution at position 160, and CYP2A6*4A, which is a complete deletion of the CYP2A6 gene. The former allele is found at a frequency of 1%–3% in Europeans, and the latter allele is more prevalent in Asians. Because of the role of CYP2A6 in nicotine metabolism, polymorphism of this isoform has been implicated in differences in smoking behavior and the incidence of lung cancer among smokers.

CYP2C9

CYP2C9 plays a major role in metabolism of the anticoagulant drug warfarin, and defects in this isoform can lead to excessive plasma concentrations and increased risk of

bleeding episodes in effected individuals. The major allelic variants of CYP2C9 are CYP2C9*2 and CYP2C9*3, both of which are due to missense mutations.[67,110] In Caucasians, the frequencies of these two alleles are approximately 11% and 7%, respectively, and the frequency of homozygotes, who express the PM phenotype, is around 3%–4%. For CYP2C9*2, decreases in activity between 8% and 94% have been reported, depending on the substrate used. Similarly, carriers of the CYP2C9*3 allele express significantly lower catalytic activity compared to the CYP2C9*1 genotype.[110] Polymorphisms in CYP2C9 can significantly effect therapeutic responses to a number of commonly prescribed pharmaceuticals, the most important of which may be warfarin, due to its narrow therapeutic window. Since 2007, warfarin has included pharmacogenetic information in its label, including a dosing table based on genotype, added in 2010.[111]

CYP2C19

CYP2C19 is responsible for metabolism of approximately 10% of marketed drugs.[67] Major polymorphisms of CYP2C19 are associated with deficient 4-hydroxylation of *S*-mephenytoin and the sensitivity of affected individuals to excessive sedation by this anticonvulsant agent. Deficient mephenytoin metabolism is found in 2%–5% of Caucasians but is much more prevalent in Asians, with a frequency of 18%–23%. Two major allelic variants were identified as CYP2C19*2 and CYP2C19*3.[112] CYP2C19*2 is a splice mutant that codes a truncated protein, and CYP2C19*3 contains a premature stop codon in exon 4. Both mutations lead to nonfunctional proteins. Gain of function alleles are also known. CYP2C19*17 results from mutations in the promoter region, which lead to increased gene transcription, and the CYP2C19*1/*17 and *17/*17 genotypes are considered UMs of effected substrates. In addition to mephenytoin, CYP2C19 is involved in bioactivation of clopidogrel, an antiplatelet agent used extensively in patients with cardiovascular disease. PMs are at higher risk for thrombosis and myocardial infarction during clopidogrel therapy, whereas UMs have an increased bleeding risk. CYP2C19 mediates metabolism of a variety of other drugs including proton pump inhibitors such as omeprazole and antiviral compounds such as nelfinavir, both of which show greater efficacy in PM phenotypes.

CYP2D6

High interindividual variability in the metabolism of the antihypertensive agent debrisoquine led to the discovery of one of the first CYP polymorphisms. Since its initial discovery, CYP2D6 polymorphism has been the subject of intensive research efforts and is probably the most thoroughly characterized CYP polymorphism. CYP2D6 plays a major role in the metabolism of tricyclic antidepressants and antipsychotics, and PM status has been associated with a higher incidence of extrapyramidal and other side effects, while UM status is associated with a lack of therapeutic effect of these agents. For example, the antipsychotic agent thioridazine is associated with higher risk of ventricular arrhythmia related to QT prolongation in CYP2D6 PM phenotypes.[113] One of the major defective CYP2D6 variants is CYP2D6*4, a splice mutation that is found in Caucasians at a frequency of almost 21%. Another common variant is CYP2D6*5, in which the entire gene is deleted. The frequency of this allele is 4%–6%. The UM phenotype is due to gene duplication, and frequencies for this phenotype range from 1% to 2% in Caucasians to almost 30% in Arabian and North African populations.[70]

CYP3A5

Functional CYP3A5 is expressed in approximately 20% of Caucasians and about 67% of African Americans. Although this isoform contributes to total CYP3A activity in these individuals, the clinical implications of CYP3A5 status are unclear in most cases. Recently, a specific role for CYP3A5 in the metabolism of tacrolimus has been described, suggesting that CYP3A5 expression status may play a role in the high interindividual variability seen with many CYP3A substrates.

Species, Strain, and Gender Differences in Monooxygenase Activity

The activities of cytochromes P450 play a central role in the expression of the toxicity of many xenobiotics. One factor that complicates extrapolation of toxicity between species is the quantitative and qualitative differences in how species metabolize xenobiotics. Generally, the basic reactions and major metabolites of a xenobiotic are similar between species; however, subtle differences in metabolism can lead to major differences in susceptibility to the toxicity of a xenobiotic. Mechanisms that may account for species differences include the following: (1) lack of a metabolic pathway or a genetic defect in a particular metabolic pathway; (2) differences in the K_m and V_{max} (i.e., level of expression) of specific enzymes; (3) the existence of different isozymes and differences in the ratios of specific isozymes of important enzymes, such as P450; and (4) differences in the ratio of activities of separate enzyme systems that act together to metabolize a specific xenobiotic.

When one metabolite represents a metabolically activated form and another a detoxified form, the ratio of these metabolites can dictate a species susceptibility to a xenobiotic. This type of species difference is most commonly encountered when the P450-dependent monooxygenase acts in coordination with another pathway. Species may differ in either the initial monooxygenase functionalization reaction or in the activity of the secondary pathway. This is illustrated by the metabolic activation of BP (see Figure 4.4) in rats and mice. The metabolic activation of BP requires initial epoxidation by the P450-dependent monooxygenase at the 7,8 position, followed by hydration of the epoxide by epoxide hydrolase to yield the 7,8-diol. This diol is then epoxidated by the monooxygenase to yield the ultimate carcinogen of BP, the 7,8-dihydrodiol 9,10-oxide. When mouse hepatic microsomes were used for metabolic activation in the Ames assay for mutagenicity, BP was highly mutagenic, indicating a high degree of

metabolic activation; however, when rat hepatic microsomes were employed in the same assay, only slight mutagenicity was evident. This indicates a significantly lower ability for the rats to metabolically activate BP in vitro.[114] Although mice do metabolize BP to a greater extent than rats, rats have six- to sevenfold more epoxide hydrolase activity. Further studies[114,115] indicated that both species have adequate monooxygenase to metabolically activate BP and that higher epoxide hydrolase activity in the rat may have been responsible for the lower mutagenicity; therefore, the species differences in the secondary pathway, epoxide hydrolase, may have controlled the mutagenicity, as opposed to differences in the monooxygenase activity.

Different strains of the same species may demonstrate differences in metabolism; for example, if a different strain of mouse had been used in the studies described earlier, the conclusions may have been different. It is important to recognize these strain differences when designing toxicological studies. The mechanisms associated with strain differences may be diverse. Without an understanding of these species and strain differences, it will be difficult to extrapolate toxicological studies performed with animals to humans.

Studies of species differences in animals are difficult to design and interpret, and those involving humans are even more complex. This complexity results from the large differences in xenobiotic biotransformation found in humans. Many factors contribute to these individual differences in metabolism, including the following: (1) humans are free-living and have few restraints to reproductive diversity, diminishing the development of small genetic pools that result in genetically less diverse, more homogeneous control of metabolism; (2) environmental factors, such as diet, nutrition, and xenobiotic exposure, are diverse among humans; and (3) humans generally have more control and probably more interest in consumption of varied nonnutritive materials, such as alcohol or drugs. These as well as other factors result in a large diversity in susceptibility to xenobiotic exposure. This is, in part, why such large safety factors are employed in risk or hazard assessments of xenobiotics to which humans may be exposed. These safety factors are used to attempt to protect the vast majority of individuals at risk. For further discussions of species differences, the reader is directed to several recent review articles.[116–118]

Gender differences in xenobiotic metabolism may be an important factor in gender-dependent differences in toxicity. The best example of gender differences in xenobiotic metabolism, especially cytochrome P450-mediated metabolism, is the rat. Because the rat is commonly used in toxicological safety assessments, it is important to realize the gender differences in this species and understand how they relate to the extrapolation of rat data to humans.

Generally, male rats have a higher capacity to metabolize xenobiotics than females. This difference is in large part related to the CYP content and its temporal pattern of growth hormone-regulated expression. Although females have 10%–30% less total P450 than males, this difference is not high enough to explain the 2- to 20-fold differences seen in metabolism. Much of the differential seen between males and females can be explained by differences in P450 isozymes between the sexes; for example, males express CYP2C11 but females do not. Isozymes that predominate in males are CYP2A1, CYP2A2, and CYP3A2. Adult females also have predominant P450s, such as CYP2C12, which occurs in juvenile and older males but not in young adult males. These differences are under hormonal control and can be altered by procedures such as castration and administration of sex hormones. They also are developmentally controlled, and the stage of life at which these procedures are done can influence their outcome. Neonatal castration of male rats results in different expression of P450 when they become adults. The adult expression of P450s can actually be imprinted during the neonatal period. Although sex hormones play an important role in the expression of P450 in rats, other hormones, including growth hormone, thyroxine, insulin, and somatostatin, may play important roles.

These differences between male and female rats also show an age dependency. As male rats age, their P450 isozyme profiles begin to appear more like females. Toxicologists using rats as a model in safety assessments need to be cognizant of these gender- and age-dependent changes. The toxicity data from young rats, generally used in toxicity studies, may not reflect the toxicity seen in old rats. During chronic toxicity and carcinogenicity studies, the response of rats to the toxicity of a test material may change as the study progresses. This is especially true of carcinogenicity studies that begin with in utero exposure. Early developmental changes in P450 in animals and humans may be important in responses to teratogens and embryotoxic compounds.[117]

If gender differences in xenobiotic metabolism in rats can complicate toxicity assessments, what about other species, including humans? Rats have been the most intensively investigated species in regard to gender differences; however, studies with other species suggest that they generally do not demonstrate such large gender differences. Mice, another species important in toxicology studies, generally do not show the exaggerated gender differences in xenobiotic metabolism seen in rats. Gender differences in mice seem to be dependent on the specific strain of mouse investigated. Where gender differences do exist in mice, it is generally the female that has the higher metabolic capacity, but the differences are not as great as that seen in rats.

Other species used in toxicological investigations, such as dogs, appear to demonstrate some gender differences in the expression of P450 isozymes, but, again, they are not as exaggerated as in rats. Although there are few reported studies, monkeys have not been reported to demonstrate significant gender differences in xenobiotic metabolism.

Humans have not been shown to demonstrate gender-dependent differences in the expression of P450 isozymes. Although there can be significant differences between human males and females in xenobiotic biodisposition, these appear to be more based on anatomical and physiological differences that affect absorption, distribution, and excretion. Individual humans can display large differences based

on lifestyle factors and exposure to environmental chemicals, foods, and drugs; however, inherent gender differences in the expression of P450 are not apparent.

This raises the question as to how species, such as rats, can be used to predict toxicity in humans. With care and knowledge of the differences between rats and humans, the rat can serve as a useful model for human toxicity. This has been shown through decades of use; for example, rats and humans share similarities between the CYP isozyme subfamilies CYP1A1, CYP1A2, and CYP2E1, and these subfamilies are not expressed in a highly gender-dependent manner in rats. Xenobiotics metabolized by CYP1A or CYP2E may reflect human metabolism because regulatory control over these isoforms has been highly conserved between rodents and humans; however, gender-dependent differences are generally not reflected when extrapolating from rats to humans.[68] Gender-dependent differences in xenobiotic metabolism are but one of the reasons toxicologists must use both sexes in the safety assessment of chemicals. Gender differences in xenobiotic metabolism have been reviewed.[68]

Microsomal Flavin-Containing Monooxygenase

Since 1960, it has been apparent that a microsomal monooxygenase other than P450 could catalyze the oxygenation of nucleophilic nitrogen, sulfur, and phosphorus compounds. Purification to homogeneity indicated that the enzyme was an NADPH-dependent, FMO distinct from P450. This monooxygenase has been referred to as amine oxidase, Ziegler enzyme, dimethylaniline monooxygenase, and FMO. This enzyme may be a good example of proteins involved in normal anabolic and catabolic metabolism being recruited for xenobiotic metabolism. The flavin prosthetic group that is characteristic of these enzymes is especially versatile at carrying out redox functions.

The catalytic cycle for the FMO is shown in Figure 4.7. NADPH reduces the FAD of the enzyme, and the oxidized $NADP^+$ remains bound (Figure 4.7A). Oxygen then binds to yield FAD peroxide (Figure 4.7B and C) followed by substrate binding (Figure 4.7F). Prior to substrate binding, the peroxide complex can decompose, releasing superoxide (Figure 4.7D). One oxygen atom from the peroxide is transferred to the substrate, leaving the hydroxyflavin (Figure 4.7G). The final and rate-limiting step of the cycle is the dehydration to regenerate FAD, yield water, and release the bound $NADP^+$ (Figure 4.7H and I). NADH can substitute for NADPH, but with lower affinity and activity.

The FMO has at least five isoforms (designated as FMO1–FMO5), whose genes are expressed across several species and tissues. These forms have different substrate specificities and are probably related to the species-dependent toxicity of certain substrates, such as the pyrrolizidine alkaloids. In addition to the five functional forms of FMO, at least six other FMO genes have been identified, all of which appear to be pseudogenes.[118] Humans, rats, and mice have relatively high activity of FMO1 in kidney. Humans and mice have low activity for this form in the liver, whereas the rat has high activity. This isoform is also expressed in human lung and brain. FMO2 is the primary isoform expressed in human

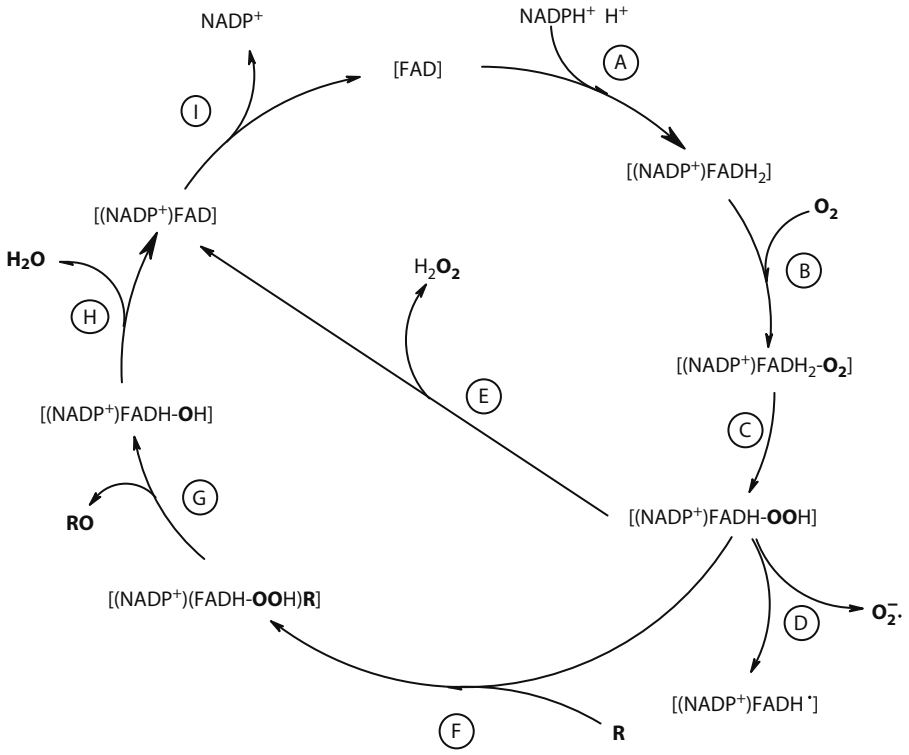


FIGURE 4.7 Catalytic cycle of the FMOs. See text for details. (Adapted from Masnil, M. and Testa, B., in *Advances in Drug Research*, Vol. 13, Testa, B., ed., Academic Press, London, U.K., 1984, pp. 95–207.)

lung, with lower amounts found in kidney, liver, small intestine, and brain. Humans and mice have high activity of FMO3 in the liver, whereas the rat has low activity. FMO3 activity has also been detected in human kidney, lung, and small intestine, and rat and mouse kidney both show high activity. In mice, only females express FMO3 and have two- to threefold higher activity of FMO1 compared to males. Human FMO4 expression levels are highest in liver and kidney, with lesser amounts in small intestine and lung and very low levels in brain. FMO5 is quantitatively the most important isoform in human liver and is also expressed in small intestine, kidney, and lung to a lesser, though significant, degree. FMO5 shows no gender differences. Male rats have higher total FMO than females and two- to threefold more FMO1 than females, but no differences are seen in FMO3.[119] Humans can show considerable individual differences, but no gender differences have been demonstrated. This information illustrates the species, gender, and tissue differences that can be encountered with this monooxygenase and emphasizes the importance of choosing an appropriate animal model for toxicological studies of compounds that are potential substrates for this enzyme.

FMOs catalyze the oxidative attack on the nucleophilic nitrogen and sulfur heteroatom of a variety of xenobiotics (Figure 4.8).[120] It was once believed that oxidations of basic aliphatic and tertiary aromatic amines were carried out by the FMO, primary aromatic amines and the acidic nitrogens of amides were catalyzed by P450, and secondary amines were oxidized by both enzyme systems. More recent studies with the purified enzymes have demonstrated no clear division between the types of substrates preferred by the two enzymes; therefore, the metabolism of each nitrogen-containing xenobiotic must be considered on an individual basis. The thermal instability of the FMO in the absence of NADPH (above 50°C) has provided a tool to separate the activity of this enzyme in vitro from that of P450; however, defining the relative contribution of the FMO and P450 to the metabolism of many xenobiotics is difficult because some inhibitors of P450, such as SKF-525A, are substrates for the FMO. More selective inhibitors of P450, such as *N*-benzylimidazole and aminobenzotriazole, are a better choice for distinguishing these two enzymes. Antibodies to specific P450 isozymes can also be used to inhibit P450 and determine the role of the FMO.

Many nitrogen- and sulfur-containing xenobiotics are metabolized by FMOs, as seen in Figure 4.8. N-Oxidation of nucleophilic tertiary amines yields N-oxides, and primary and secondary amines are oxidized to hydroxylamines. In addition, primary amines can be oxidized to oximes and secondary amines to nitrones. Thiols, thioethers, and other xenobiotic-containing sulfur can be oxidized to sulfur oxides. In addition to the functional groups shown in Figure 4.8, FMOs are also capable of oxidizing organic phosphines, boronides, selenides, and iodides. The flavin-containing monooxygenases have broad substrate specificity, but individual isozymes demonstrate some specificity. Broad substrate specificity and its occurrence in several tissues indicate that it can be a major determinant in oxidative xenobiotic metabolism.

Transcriptional regulation of FMOs has received much less attention than the regulation of P450s, and the mechanisms governing the expression of FMOs are unclear. Basal expression of FMOs is under hormonal control. To date, little evidence exists to suggest xenobiotic-mediated induction of FMOs. Few isoform-selective FMO substrates are known, although stereoselective N-oxidation of nicotine and S-oxidation of cimetidine have been used as marker activities for FMO3. Further, N-oxidation of trimethylamine is catalyzed by FMO3, and a genetic defect in this isoforms results in trimethylaminuria (fish odor syndrome). Few selective inhibitors of FMOs are known, although indole-3-carbinol and *N,N*-dimethylaminostilbene carboxylates have been used for this purpose. It has been suggested that targeting drugs for FMO-mediated metabolism may result in fewer adverse drug reactions, due to the lack of induction and selective inhibition of FMOs.[118] Numerous single nucleotide polymorphisms (SNPs) for FMOs have been identified, but because of the limited role of FMO in drug metabolism, the clinical significance of polymorphic FMO expression is uncertain.

Xanthine Oxidoreductase

Xanthine oxidase and xanthine dehydrogenase are members of the molybdenum hydroxylase flavoprotein family commonly referred to as the xanthine oxidoreductase (XOR) family.[121] Oxidation reactions carried out by xanthine oxidase and aldehyde oxidase are different from that of cytochrome P450-catalyzed oxidations in that hydroxylation of the substrate is derived from water rather than molecular oxygen.

Xanthine oxidase and xanthine dehydrogenase are actually different enzymes derived from the same gene product. Conversion of the dehydrogenase to the oxidase involves oxidation of critical protein thiol groups followed by the cleavage of a 20 kDa fragment from each of two subunits. Although both forms of the enzyme have been recognized for years, comparatively little information exists for this enzyme system, especially the dehydrogenase. XOR has been identified in tissues from all species studied to date, and among mammals, the highest activity is found in lactating mammary gland and cow's milk, liver, and intestine. The predominant form is the dehydrogenase, and both forms are localized in the cytoplasm. Human XOR is generally less active than that of other species.

XOR catalyzes oxidation of electron-deficient carbons, frequently adjacent to nitrogen in heterocyclic ring systems. XOR can also catalyze oxidation of aldehydes to carboxylic acids, though with much lower affinity than ALDHs. The XOR enzyme system is the rate-limiting enzyme in purine catabolism but is also well known to metabolize xenobiotics. XOR carries out the oxidation of hypoxanthine to xanthine and xanthine to uric acid (Figure 4.9). Anticancer drugs including substituted and unsubstituted purines, pyrimidines, pteridines, and azopurines and heterocyclic compounds such as doxorubicin and menadione are well-known substrates of xanthine oxidase. More recently, the generation of nitric oxide from *S*-nitrosothiols and nitrite by XOR has been demonstrated.

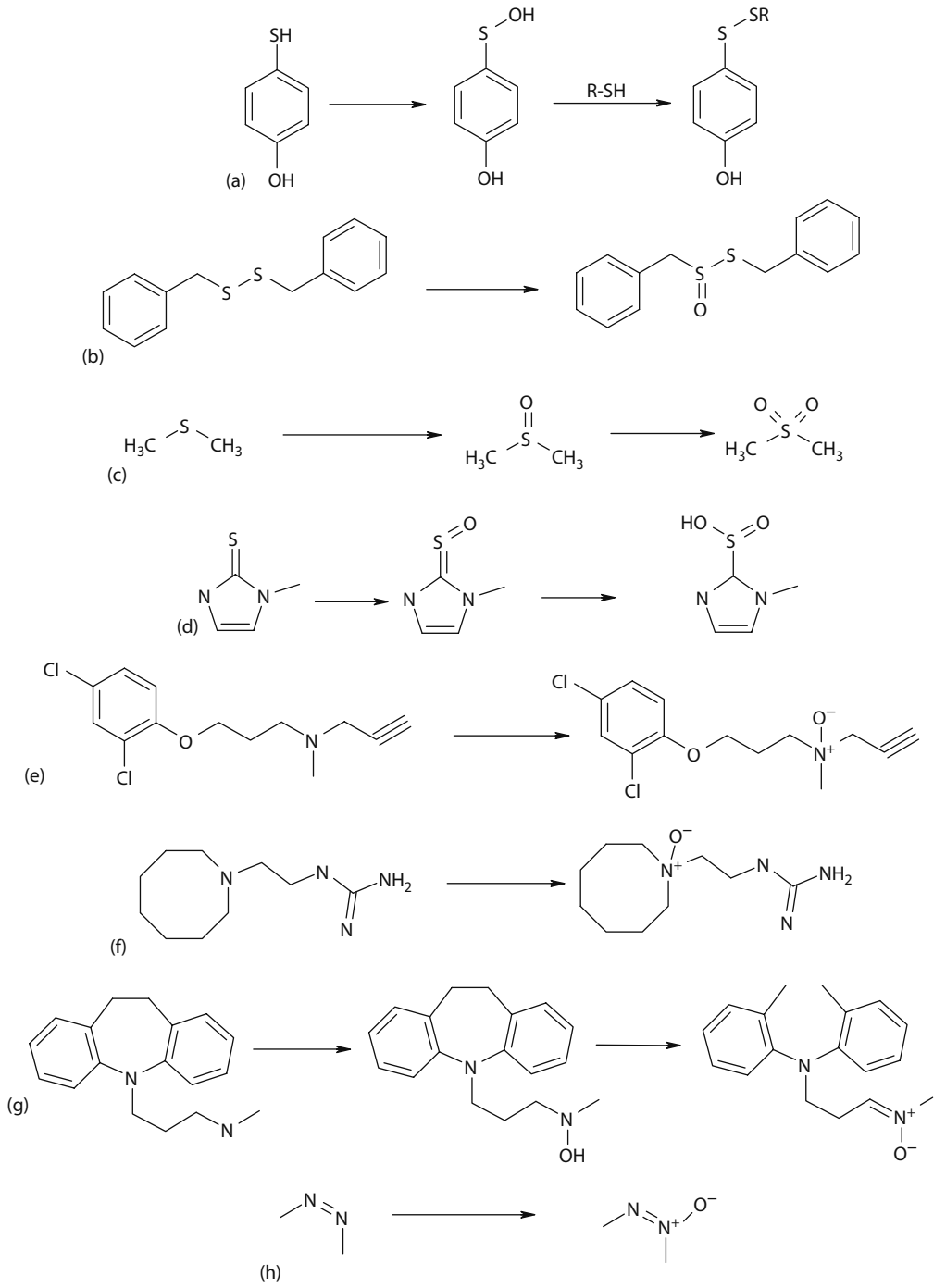


FIGURE 4.8 Examples of reaction types catalyzed by FMOs. Specific substrates are indicated in parentheses following the reaction type: (a) S-oxidation of thiols (thiophenol); the second step forming the disulfide is nonenzymatic. (b) S-oxidation of disulfides (benzyl disulfide). (c) S-oxidation of thioethers (dimethylsulfide). (d) S-oxidation of thiocarbamates, thiocarbamides, and thioureas (methimazole). (e) N-oxidation of aliphatic tertiary amines (clorgyline). (f) N-oxidation of alicyclic tertiary amines (guanethidine). (g) N-oxidation of aliphatic secondary amines (desipramine). (h) N-oxidation of azo compounds (azomethane).

Both the oxidase and reductase forms are capable of metabolizing xenobiotics with the preference determined by the specificity of each enzyme for a different electron acceptor.[121] The oxidase utilizes molecular oxygen as an electron acceptor with negligible reactivity toward NAD^+. Reoxidation of the oxidase enzyme takes place via two one-electron reductions of molecular oxygen to yield hydrogen peroxide. On the other hand, the dehydrogenase utilizes NAD^+ as an electron acceptor to produce NADH through a two-electron reduction. Thus, the xanthine dehydrogenase enzyme has been shown to participate in the redox cycling of doxorubicin and menadione, resulting in the formation of their hydroquinones, which are generally unstable and generate oxygen radicals. The efficient utilization of oxygen and

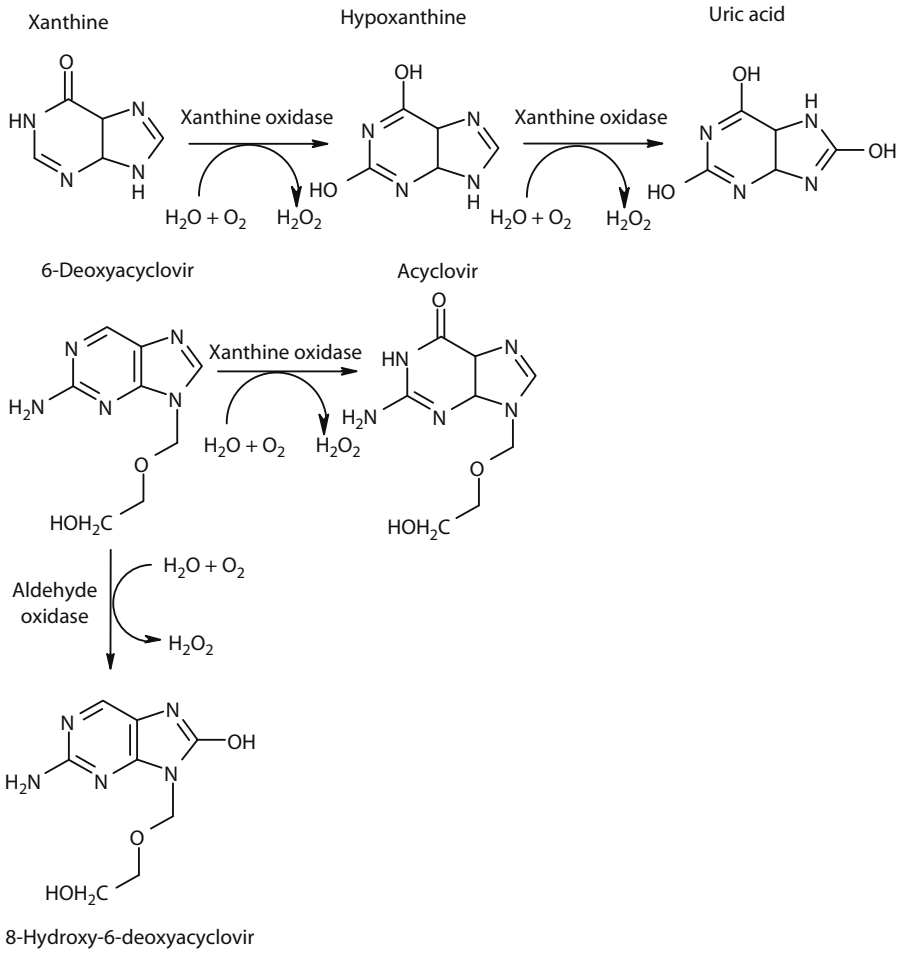


FIGURE 4.9 Oxidation of xanthine, hypoxanthine, and 6-deoxyacyclovir by xanthine oxidase or aldehyde oxidase using molecular oxygen as the electron acceptor.

the production of oxygen radicals have been proposed to contribute to the cytotoxic action of these drugs.[122]

Amine Oxidases

Amine oxidases can play a significant role in the metabolism of specific xenobiotics.[126] Monoamine oxidase (MAO) and related amine oxidases catalyze the oxidative deamination of endogenous amines. They can also be involved in the metabolism of primary, secondary, and tertiary xenobiotic amines. Two of the amine oxidases (MAO and semicarbazide-sensitive amine oxidase [SSAO]) will be used as examples of amine oxidases.

Most tissues express two forms of the mitochondrial enzyme MAO (termed MAO-A and MAO-B), each being expressed by a separate gene. Although MAOs are expressed in most tissues, expression in various tissues is isoform specific. The highest concentrations of both isoforms are found in the liver and gastrointestinal tract. Only MAO-B is expressed in human platelets. MAO is a flavoprotein capable of oxidative deamination of primary, secondary, and tertiary amines. Metabolism of primary amines yields an aldehyde and ammonia, whereas secondary amines yield an aldehyde and a primary amine. The aldehyde products may be further metabolized by other enzymes to carboxylic acids or alcohols. Unlike the monooxygenases, the oxygen used in the reaction is derived from water. During the oxidation, the FAD prosthetic group is reduced (FADγFADH2) then reoxidized by oxygen with the production of hydrogen peroxide. A number of amine drugs have been shown to be substrates for MAO. Some of these act as prodrugs and require MAO metabolism to produce the active form; others have their activities limited by MAO metabolism. MAO-A and MAO-B have different substrate specificities, but there can be overlap in specificity.

Induction of MAO by drugs or other xenobiotics has not been observed. Basal expression of MAOs is under hormonal control and can be perturbed by steroid analogs such as prednisone. Nonselective inhibitors of MAO include iproniazid and phenelzine, and selective inhibition of MAO-A and MAO-B can be achieved with clorgyline and pargyline, respectively. Although these inhibitors are useful for in vitro diagnostic studies of drug metabolism, they also inactivate some CYP isoforms, diminishing their usefulness as in vivo probes of MAO involvement. Polymorphisms of both MAO isoforms are known, although the consequences for drug metabolism and toxicity are not well understood. MAOs play a role in the metabolism of a variety of compounds, including

β-adrenergic agonists/antagonists and phenylethylamine derivatives such as mescaline. The quantitative contribution of MAOs to xenobiotic clearance is unknown but is likely to be low compared to P450s.

A well-publicized example of an MAO-related toxicity was initiated by individuals attempting to synthesize a narcotic related to demerol. Instead of the intended product, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) resulted from the synthesis. Individuals who self-administered MPTP demonstrated Parkinson's disease-like symptoms. This was related to neurocytotoxicity in dopaminergic neurons produced by brain MAO-B metabolism of MPTP to 1-methyl-4-phenyl-2, 3-dihydropyridine ($MPDP^+$), which oxidizes to the neurotoxic 1-methyl-4-phenylpyridine (MPP^+). The cytotoxicity of MPP^+ results from its inhibition of mitochondrial respiration.

SSAOs, like MAO, catalyze the oxidative deamination of endogenous amines but can also metabolize xenobiotic amines.[123,124] The SSAOs do not contain a flavin but do contain copper. They demonstrate a more limited activity than MAO by only catalyzing deamination of primary aliphatic and aromatic monoamines. They are sensitive to inhibition by semicarbazide but insensitive to the classic MAO inhibitors. The products of their reaction are an aldehyde, ammonia, and hydrogen peroxide. They occur in most species, including bacteria, fungi, plants, and animals. In animals, they occur in plasma and may be bound to tissues. Although they can metabolize several endogenous substrates,[125] their exact physiological role is currently unknown.

Considerable species differences exist for SSAO; for example, rats have relatively low concentrations of plasma SSAO compared to humans. SSAO can metabolize certain xenobiotics to more toxic metabolites. 3-Aminopropene has been used in the manufacture of pharmaceuticals and in rubber vulcanization; chronic exposure to this compound can produce lesions similar to acute myocardial necrosis and atherosclerosis. SSAO appears to metabolize 3-aminopropene to 2-propenal (acrolein), which alkylates and inactivates GST and allows excessive peroxidative damage.[126] Damage occurs in the heart and aortic tissue, which have high SSAO activity.[127] The tissue specificity of this effect is related to relatively high expression levels of SSAO in heart. This is in contrast to acrolein produced from allyl alcohol by alcohol dehydrogenase (ADH), which results in liver toxicity.

Aldehyde Oxidase

Aldehyde oxidase is similarly a cytosolic, sulfur-containing molybdenum hydroxylase, is closely related structurally and catalytically to xanthine oxidase, and is present in highest quantities in liver, particularly of the rabbit. Despite its ability to oxidize aldehydes in vitro, especially aromatic aldehydes (e.g., vanillin to vanillic acid), this misnamed enzyme preferentially catalyzes the oxidation of purines and other heterocyclic amines. Unlike xanthine oxidase, aldehyde oxidase is able to catalyze hydroxylations at the C-8 position of purines (Figure 4.9); thus, the prodrug 6-deoxyacyclovir undergoes hydroxylation at the C-8 position by aldehyde oxidase to yield active acyclovir.[128] The most efficient substrates for aldehyde oxidase are aromatic heterocycles with two fused six-membered rings.[129] There is also evidence that iminium ions can be metabolized to lactams by aldehyde oxidase.[130]

Cooxidation of Xenobiotics by Prostaglandin H Synthase and Other Peroxidases

Pathways other than the monooxygenases may be involved in xenobiotic oxidation. These include myeloperoxidases (MPOs), eosinophil peroxidase (EPO), uterine peroxidase, lactoperoxidase (LPO), thyroid peroxidase (TPO), and the prostaglandin synthases. Prostaglandin synthase, also known as cyclooxygenase, is the initial enzyme in arachidonate metabolism and the formation of prostanoids such as prostaglandins, prostacyclins, and thromboxanes. Marnett and Reed[131] demonstrated that prostaglandin H synthetase, an enzyme system responsible for prostaglandin biosynthesis, was capable of oxidizing BP to quinines (Figure 4.4). The following cycle of reactions is involved in the oxidation of xenobiotics[132]:

$$\text{Peroxidase} + \text{ROOH} \rightarrow \text{Compound I} + \text{ROH}$$

$$\text{Compound I} + \text{ROH} \rightarrow \text{Compound II} + \text{RO}-$$

$$\text{Compound II} + \text{ROH} \rightarrow \text{Peroxidase} + \text{RO}- + H_2O$$

The cyclooxygenase activity of prostaglandin synthase catalyzes the oxygenation of arachidonic acid to form the hydroperoxy endoperoxide prostaglandin G_2 (PGG_2). With a xenobiotic acting as an electron donor, PGG_2 is reduced to the hydroxyl endoperoxide PGH_2, with the coordinate oxidation of the hydroxyl group of the xenobiotic as illustrated in Figure 4.7, for acetaminophen cooxidation.

Two prostaglandin H synthase (PHS) enzymes, PGHS-1 and PGHS-2, have been characterized, and both are homodimeric integral membrane proteins. PGHS-1 and PGHS-2 are localized to the luminal surface of the endoplasmic reticulum, and PGHS-2 is also found in the inner and outer membranes of the nuclear envelope. Both enzymes share about 60% primary sequence identity but are encoded by separate genes on separate chromosomes. They are under regulatory control of cytokines and growth factors.[133] Two catalytic activities copurify with the synthase: fatty acid cyclooxygenase and prostaglandin hydroperoxidase. The cyclooxygenase catalyzes arachidonic acid oxidation to (PGG_2), and the hydroperoxidase reduces the hydroperoxide (–OOH) to the corresponding alcohol in prostaglandin H_2, as shown in Figure 4.10. Oxidation of xenobiotics results from a one-electron pathway involving an oxidizing agent produced during the hydroperoxidase-catalyzed reduction of PGG_2 to the hydroxyl endoperoxide PGH_2. Prostaglandin synthetase is a major source of alkyl hydroperoxides produced during normal metabolism. Most tissues possess prostaglandin synthetase activity and are capable of oxidizing certain xenobiotics, even if the tissue is low in P450 content. In fact,

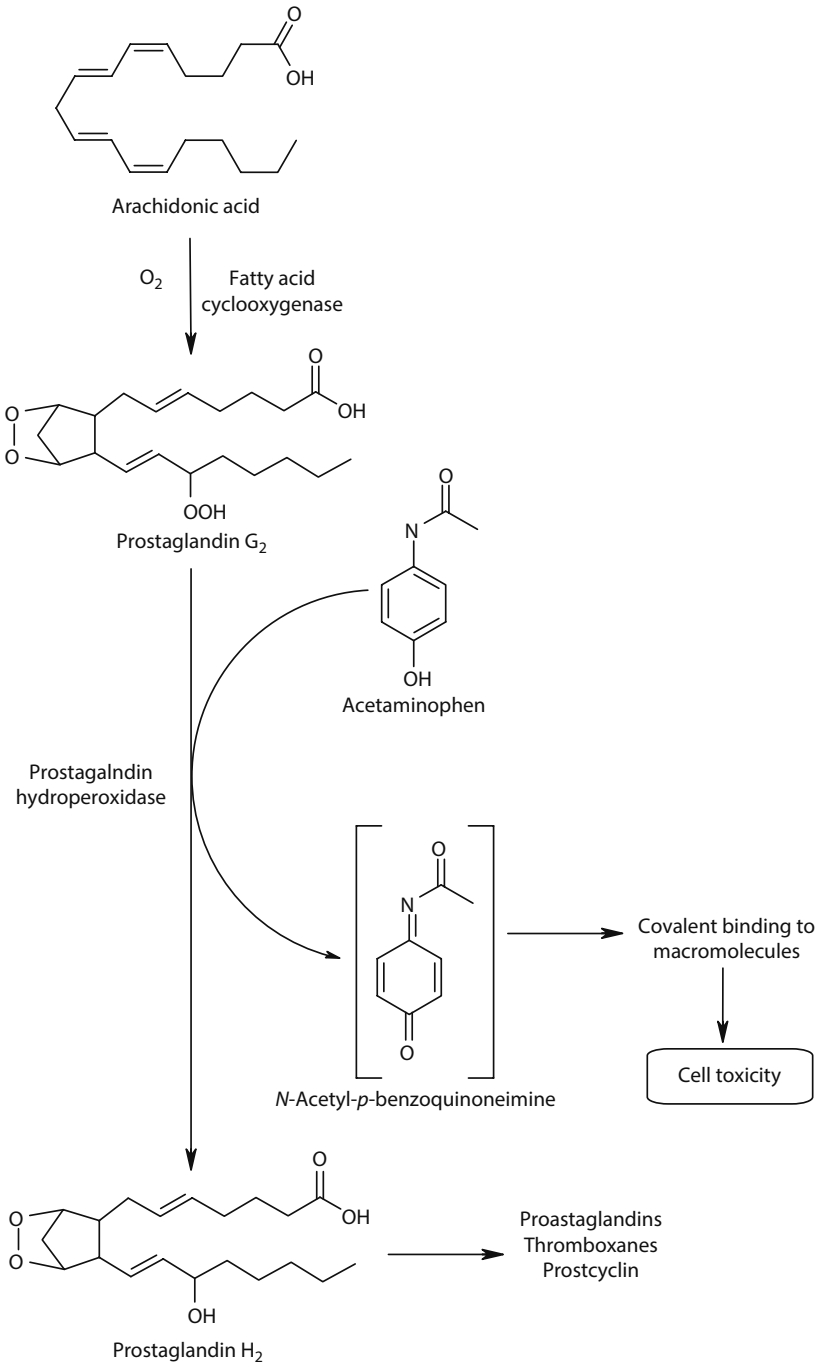


FIGURE 4.10 Cooxidation of acetaminophen by prostaglandin endoperoxide synthase.

acetaminophen, which is activated to a reactive intermediate by P450, can also be activated by prostaglandin synthetase in the medulla of the kidney. This tissue is low in P450 activity, but in the presence of arachidonic acid, the medulla activates acetaminophen to a reactive intermediate that covalently binds to tissue macromolecules.[133] The localization of prostaglandin synthetase in the inner medulla and papilla may be a contributing factor to the toxicity produced by other chemicals in this region of the nephron.[134] Other compounds that undergo cooxidation include aminopyrine, benzphetamine, oxyphenbutazone, benzidine, and BP.

In addition to kidney, other extrahepatic tissues including bladder, intestinal mucosa, spleen, and blood cells, such as peripheral blood mononuclear cells, and macrophages possess prostaglandin synthase activity. The bladder also possesses high prostaglandin synthetase activity. Mattammal et al.[135] proposed that several structurally diverse renal and bladder carcinogens are metabolically activated by prostaglandin synthetase; for example, the bladder carcinogen 2-amino-4-(5-nitro-2-furyl)thiazole is believed to be activated by prostaglandin synthetase cooxidation in bladder transitional epithelium to metabolites capable of covalently

TABLE 4.4
Human Peroxidases

Peroxidases	Cells	Subcellular Location
MPO	Neutrophils, leukocytes	Lysosomes
EPO	Eosinophils	Lysosomes
LPO	Mammary ductal epithelial cells, secretory cells of exocrine glands	Extracellular milk, saliva, tears
TPO	Thyroid follicular cells	Rough endoplasmic reticulum, Golgi apical membrane, perinuclear membrane
PGHS-1	Platelets, seminal vesicles	—
PGHS-2	Inflammatory cells	—

Source: Adapted from O'Brien, P.J., *Chem. Biol. Interact.*, 129, 113, 2000.
Notes: MPO, myeloperoxidase; EPO, eosinophil peroxidase; LPO, lactoperoxidase; TPO, thyroid peroxidase; PGHS-1, prostaglandin H synthase 1; PGHS-2, prostaglandin H synthase 2.

modifying RNA and DNA. Feeding aspirin to rats can inhibit the bladder lesion induced by 5-nitrofuran, the ultimate carcinogen. This suggests that prostaglandin synthetase is involved in the metabolic activation, as aspirin is a specific inhibitor of prostaglandin synthetase.

Use of the analgesic *p*-phenetidine has declined because of reports of kidney damage in humans following prolonged use of the drug. Andersson et al.[136] proposed a mechanism by which phenetidine is activated by prostaglandin synthetase in the kidney. The primary amine nitrogen of phenetidine undergoes a one-electron oxidation similar to that shown in Figure 4.10 for acetaminophen. This leads to hydrogen abstraction, yielding a reactive nitrenium radical. A radical intermediate is postulated based on its rate of reaction phenacetin with reduced GSH in the presence of arachidonic acid and microsomes from sheep seminal vesicles. Benzene can be hydroxylated to phenol in the liver by P450, and the phenol can be further oxidized to hydroquinone. The phenol and hydroquinone can enter the blood stream and be distributed to other tissues. In the bone marrow, the phenol stimulates prostaglandin synthetase peroxidative activation of hydroquinone to reactive metabolites that form adducts with nucleophiles, such as protein and DNA. This is believed to result in the bone marrow suppression seen with chronic exposure to benzene. Phenolic compounds may be converted to reactive phenoxyl radicals by the one-electron oxidative process.

Another example of a PGHS-2-mediated metabolic activation reaction is the biotransformation of procainamide. Drugs such as procainamide undergo an N-acetylation reaction as a primary means of elimination; however, as discussed in another section, individuals with the slow acetylator phenotype do not readily eliminate procainamide via the N-acetylation pathway, leaving more drug to reach extrahepatic tissues. It is in tissues such as monocytes and macrophages that PGHS-2 can oxidize procainamide to the hydroxylamine and nitroso derivatives. These reactive molecules are proposed to form haptens and subsequently sensitize T-cells. The enhanced neoantigen formation and T-cell sensitization seen in slow acetylators might be explained by the higher concentration of procainamide that is available for extrahepatic N-oxidation in antigen-presenting cells.[137]

In the developing embryo and other conceptal tissues, levels of cytochrome P450 expression are very low, especially in the first trimester. Oxidative metabolism, however, can proceed through peroxidative mechanisms dependent on prostaglandin synthase as well as lipoxygenase, peroxidase, and lipid peroxidation-coupled cooxidation.[132] Metabolic activation of xenobiotics to toxic intermediates through these mechanisms may be responsible for certain terata. Phenytoin, an antiepileptic drug and known teratogen, was shown in vitro to be less effective when mouse embryos were cultured in the presence of inhibitors of the prostaglandin synthase and lipoxygenase pathways.

MPO, EPO, and LPO are unique among the peroxidases in that they are primarily found in lysosomes of neutrophils, eosinophils, and secretory cells of the exocrine glands, respectively (Table 4.4).[132] Neutrophil MPO catalyzes the oxidation of halides by hydrogen peroxide to produce hypohalous acid. Leukemias induced by benzene exposure have been attributed to DNA prooxidant phenoxyl radicals formed by the MPO/H_2O_2-mediated oxidation of the benzene CYP2E1 product phenol. TPO is a membrane-bound enzyme localized to the thyroid follicular cells and is under regulatory control of thyroid-stimulating hormone. TPO catalyzes the iodination of thyroglobulin tyrosine residues.

Alcohol and Aldehyde Dehydrogenases

Alcohol Dehydrogenase

ADHs catalyze the NAD^+-dependent oxidation of primary and secondary alcohols to aldehydes and ketones, respectively.[139] ADHs are dimeric cytosolic proteins with a molecular weight of approximately 40,000 and contain one structural and one catalytic zinc atom. Mammalian ADHs are encoded by six different genes (ADH1–ADH6), each of which codes for an individual subunit (designated α, β, γ, π, χ, and σ). Only five genes (ADH1–ADH5) are found in primates, including humans.[71] The α, β, and γ subunits have >90% sequence

homology and can thus form both homodimers and heterodimers. The π and χ subunits have lower homology and can only form homodimers. ADHs are divided into four classes (I–IV) based on their subunit composition. Class I ADHs, composed of α, β, and γ hetero- and homodimers, are the most important isoforms involved in ethanol metabolism. Class I ADHs are expressed at high levels in liver and adrenal gland and at lower levels in a variety of other tissues. Class IV ADH, composed of σ homodimers, is expressed primarily in the gastrointestinal tract in adult humans and is responsible for first-pass ethanol metabolism as well as extrahepatic oxidation of retinoids.[139] Because of its localization and activity in ethanol metabolism, this isoform is thought to be involved in the induction of gastrointestinal cancer following chronic alcohol abuse.[140]

The catalytic mechanism involves initial deproteination of the hydroxyl group, followed by hydride transfer to the NAD^+ cofactor (Figure 4.11). Enzyme activity can be followed by monitoring formation of NADH spectrophotometrically, and this has been used as a convenient nonspecific assay for ADH activity. ADHs have wide substrate specificity and can catalyze the dehydrogenation of a variety of primary and secondary aliphatic alcohols and aromatic alcohols, as well as diols and aminoalcohols.[141–143] Primary alcohols are more readily dehydrogenated compared to secondary alcohols, and within a series, catalytic efficiency appears to be correlated with lipophilicity. Pyrazole and 4-methylpyrazole have been used as selective inhibitors of ADH, although some isoforms such as ADH2 are resistant to these inhibitors[71] and at higher concentrations, these compounds can inhibit P450 activity as well.

ADHs are active in the metabolism of a variety of drugs and other xenobiotics. The most obvious example is conversion of ethanol to acetaldehyde, which is detoxified by ALDHs (discussed in the following). ADH mediates toxicity of a number of alcohol-containing toxicants; for example, ADHs oxidize methanol to formaldehyde, which is converted to formic acid, resulting in metabolic acidosis. Interestingly, formaldehyde can be scavenged by reduced GSH, and the resulting conjugate, *S*-hydroxymethylglutathione, is a major substrate for ADH3.[144] Similarly, ADH-mediated oxidation of ethylene glycol to glyoxal, which is ultimately converted to oxalic acid, results in kidney toxicity.[145,146] The ADH inhibitor 4-methylpyrazole is used to treat accidental and intentional ingestion of methanol and ethylene glycol. A third example is the ADH-mediated dehydrogenation of allyl alcohol to the hepatotoxin acrolein.[147]

Expression of ADH is polymorphic, with allelic variants occurring at the ADH2 and ADH3 loci (β and γ subunits). Humans express 3 isoforms of ADH1 (ADH1A–ADH1C), with broad and overlapping substrate specificity. Bile acids are exclusively metabolized by ADH1C.[71] ADH2*2 isoforms, containing at least 1 β_2 allele and collectively known as atypical ADH, occur at a frequency of approximately 90% in Pacific rim Asian populations and are responsible for the rapid oxidation of ethanol to acetaldehyde in these

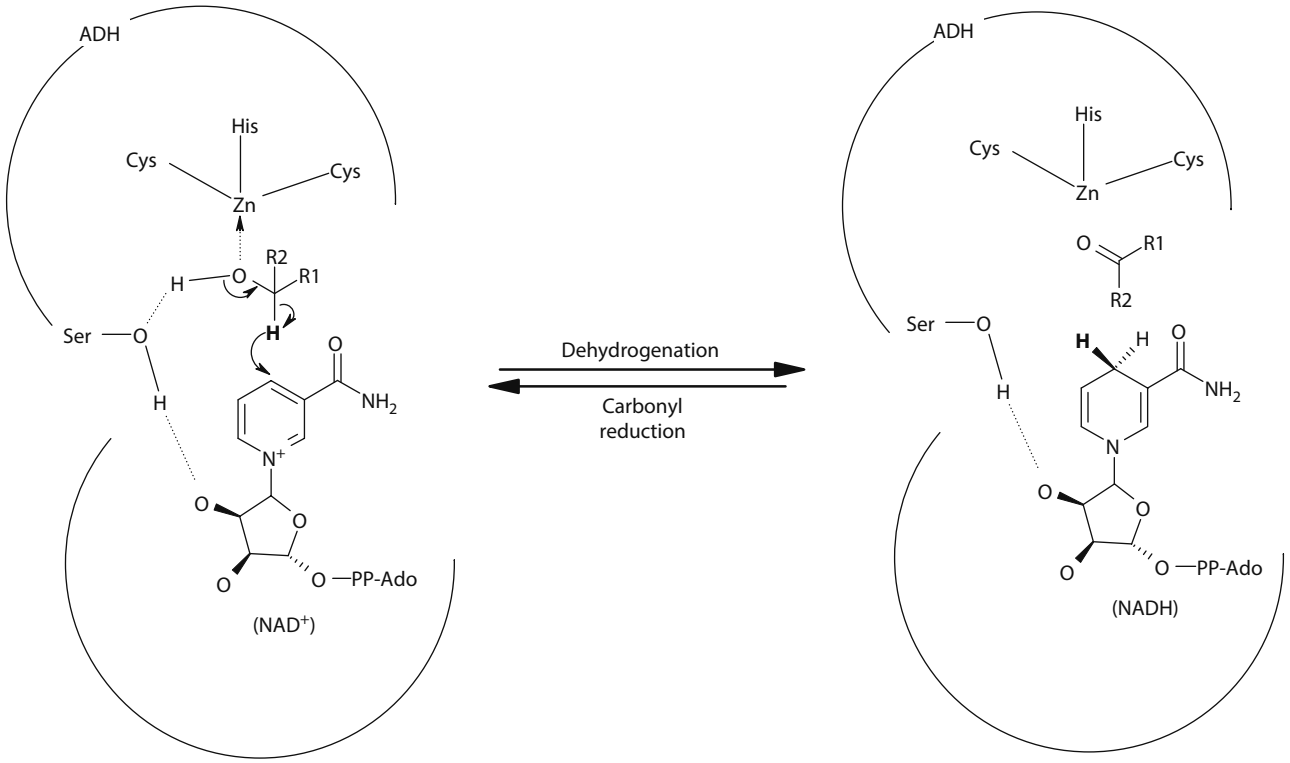


FIGURE 4.11 Catalytic mechanism of cytosolic ADH. Initial deproteination of the alcohol group is accomplished by a proton shuttle involving an active site serine, the ribosyl group of the cofactor, and an active site histidine residue (not shown). As shown, the reaction is reversible so ADH can also function as a carbonyl reductase.

individuals.[148] These isoforms are found at a much lower frequency in Caucasians and African Americans. ADH3*1 and ADH3*2 variants also occur at a high frequency in Pacific rim Asians, but these variants have little impact on ethanol metabolism.

Aldehyde Dehydrogenase

ALDHs are the major enzymes responsible for oxidation of aldehydes to carboxylic acids. ALDHs are found in the cytosol, mitochondria, and endoplasmic reticulum, and both constitutive and inducible forms are expressed in numerous tissues.[139] ALDHs exist as tetramers with an approximate molecular weight of 200,000–250,000 Da. ALDHs can use either NAD^+ or $NADP^+$ as a cofactor, and cofactor preference is isoform specific. ALDH genes are classified into families and subfamilies based on sequence homology. Isoforms with >40% homology are assigned to the same family (designated by an Arabic number), while enzymes with >60% similarity are assigned to the same subfamily (designated by a letter). Individual subfamily members are designated by an Arabic number. At least 17 ALDH genes have been identified in humans. These are arranged into 10 families and 13 subfamilies.[149]

The mechanism of ALDH catalysis is illustrated in Figure 4.12. In the initial step, the NAD(P) cofactor binds with the enzyme, followed by binding of the aldehyde substrate, which forms a covalent bond with an active site cysteine sulfhydryl group. The second step is the transfer of a hydride ion from the substrate to the pyridine moiety of NAD(P), which effectively oxidizes the substrate to an acyl compound. In the final step, the acyl compound is hydrolyzed, giving rise to a carboxylic acid.

Similar to ADH, ALDH can oxidize a broad array of substrates, including aliphatic and aromatic aldehydes and dialdehydes. ALDHs are involved in a number of endogenous biosynthetic pathways, such as the synthesis of retinoids, amino acids, and neurotransmitters; metabolism of folate and fatty aldehydes; and detoxification of aldehydes generated by lipid peroxidation. Xenobiotic substrates include acetaldehyde (from metabolism of ethanol), *p*-nitrobenzaldehyde, acrolein, and aldophosphamide (from metabolism of cyclophosphamide). Metabolism of acetaldehyde is mediated by ALDH1A1, ALDH1B1, and ALDH2 in humans, with the latter isoform taking the predominant role. ALDH1A1 also catalyzes oxidation of retinal, and competition between this substrate and acetaldehyde may be involved in the deficiencies in vitamin A metabolism observed in alcoholics.[150] The dithiol compound disulfiram is a potent inhibitor of ALDHs and has been used as a deterrent in the treatment of alcoholism; however, disulfiram has limited value as a probe inhibitor of ALDH in vivo, as its metabolite diethyldithiocarbamate also irreversibly inhibits CYP2E1. ALDHs are induced by PAHs and TCDD by an Ah-receptor-mediated mechanism. PB also induces some ALDHs, although the specific mechanism underlying this effect has not been investigated.

Many allelic variants of ALDH have been identified, although only a few are thought to play a role in polymorphic xenobiotic metabolism. The most thoroughly studied ALDH polymorphism involves a deficiency in ALDH2. The ALDH2*2 variant is caused by a point mutation in exon 12,

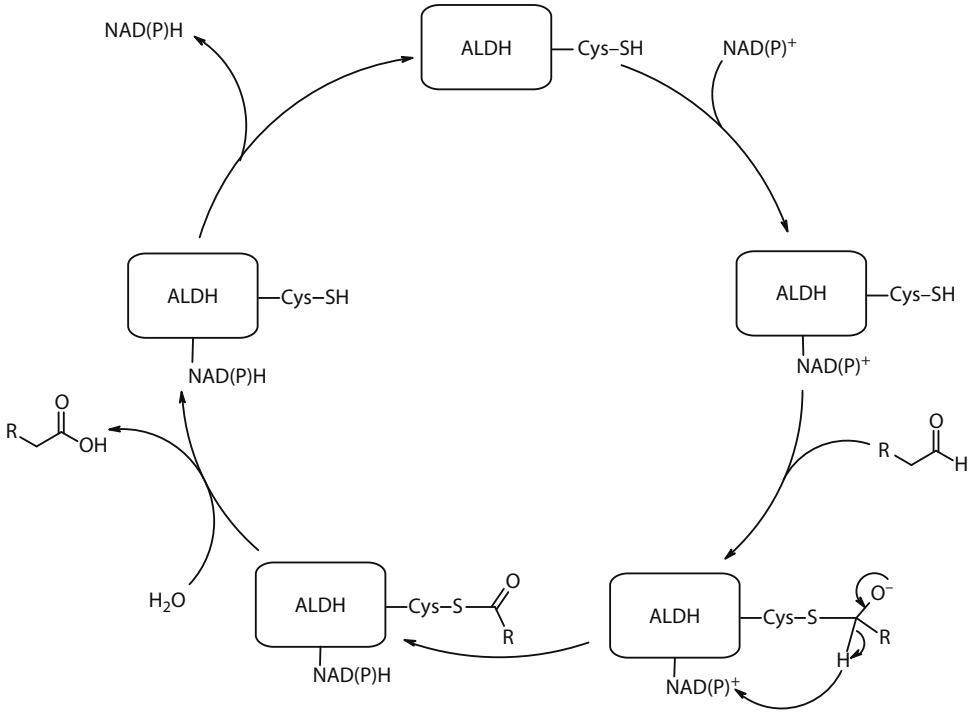


FIGURE 4.12 Catalytic mechanism of cytosolic ALDH. The oxidation of aldehydes to carboxylic acids occurs in two steps: dehydrogenation and thioester hydrolysis. By virtue of the second activity, ALDH can also function as an esterase.

resulting in synthesis of an inactive enzyme. This allele is common in Asian populations, and individuals carrying this variant have a compromised ability to detoxify acetaldehyde following consumption of ethanol. This deficiency is manifested as the so-called flushing syndrome common in Asians following ethanol consumption. ALDH1A1 polymorphism may also play a role in ethanol tolerance, although to a lesser degree.

Quinone Oxidoreductases

NAD(P)H:Quinone Oxidoreductase 1

NAD(P)H:quinone oxidoreductase 1 (NQO1; also known as DT diaphorase) catalyzes the two-electron reduction of quinones to the corresponding hydroquinones.[151,152] Functional NQO1 is a cytosolic homodimer containing two FAD prosthetic groups. High levels of NQO1 are found in liver of rodents and other common laboratory species. In contrast, human NQO1 was detected in lung, breast, and gastrointestinal epithelium; vascular endothelium; adipocytes; bone marrow; and several areas of the eye but not in liver. Thus, this enzyme does not appear to play a role in hepatic metabolism of xenobiotics. In addition to quinones, NQO1 can catalyze two-electron reductions of a wide variety of functional groups including quinone imines, GSH conjugates of naphthoquinones, azo compounds, and aromatic nitro compounds. NQO1 can also catalyze four-electron reductions of aromatic azo and nitro compounds. Dicumerol is a potent inhibitor of NQO1.

Quinone reduction by NQO1 can result in either detoxification or bioactivation, depending on the stability of the resulting hydroquinone; thus, NQO1 detoxifies redox-active quinones such as menadione by converting them to stable hydroquinones that are substrates for phase II conjugation reactions. Less stable hydroquinones can undergo autooxidation to semiquinone radicals or rearrange to alkylating species.

NQO1 can be induced by PAHs, azo compounds, and phenolic antioxidants, as well as by oxidative stress. The 5′ flanking region of the NQO1 gene contains both an antioxidant response element (ARE) and an XRE. Induction controlled by the XRE is mediated by the Ah receptor as described for P450 induction. Induction of NQ1 by the ARE may be mediated by a variety of transcription factors, including Jun, Fos, and Nrf, among others.[116]

Several polymorphisms of NQO1 have been identified. The most striking of these is NQO1*2*2, in which no active protein is produced. The frequency of this genotype varies from about 4% in Caucasians to over 20% in East Asian populations. The presence of this genotype has been shown to be a risk factor for the development of hematotoxicity of benzene.

NAD(P)H:Quinone Oxidoreductase 2

NAD(P)H:quinone oxidoreductase 2 (NQO2) has approximately 82% sequence similarity to NQO1, although it is 43 amino acids shorter at the carboxy terminal of the protein. In humans, NQO2 is expressed in a variety of tissues, including liver, kidney, and lung, although the highest expression levels were found in skeletal muscle. NQO2 is also similar to NQO1 in many respects, such as substrate specificity and response to inducers; however, these enzymes are different in a number of important ways. For example, NQO2 uses dihydronicotinamide riboside as a cofactor instead of NAD(P)H. Inhibitor selectivity is also different; NQO2 is inhibited by quercetin and BP but not by dicoumarol. Despite gains in understanding the expression and functional activity of this enzyme, the significance of NQO2 for endobiotic and xenobiotic metabolism remains to be demonstrated.[151,153]

BIOCHEMICAL CONJUGATIONS

Mammals can synthesize xenobiotic conjugates that are more polar and readily excreted compared with the parent compound. Two major reactants are required for conjugate synthesis: a xenobiotic with the appropriate functional group and a cosubstrate that can be conjugated with the xenobiotic. If the xenobiotic does not have a functional group amenable to conjugation, such as a hydroxyl group, it may be oxidized (functionalized) by cytochromes P450. The oxidized product and the cosubstrate must be simultaneously available for conjugation. Both functions must be tightly integrated for rapid excretion of the xenobiotic. Although the forthcoming sections will discuss each conjugation system as a separate entity, it must be emphasized that in vivo metabolism is integrated. Examples showing the integration of the conjugation systems with related pathways will be presented.

Glucuronidation: Uridine Diphosphoglucuronosyltransferases

P450s are the principle phase I oxidative enzymes. Similarly, uridine diphosphoglucuronosyltransferases (also known as UDP-glycosyltransferases [UGTs]) are the principal phase II enzymes. Glucuronosyltransferases can use monooxygenase products to form glucuronides; however, it is not a necessity for substrates of the glucuronosyltransferase to be monooxygenase products. Significant numbers of xenobiotics and certain endobiotics possess the necessary functional groups for glucuronidation and do not require functionalization. This pathway has been estimated to account for 35% of all drugs metabolized by phase II drug-metabolizing enzymes.[154] UDPGTs occur in several tissues, but their highest activity is found in the liver. Most isoforms have a distinct hepatic and/or extrahepatic expression resulting in significant expression in kidney, intestine, and steroid target tissues.[155] As such, glucuronosyltransferase is a significant metabolic pathway contributing to the elimination of xenobiotics and affecting poor bioavailability of orally administered drug substrates.[156]

Whereas the multienzyme complex of the P450 monooxygenase is termed a system because the enzymes are closely linked, the multiple enzymes of glucuronidation are not linked but are interdependent. The general reaction mechanism of the conjugating enzymes involves the activation of

an endogenous molecule. Subsequent reaction of this activated form of the endogenous molecule with the xenobiotic produces the conjugate. Activation may occur in a different cellular compartment than conjugation, as is the case with glucuronidation. Activation of glucose occurs in the cytosol, whereas conjugation occurs in the lumen of the endoplasmic reticulum.

Although the products of P450 are more water soluble than their parent compounds, some still possess considerable lipophilicity. Subsequent conjugation produces metabolites with higher water solubility. These metabolites can generally be readily excreted in the bile or urine. Transport proteins recognize the glucuronic acid moiety of the glucuronide and aid in excretion from the liver and kidney. An additional method by which glucuronidation produces less toxic metabolites is via the addition of a bulky moiety to the xenobiotic. This can result in both the shielding of reactive portions of the xenobiotic and in the blocking of reactions between the xenobiotic and the site responsible for the toxicological sequelae. In some cases, the product of glucuronidation has more toxicological activity than the parent compound, and conjugation can be considered metabolic activation, although examples are far fewer than with P450 oxidation. Similarly, conjugation with glucuronic acid results in significant structural change so pharmacologic activity is generally abolished, although in a few cases, glucuronidation will result in a molecule with similar or even greater pharmacologic activity.[157]

Recent findings on regulation of P450, UDPGTs, and transporters suggest that although nuclear receptor signaling induces different cytochromes P450, regulation may converge on single UGTs and transporters.[158] The nuclear receptors CAR, PXR, and AhR coordinate the induction of several CYP, UGT, and drug transporters (Table 4.5) and thus lead to differential expression of various UGT forms. As an example, rifampicin induction of CYP3A4 is PXR mediated and is responsible for the conversion in the liver of lithocholic acid to the less toxic form hyodeoxycholic acid. Hyodeoxycholic acid is in turn conjugated by several UGTs, and transport out of the hepatocyte is mediated by the rifampicin-inducible transporter multidrug resistance protein 2 (MRP2). Similarly, comedication with rifampicin leads to reduced effects of ezetimibe, an inhibitor of the cholesterol uptake transporter, by faster elimination via glucuronidation and subsequent intestinal or hepatic secretion via the efflux transporter P-glycoprotein (P-gp) and MRP2.[159]

Glucuronides are secreted either by the liver into the bile and consequently found in the feces or by the kidney into the urine. The excretion route is generally dependent on the molecular weight of the xenobiotic. In both cases, secretion is via specific organic anion transporters, members of the adenosine triphosphate (ATP)-binding cassette superfamily such as MRP2, at the apical plasma membrane, and MRP3, at the basolateral membrane of hepatocytes and enterocytes. The rat excretes glucuronides of xenobiotics with molecular weights greater than about 250–300 into the bile and those with lower molecular weights in the urine. Higher molecular weight xenobiotics, such as morphine, chloramphenicol, and endogenous steroids, are excreted in bile and enter the intestine. Biliary excretion can result in enterohepatic circulation, which can cause prolonged plasma half-lives for some compounds. Intestinal microflora express the enzyme β-glucuronidase, which catalyzes the hydrolysis of glucuronide conjugates. This releases the xenobiotic (referred to as the *aglycone*) in the intestine, where it can be absorbed into the blood. The xenobiotic can then be taken up by the liver, where it is reconjugated and excreted into the bile, where the cycle is again initiated. This can cause prolonged exposure to target organs, such as the liver, and result in unanticipated toxicity.

Nomenclature for UDP-Glucuronosyltransferase Gene Superfamily

Nomenclature for the UDPGTs has progressed similarly to that for the P450 superfamily.[160,161] It has been proposed that each gene be identified by the root symbol UGT for UDPGT. The gene family is identified by a number, and a letter is added to designate the subfamily (e.g., UGT2B) followed by a number to identify the gene (e.g., UGT2B1). This system, as with the P450 nomenclature, is an attempt to provide isoforms with a name that is not only specific but also reflects the evolutionary divergence of the genes.

There are four main gene families denoted UGT1, UGT2, UGT3, and UGT8.[162] Unlike UGT1 and UGT2, neither UGT3 nor UGT8 use UDP-glucuronic acid. UGT1 and UGT2

TABLE 4.5
Selected Human and Rodent CYPs, UGTs, and Glucuronide Transporters Induced by the Ah, CAR, and PXR Receptors

Nuclear Receptor	Uptake Transporter	CYPs	UGTs	Export Transporter
AhR	Not determined	CYP1A1	UGT1A1	Not determined
	CYP1A2	UGT1A6 (rUGT1A7)		
CAR	OATP2 (mOatp2)	CYP2B6 (rCYP2B6, mCyp2b10)	UGT1A1 (rUGT2B1)	MPR2 (mMrp2)
PXR	OATP2 (mOatp2)	CYP3A4 (rCYP3A23, mCyp3a11)	UGT1A1 (mUgt1a6, mUgt1a9)	MRP2
				MRP3

Source: Bock, K.W. and Köhle, C., *Drug Metab. Rev.*, 36, 595, 2004. With permission.
Note: Inducible rodent enzymes are listed in parentheses.

families are solely responsible for drug glucuronidation and therefore the discussion here is restricted to those enzymes. These two families are further divided into UGT1A, UGT2A, and UGT2B. The entire UGT1A family is derived from a single-gene locus and encodes eight different proteins via alternative splicing of the UDPGA-binding domain with different substrate-binding domains. Most of the UGT1A isoforms have been isolated from human liver whereas three isoforms have been isolated from extrahepatic sources such as bile ducts, tissues of the entire gastrointestinal tract, olfactory epithelium, brain, and fetal lung. UGT2 genes encode seven proteins and have also been isolated from liver, gastrointestinal tract, mammary gland, prostate, and adrenal tissues.

Biochemistry of Glucuronidation

Glucuronidation (illustrated in Figure 4.13) requires the availability of three reactants:

- UDP-α-D-glucuronic acid (UDPGA), generated in the cytoplasm
- UDPGT, bound to the endoplasmic reticulum
- Substrate with the requisite functional group and some hydrophobic character

Maximal enzyme activity is dependent on optimal concentrations of these reactants at the membrane site of catalysis.

As seen in Figure 4.13, D-glucose is the original precursor of UDPGA. During anabolic metabolism, D-glucose is converted to β-D-glucose-1-phosphate. This compound serves as substrate for UDP-glucose pyrophosphorylase, which catalyzes its reaction with uridine triphosphate to yield the high-energy phosphate-containing UDP-D-glucose and pyrophosphate. UDP-D-glucose then reacts with nicotinamide adenine dinucleotide (NAD) catalyzed by UDP-glucose dehydrogenase to yield UDP-D-glucuronic acid, which completes glucose activation. This compound is termed the glycone, indicating its source. The xenobiotic that is conjugated is termed the aglycone. Glucose activation occurs within the cytoplasm, whereas glucuronidation of the aglycone occurs at the endoplasmic reticulum. Because UDP-D-glucose is also used in glycogen synthesis, it generally is available in the cell. This is not true for all conjugation reactions and may be one of the reasons why glucuronidation is a major conjugation pathway.

UDPGTs are oriented in the endoplasmic reticulum in such a way that the majority of the protein protrudes into the lumen of the endoplasmic reticulum. The intraluminal portion of the protein possesses the UDP-glucuronic acid-binding domain as well as the xenobiotic- or endobiotic (endogenous substrates)-binding domain. This means that UDP-glucuronic acid must pass through the membrane, possibly by carrier mediation, and that the substrate must also pass through the membrane.[163] Molecular biology studies indicate that the C-terminal half of the protein is highly conserved among different UDPGTs, whereas the N-terminal region is highly variable. The C-terminal half of the protein contains the transmembrane sequences that anchor the enzyme within

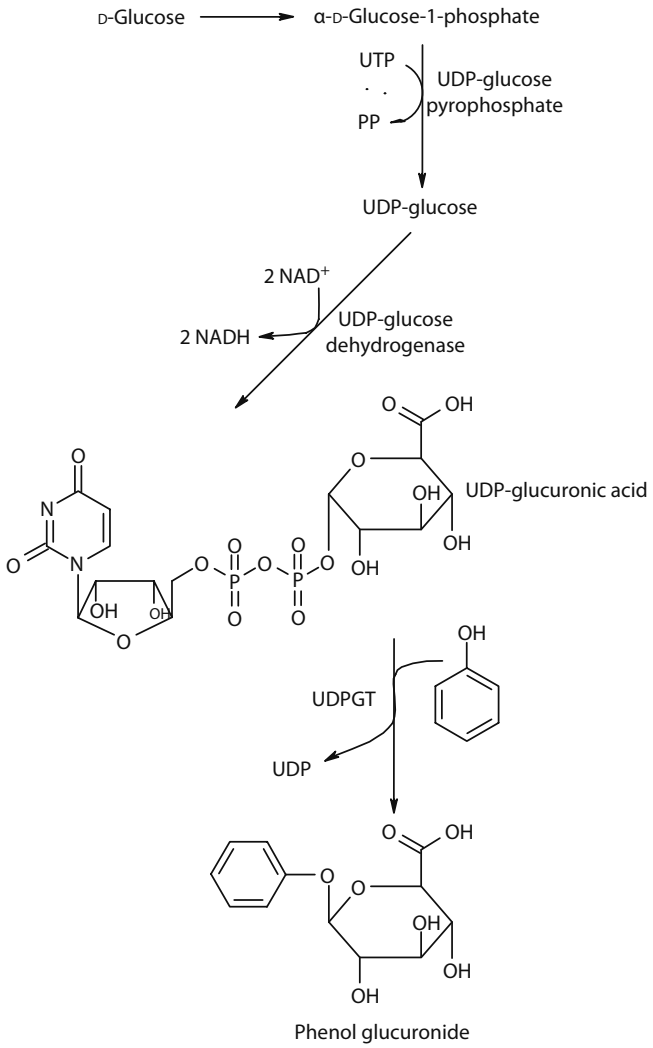


FIGURE 4.13 Glucuronidation of phenol; an example of the pathway leading to the production of glucuronic acid conjugates.

the membrane and the short portion of the C-terminus that protrudes from the outside surface of the endoplasmic reticulum into the cytoplasm. The C-terminal half of the enzyme may contain a UDP-glucuronic-acid-binding site. The broad substrate specificity is believed to come from variation in the primary sequence of the N-terminal region where the substrate-binding domain resides.[164]

UDP-glucuronic acid and the aglycone (xenobiotic or endobiotic) must be present for the conjugation reaction to be initiated. The number of xenobiotics that have been shown to be substrates for UDPGTs is large and continues to grow.[165] The major functional groups forming glucuronides are (1) hydroxyl, (2) carboxyl, (3) amino, and (4) sulfhydryl. The substituents to which these functional groups are attached can be quite variable (see Table 4.6). Similar to the substrate requirements for monooxygenases of the endoplasmic reticulum, the aglycone must be somewhat lipid soluble to be a substrate for the UDPGTs. This requirement reflects the need for the xenobiotic to penetrate the endoplasmic reticulum to gain access to the active site. All of the endobiotics

TABLE 4.6
Xenobiotic Substrates Glucuronidated by Expressed Human UDP-Glucuronosyltransferases

Human Glucuronides	Substrates
Linkage through –O–	
Aryl hydroxyl (ether)	Simple and complex phenols, anthraquinones and flavones, opioids and steroids, hydroxylated coumarins
Aryl or alkyl enolic	Coumarins, steroid-dione structures
Alkyl hydroxyl	Primary, secondary, tertiary alcohols
Acyl hydroxyl (carboxylic esters)	Bilirubin, carboxylic acids
Linkage through –S–	
Aryl and alkyl thiols	No examples reported
Linkage through –C–	No examples reported
Linkage through –O–	
Sulfonamides	No examples reported
Nonquaternary	Primary and secondary amines, arylamines, arylamine N–OH, tetrazoles
Quaternary	Cyclic tertiary, alicyclic tertiary, imidazoles, pyridines, triazoles

Source: Tukey, R.H. and Strassburg, C.P., *Annu. Rev. Pharmacol. Toxicol.*, 40, 581, 2000. With permission.

associated with normal metabolism and homeostasis that are substrates for the UDPGTs are lipid soluble and include bilirubin, catechols such as 3-*O*-methyladrenaline, serotonin, and 17-hydroxy-containing steroids.

Reactions Catalyzed by the UDP-Glucuronosyltransferases

As with many of the enzymes of detoxification, the glucuronosyltransferases have a low order of substrate specificity. This lack of substrate specificity makes them ideally suitable as detoxification enzymes. Whether or not they evolved as detoxification enzymes or represent enzymes of normal metabolism whose lack of specificity makes them suitable for detoxification is open to debate. Of interest in this respect is that they occur only in higher organisms. Glucuronosyltransferases have been found in all mammals, birds, and reptiles that have been investigated, although their specific activities toward specific substrates may vary among different species and strains. Unlike the monooxygenase, they have not been found in bacteria and other lower species. This fact suggests that these transferases evolved to metabolize endogenous compounds, such as bilirubin, catecholamines, and steroids, and not as detoxification enzymes.[166]

Table 4.6 illustrates the functional groups, generally nucleophilic heteroatoms, that form glucuronides and examples of the reactions. The glucuronides formed from these functional groups have different properties. Stability is among the most important with respect to detoxification. Breakdown of the glucuronide can lead to reformation of the parent compound and in certain cases the production of highly reactive electrophilic species. These reactive species may be responsible for the production of acute and chronic toxicity by covalent binding to nucleophilic sites on tissue macromolecules.

Among the most commonly encountered glucuronides are those involving linkage of glucuronic acid and the xenobiotic through an oxygen atom. These O-glucuronides may form with a number of chemical classes, including aryl, alkyl, and acyl compounds, as illustrated in Table 4.6.

The alkyl-O-glucuronides are ether-linked glucuronides that can form from a variety of primary, secondary, and tertiary alcohols. Although generally stable at physiological conditions, they can be hydrolyzed under acidic conditions.

The enolic glucuronides are formed from aglycones without a free hydroxyl group. Glucuronides are formed from the enolized keto group. These conjugates lack the stability of the ether glucuronides and are susceptible to both acid and alkaline hydrolysis. They are more stable at neutral and alkaline pH than in acid conditions. Ester glucuronides can be produced from a variety of carboxylic acids, including primary, secondary, and tertiary aliphatic acids and both aryl and heterocyclic compounds. They generally are stable in acidic conditions but are susceptible to alkaline hydrolysis.

The chemical properties of N-glucuronides are different from those of O-glucuronides. One of the most important of these is their lack of stability. They are especially unstable at pH below neutrality. The instability of these compounds may have important biological consequences; examples are discussed in more detail later. Quaternary ammonium N-glucuronides are formed by N-glucuronidation of cyclic and acyclic tertiary amines. These charged metabolites may be formed in higher primates while not being found in other animal models, such as the rat.

The S-glucuronides are not as commonly encountered as the O-glucuronides, but they represent important detoxification pathways for thiolic compounds. Their stability is similar to that of the O-glucuronides.

The C-glucuronides represent recently recognized conjugates, and only a few examples are known, such as phenylbutazone. Generally, they appear to be formed by the transferase, but other possible mechanisms of formation have been suggested.

Role of UDP-Glucuronosyltransferases in Detoxification and Metabolic Activation

The foregoing discussion indicates that the UDPGTs play a critical role in the metabolism and detoxification of xenobiotics. Some substrates require functionalization by the monooxygenase before metabolism by the transferase, whereas others can be directly conjugated. The conjugates are more water soluble than the parent xenobiotic, and some readily form salts. Addition of the glycone may enable some of the conjugates to be more readily excreted through carrier-mediated mechanisms. Mechanisms other than increased excretion rates may also be important. The addition of the relatively bulky glycone may hide or hinder the biological

reactivity of particular functional groups on the xenobiotic. In addition, binding of the toxicant to particular receptors responsible for toxicity may be blocked. Overall, these mechanisms represent an efficient system for detoxification. On the other hand, glucuronidation of certain compounds represents a metabolic activation where the product is more toxic than the parent compound.

Aromatic amines are among the most studied examples of the role glucuronidation plays in metabolic activation of carcinogens. These glucuronides transport the proximate carcinogen to the target site, where it decomposes to the species that react with cellular macromolecules producing the biochemical lesion responsible for generating the pathological lesion.

Several of the arylamines are potent bladder carcinogens, including 4-aminobiphenyl, 1-naphthylamine, and benzidine. Metabolic activation of these carcinogens to the ultimate carcinogen appears similar and requires the action of UDPGT. Metabolic activation begins with P450-dependent activation of the arylamine to the proximate carcinogen, an *N*-hydroxyarylamine. Other specific ring-hydroxylated forms may be produced and may represent more stable products. The unstable *N*-hydroxyarylamines are then converted to stable N-glucuronides. These N-glucuronides are transported to the bladder. In the bladder, the N-glucuronides are subject to β-glucuronidase activity, which splits off the aglycone. They are also subject to hydrolysis in acidic urine producing the *N*-hydroxyarylamine. The *N*-hydroxyarylamine spontaneously converts to the electrophilic arylnitrenium ion. A similar mechanism involving sulfonation-mediated formation of this reactive species is illustrated later in the chapter. The electrophilic arylnitrenium ion can then react with nucleophilic centers on macromolecules of the bladder epithelium, especially DNA, to initiate tumor formation. The concentration of the glucuronide in the bladder, in combination with the time the glucuronide remains in the bladder, can modify the potential for tumor formation. Glucuronides may function in this manner with a number of carcinogens and be important in explaining why certain target organs are susceptible to a specific carcinogen and others are not susceptible. In the earlier example, glucuronidation may protect the liver but makes the bladder, the target organ, susceptible.

Glucuronidation has also been implicated in adverse drug reactions of certain carboxylic drugs that have resulted in a toxic immunological response. It is believed that a reactive glucuronide covalently binds to cellular proteins that act as haptens, producing an anaphylactic reaction. Glucuronidation of the carboxylic acid moiety of drugs such as diclofenac, a nonsteroidal anti-inflammatory drug, leads to an unstable and reactive acyl glucuronide metabolite. The conjugate then undergoes transacylation of protein nucleophiles by the 1-*O*-acylglucuronide or glycation of proteins via mechanisms that involve open-chain aldehyde reactions with protein amino groups. These drug–protein adducts are believed to be recognized as foreign by the immune system, resulting in an immune response and thereby leading to the associated idiosyncratic hepatoxicity.[167]

Species, Gender, and Genetic Differences in UDP-Glucuronosyltransferase Activity

Studies of species, strain, and gender differences in glucuronidation are complicated by a number of factors. Activity may be affected by age, hormonal status, environmental exposure to xenobiotics in the diet and other sources, and nutritional status. Factors associated with the methodology to determine differences in glucuronidation also play a role, including substrate, assay method, method of freeing latent activity, and the method of isolating the preparation employed to measure activity. This has led to a number of reports of differences in activity that could be artifactual; however, the large number of reports concerning differences in glucuronidation among species, strains, and the sexes indicate that certain of these differences are real and may have a genetic basis.

As mentioned previously, lower animals, including prokaryotes and invertebrates, do not produce glucuronides. Fish and reptiles do demonstrate glucuronidation of xenobiotics but vary dramatically in activity, which is generally at least 10-fold lower than mammalian activity. Birds have glucuronidation ability similar to that of mammals.

Differences among mammalian species in their ability to glucuronidate a xenobiotic may be quite large, as is the case for the human immunodeficiency virus (HIV) drug zidovudine. This drug is eliminated in the rat and dog primarily unchanged, whereas the glucuronide represents the majority of metabolites in monkeys and humans.[168] The guinea pig generally has higher activity than most other laboratory species. This higher activity may be associated with less latent enzyme activity, as its UDPGTs can be activated by much gentler methods than other species. Cats are well known for their extremely low transferase activity. Although capable of forming glucuronides with endogenous compounds, they form only low levels of or no glucuronides with xenobiotics.

Glucuronidation of amines is divided into two groups: nonquaternary N-conjugates and quaternary N-conjugates. Major qualitative species differences do not appear to exist in the conjugation of the primary and secondary amines, sulfonamides, arylamines, and cyclic and heterocyclic amines to form nonquaternary N-conjugates, although quantitative differences do exist. Quaternary glucuronidation occurs in primates, including humans, but not in other species. In humans, quaternary-ammonium-linked glucuronides of aliphatic amines appear to be produced by UGT1A3 and UGT1A4.[170,171]

A well-known example of a strain difference is the almost complete lack of bilirubin glucuronidation in the Gunn rat. This rat strain also has low activity toward a number of xenobiotic substrates but normal activity toward others. There is a genetic component to this, with the low activity being autosomally recessive. The mutation in the Gunn rat responsible for its lack of bilirubin conjugation occurs in the UGT1 family and affects this entire group of isozymes. A frameshift mutation occurs because of a deleted guanine that results in a TGA stop codon occurring sooner than normal. This mutation results in a protein missing 115 amino acids that

constitute a hydrophobic region associated with insertion of the protein into the membrane. Lack of insertion negates the activity of this enzyme form and results in degradation of the incomplete protein. The genes in the UGT2 family are normally expressed in the Gunn rat.

Similar defects occur in humans and result in unconjugated hyperbilirubinemias. Gilbert's syndrome is a milder form of the disease that occurs in 2%–5% of the population. This large prevalence in the population makes it an important human genetic deficiency when considering interindividual variation in xenobiotic metabolism. These patients are characterized by mild, chronic, unconjugated hyperbilirubinemia that produces jaundice and an impaired ability to metabolize menthol. Decreased clearance of several drugs, including tolbutamide, rifamycin, josamycin, and paracetamol, has been observed. Crigler–Najjar syndrome is a familial form of severe unconjugated hyperbilirubinemia. Infants often develop severe neurological damage from bilirubin encephalopathy (kernicterus). Patients are divided into two types. Type I is more severe (unconjugated bilirubin, >20 mg/dL) and not responsive to barbiturate or glutethimide therapy. Type II patients respond to induction by PB, which suggests a fundamental difference from type I in the molecular basis of the genetic defect. Type I results from mutations in the UGT1 family that produces a loss of bilirubin conjugation,[172] whereas less severe mutations occur in type II that produce a decrease, but not a loss, of activity.

Gender differences appear hormonally related[173] and can be substrate dependent. Although it is sometimes stated that males have higher glucuronidation activity than females, this is substrate dependent, and no general classification should be made. Like monooxygenase activity, activity may be sensitive to imprinting or programming during the neonatal period. As with species and strain differences, care must be taken when extrapolating data obtained with one substrate to other substrates. Glucuronidation of estradiol and estrone is higher in female rats than male rats.[174] Paracetamol, oxazepam, and diflunisal are cleared 30%–50% faster in males, due primarily to enhanced glucuronidation.

Induction of the Glucuronosyltransferases

UDP-glucuronosyltransferases are inducible enzymes, much like cytochrome P450, and are inducible by some of the same chemicals. Evidence of a true induction process involving de novo protein synthesis and increases in mRNA has been observed for induction of the UDPGTs by PB. Most inducers of CYP1A, CYP2B, CYP3A, and CYP4A can induce these transferases. Rat UGT1A6 and UGT1A7 and human UGT1A6 and UGT1A9 are polycyclic-hydrocarbon-inducible transferases. Induction appears mediated by the Ah receptor. Rat UGT1A7 and human UGT1A9 have high activity toward the phenolic and diphenolic metabolites of polycyclics, such as BP.[175] Few specific inducers of the transferases that do not also induce the monooxygenase are known. For example, trans-stilbene oxide and ethoxyquin appear to only induce the transferases, but more studies are needed to determine if this is a true induction. Induction of the transferases modifies the toxicity of xenobiotics in a manner similar to induction of P450, as previously discussed.

Sulfonation: Sulfotransferases

Sulfonation of xenobiotics and endobiotics is catalyzed by a set of enzymes called *sulfotransferases* (SULTs). These enzymes belong to a multigene family and occur in prokaryotes, plants, and animals. Some of the enzymes are membrane bound and others occur in the cytosol. The membrane-bound SULTs are found in the Golgi membranes and are involved in the sulfonation of endogenous compounds, such as glycosaminoglycans, glycoproteins, and proteins, and peptides secreted by the Golgi apparatus; they are not involved in xenobiotic metabolism. The soluble or cytosolic SULTs catalyze the sulfate conjugation of a variety of substrates, including steroid hormones such as 17β-estradiol and dehydroepiandrosterone; thyroid hormones; catecholamines and xenobiotics, such as *N*-hydroxy-2-acetylaminofluorene; isoflavones; and many drugs, including acetaminophen and minoxidil. For the most part, sulfonation of xenobiotics results in metabolites that are less toxic than the parent compound; however, the SULTs, like many xenobiotic metabolism enzymes, can produce metabolically activated products that have mutagenic and carcinogenic potential.

Until recently, the SULTs have not been as intensely investigated as some of the other xenobiotic metabolism enzymes. Lately, interest has been renewed in these enzymes, particularly their description at the gene level. Utilization of the tools of molecular biology has provided new insight into their roles in metabolism, has revealed the complexity of their gene family, and has enabled development of a nomenclature system.[176–178] The ability to sequence the SULTs, identify new isoforms of these enzymes, and measure their activity with increased sensitivity has progressed faster than our understanding of their individual roles in xenobiotic metabolism.

Biochemistry of Sulfonation

A limiting factor in the sulfonation of xenobiotics by the SULTs is the availability of 3-phosphoadenosine-5′-phosphosulfate (PAPS).[179,180] As illustrated in Figure 4.14, PAPS is synthesized in a two-step process. The first step is formation of adenosine-5′-phosphosulfate (APS) catalyzed by ATP-sulfurylase. Although the synthesis of APS from sulfate and ATP is not energetically favored, the rapid hydrolysis of pyrophosphate and the rapid utilization of APS as a substrate for APS-kinase drive the reaction toward APS synthesis. APS-kinase catalyzes synthesis of PAPS from APS and ATP. This enzyme is tightly coupled with the ATP-sulfurylase, which results in the rapid utilization of APS.

Tissue concentrations of PAPS are relatively low compared to UDPGA, the active form of glucuronic acid used in glucuronidation. During active sulfonation, PAPS becomes rapidly depleted; for example, the SULT has a high affinity for acetaminophen, which forms a sulfate conjugate. At low doses, rats excrete the sulfated acetaminophen as a major urinary metabolite. As the dose of acetaminophen is increased,

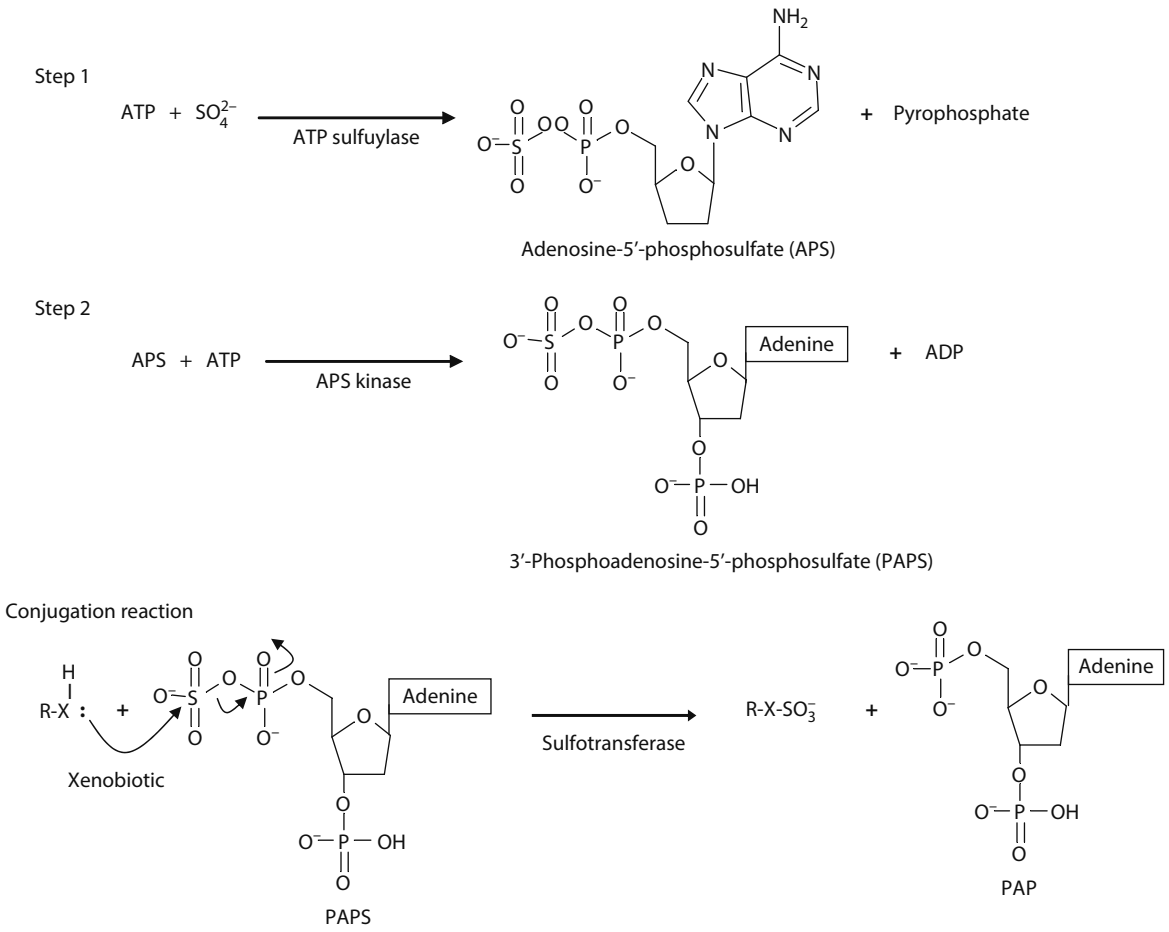


FIGURE 4.14 Reactions catalyzing the formation of PAPS and the xenobiotic–sulfate conjugate. X in the conjugation reaction represents a nucleophilic atom in a functional group such as oxygen in a hydroxyl group or nitrogen in an amine group.

the sulfate metabolite does not increase, whereas the glucuronide of acetaminophen increases dramatically; this is believed to be due to the limited availability of PAPS. The limitation in the synthesis of PAPS is sulfate. The major sources of sulfate include diet and degradation of sulfur amino acids (methionine and cysteine). These sources are inadequate to maintain sulfate concentrations for PAPS synthesis during rapid SULT activity. In the mouse, sulfonation appears more limited by SULT activity than by PAPS and sulfate.

Reactions Catalyzed by Sulfotransferases

As mentioned, SULTs esterify a variety of endogenous substrates, including steroids, carbohydrates, and proteins. Sulfonation also plays a role in the disposition of hormones. Sulfonation directs lipophilic compounds, such as the steroidal hormones, to more polar environments, including the active sites of enzymes, and to body fluids; for example, sulfonation enhances the elimination of steroids from the adrenal gland.[181] Sulfonation also facilitates deiodination of thyroid hormone and is a rate-limiting step in one of the elimination pathways of thyroid hormone.[182]

Xenobiotic conjugation with sulfate is an important route for the conversion of lipophilic xenobiotics to more readily excreted polar metabolites.[183,184] Sulfonation of xenobiotics with an aliphatic or aromatic hydroxyl group readily occurs; for example, phenol is excreted as its sulfate conjugate (Figure 4.15). Often, it is necessary for phase I metabolism to functionalize a xenobiotic with a hydroxyl group before it can be sulfated; for example, toluene is oxidized to benzyl alcohol before conjugation with sulfate (Figure 4.15).

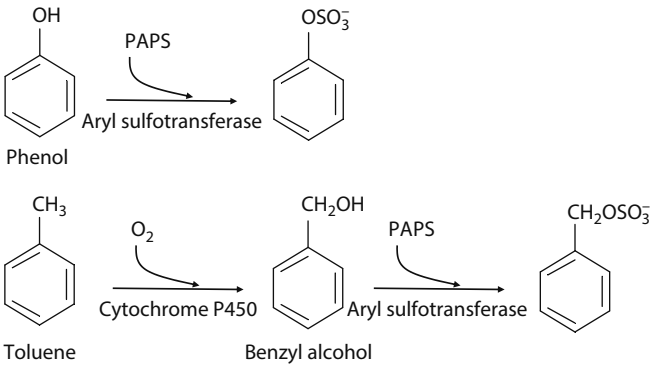


FIGURE 4.15 SULT-catalyzed sulfonation of phenol and toluene.

Role of Sulfotransferases in Detoxification and Metabolic Activation

Alcohols, phenols, aliphatic and aromatic amines, and aromatic hydroxylamines and hydroxylamides can be sulfated. These same groups can form glucuronides. At low doses, sulfonation may play an important role in detoxification of xenobiotics; however, as acetaminophen demonstrates, at high doses, glucuronidation becomes more important because of sulfate limitations. Secondary effects may be produced by the sulfonation lowering sulfate availability for the sulfonation of endogenous substrates. Depletion of sulfate pools as a result of the metabolism of high doses of drugs has been proposed to interfere with the normal biosynthesis of glycosaminoglycans during development, resulting in teratogenic effects in animals.[185] SULTs can be involved in the conversion of prodrugs to their active forms; for example, minoxidil is sulfoconjugated to its active form, which is more active as an antihypertensive and hair-growth stimulant than the parent drug.

SULTs can be involved in the metabolic activation of a number of mutagens and carcinogens. One of the best known examples is the metabolic activation of the carcinogen 2-acetylaminofluorene (2-AAF) (illustrated in Figure 4.16). N-Hydroxylation of the amide nitrogen by monooxygenases is followed by sulfonation of the N-hydroxy group. The sulfate ester is unstable and decomposes to an electrophilic nitrenium–carbonium ion, resonance ion that can form covalent adducts at nucleophilic sites on macromolecules. The support for the hypothesis that the sulfate conjugate of 2-AAF is the reactive metabolite comes from studies indicating that factors that modulate SULT activity also modulate 2-AAF carcinogenicity. Male rats have higher SULT activity and develop more 2-AAF-induced tumors than females. Reduction of SULT activity in male rats by castration, hypophysectomy, thyroidectomy, or steroid hormones reduces 2-AAF covalent adducts. These results are consistent with the hypothesis that sulfonation of 2-AAF is required for covalent modification of DNA. This mechanism is at least partially responsible for the activation of several other xenobiotics, including aromatic amines, mono- and dinitrotoluene, *N*-hydroxyphenacetin, 1′-hydroxysafrole, N^3-hydroxyxanthine, and other *N*-hydroxyarylamides.[186] Secondary nitroalkanes, such as 2-nitrobutane and 3-nitropentane, can be metabolically activated to mutagens by aryl SULT and hepatocarcinogens. Primary nitroalkanes, such as 1-butane and 1-nitropentane, are not activated by aryl

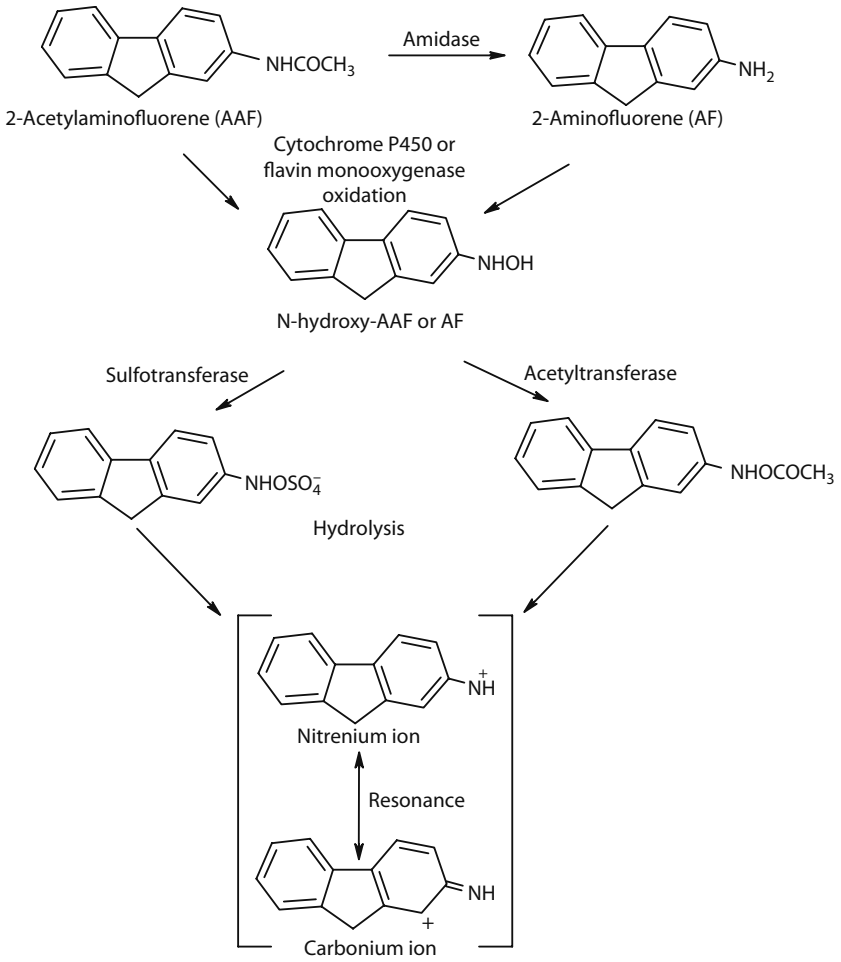


FIGURE 4.16 Metabolic activation of 2-acetylaminofluorene to a reactive metabolite capable of covalent modification of macromolecules.

SULT.[187] Mutagenicity testing is frequently hampered by the fact that phase II metabolic activation systems are typically not present in standard Ames bacterial mutagenesis assays, which do simulate phase I activation. New systems are being developed in which SULT genes are expressed in Salmonella strains and Chinese hamster V79 cells and are yielding a diverse set of structures capable of being activated by SULTs to mutagenic metabolites.[188]

SULTs can metabolically activate certain products of CYP1A1 metabolism of polycyclic hydrocarbons; for example, 9-hydroxymethylbenzo(*a*)pyrene can be sulfated to yield a highly reactive sulfate ester that is heterolytically cleaved to produce an electrophilic cation that damages DNA, RNA, and protein. In addition, 6-hydroxymethylbenzo(*a*)pyrene can be activated to the carcinogenic 6-sulfooxymethylbenzo(*a*) pyrene by rat and mouse SULT.[189] Other examples include 5-hydroxymethylchrysene and 7,12-dihydroxymethyl benz(a) anthracene.[190]

Sulfotransferase Isoforms, Genetics, and Species Differences

SULTs belong to a multigene family that produces a number of distinct enzymes that have different, but overlapping, substrate specificities. Some of these enzymes demonstrate species and tissue specificity in their expression. The nomenclature used to describe these enzymes is still evolving, but recently a system for classifying the cytosolic SULT superfamily has been proposed.[191] Membrane-bound SULTs that are localized to the Golgi apparatus exhibit a low degree of amino acid sequence identity with the cytosolic SULTs, and although they exhibit some structural similarity to cytosolic isoforms, they are generally considered a separate superfamily. The cytosolic SULTs are typically involved in xenobiotic metabolism; thus, their genetics and nomenclature is discussed here. The cytosolic form is identified by the abbreviation SULT.

More than 56 distinct eukaryotic SULT isoforms have been identified and functionally characterized.[191] SULT families are identified by the Arabic numeral immediately following the name and subfamilies identified by alphabetical categories (Figure 4.17). Unique subforms are further identified by an additional Arabic numeral; however, in some cases, such as SULT2A1, the standard nomenclature has been relaxed to accommodate more historic identifiers.

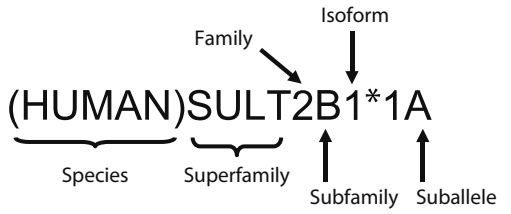


FIGURE 4.17 Naming convention illustrated for a representative cytosolic SULT allele name. A complete SULT allele name contains species, superfamily, family, isoform, allele, and suballele designations as shown. (Adapted from Blanchard, R. et al., *Pharmacogenetics*, 14, 199, 2004.)

Human genes are capitalized, while rat and mouse genes are in lowercase letters. To further facilitate the identification of orthologous SULT isoforms in different species, a three- to five-letter species code is placed in front of the SULT.

Many of the SULTs commonly encountered in xenobiotic metabolism are listed in Table 4.6. The SULT1 family, also known as the phenol SULTs, is comprised of at least 11 isoforms and is one of the most commonly encountered forms of SULT. The 1A isoforms are frequently referred to as phenol SULTs because of their high substrate specificity for phenolic xenobiotic molecules such as 17α-ethinyl estradiol, acetaminophen, minoxidil, and isoflavones but also for endogenous substrates such as 17β-estradiol, triiodothyronine, and thyroxine. The 1A isoform is highly expressed in liver and in brain, breast, intestinal epithelium, endometrium, kidney, lung, and platelets. The 1B forms also catalyze sulfate conjugation of typical phenolic substrates but are the major SULTs for thyroid hormones because of their high affinity for these substrates. The 1B form has been found in tissues such as liver, colon, small intestine, and white blood cells. The SULT1C family is involved in the sulfonation of *N*-hydroxyacetylaminofluorene, phenol, and other prototypical phenolic substrates. The SULT1E subfamily also catalyzes the sulfonation of phenolic substrates but has a much lower affinity than that of the 1A subfamily. The SULT1E isoforms are typically found in liver and small intestine.

The SULT2 family, also known as the hydroxysteroid SULTs, consists of two subfamilies, 2A and 2B, and typically catalyzes sulfate conjugation of 3β-hydroxy groups of steroids. The 2A family catalyzes the sulfonation of a range of xenobiotics, including benzylic alcohols of PAHs, and is expressed in adrenal gland, liver, brain, and intestine. 2B isoforms have only been identified in human prostate, placenta, and trachea and in mouse intestine, epididymis, and uterus.

Additional information on the SULT gene family, their nomenclature, and substrate specificities can be found in Blanchard et al.[191]

Humans demonstrate SULT genetic polymorphisms, which help explain some of the differences between individuals in response to specific xenobiotics.[192] SNPs have been identified in most isoforms but are more common in some isoforms than others. Allele frequency has been associated with certain ethnic groups and may contribute to differential drug responses in these individuals. For example, evidence is emerging that women carrying the SULT1A1*2 allele, which is associated with diminished capacity to sulfate SULT1A1 substrates such as the active antiestrogen 4-hydroxytamoxifen, show increased survival, perhaps as a result of improved drug exposure.[193] Because SULTs do not appear to be as sensitive to induction, exposure to xenobiotics may not be as important as with some of the other xenobiotic-metabolizing enzymes in producing individual variations in metabolism.

Sulfonation occurs in most species, including mammals, birds, reptiles, amphibians, fish, and invertebrates. The most notable exception to this is the low SULT activity in the pig. Members of the cat family are deficient in

glucuronyltransferase activity but have high SULT activity. This balance of glucuronyltransferase and SULT must always be kept in mind when evaluating the activity of either enzyme system. A deficiency in one pathway can shift metabolism, as similar functional groups are conjugated by the two enzyme systems. In addition, sulfonation appears to have high affinity but low capacity for phenols, whereas glucuronidation has low affinity and high capacity for these substrates.

Sulfonation of acetaminophen is limited by PAPS availability in rats. In mice, acetaminophen sulfonation is limited by lower SULT activity. Although mice have lower PAPS synthetic capability than rats, lower SULT activity is the major limiting factor in mice.[145] When the activities of acetaminophen SULT and 17α-ethinylestradiol SULT in hepatic preparations from monkeys, dogs, and humans were compared, rhesus and cynomolgus monkeys and dogs had higher acetaminophen SULT activity than humans.[194]

Factors Modifying Metabolism

SULTs are not induced by the classical inducers, PB and 3-methylcholanthrene, and these compounds may actually suppress their expression.[195] Several inhibitors of SULT have been discovered and exploited experimentally to study these enzymes. Pentachlorophenol and 2,6-dichloro-4-nitrophenol are potent SULT inhibitors. Only 0.2 μM pentachlorophenol is required for 50% inhibition of 2-dichloro-4-nitrophenol sulfonation by purified arylsulfotransferase.[196] Pentachlorophenol and 2,6-dichloro-4-nitrophenol are effective inhibitors because the ortho- and para-aromatic ring positions are substituted with electron-withdrawing groups. This effect is consistent with the mechanism whereby the SULTs facilitate electrophilic attack of the hydroxyl oxygen by the sulfur.

Gender Differences

Major gender differences have been observed in the sulfate conjugation of steroid hormones; for example, compared to female rats, male rats have a 10-fold higher expression level of SULT1C1, the isoform implicated in *N*-hydroxyacetylaminofluorene metabolism.[197] This male dominant pattern emerges at 40–50 days of age and is under gender-specific patterns of growth hormone control. Three steroid SULTs have been isolated from rat liver, and it is the relative amounts of these isozymes that account for the large gender difference. Aryl SULT concentrations in the livers of male rats were higher than in females; in contrast, hydroxysteroid SULT concentration was higher in the liver of female rats compared to males.[197,198]

Lower SULT activity observed in neonatal rats has been attributed to sexual immaturity because as gonads develop, SULT activity increases. Newborn infants, who characteristically exhibit pronounced immaturity in glucuronidation, have a fully developed phenol SULT activity; for example, newborns excrete acetaminophen as a sulfate conjugate, whereas adults primarily excrete it as a glucuronide conjugate. Chloramphenicol is extremely toxic in neonates because it is a poor substrate for SULT and is primarily cleared by glucuronidation in adults. Gender differences in clinical pharmacokinetics related to SULT activity are not widely reported.

GLUTATHIONE S-TRANSFERASES

A family of enzymes known as glutathione S-transferases (GST) is capable of conjugating relatively hydrophobic electrophilic molecules with the reduced form of the intracellular nucleophile GSH (Figure 4.18).[199] These enzymes are ubiquitously expressed in mammals, but are found in highest concentrations in the liver, kidney, intestines, and lung, but they occur in most tissues. GSH conjugates have higher molecular weights and are more water soluble and therefore more readily excreted than are the parent compounds. Further, GSH conjugates are substrates for several transporters involved in biliary and renal excretion, which facilitates their clearance from the body. In general, though not invariably, conjugation with GSH decreases the likelihood that a xenobiotic will react with toxicological targets.

Synthesis and Regulation of Glutathione

GSH, the essential cofactor for GSTs, is a tripeptide composed of glutamate, cysteine, and glycine. In contrast to the α-linkage normally found in most peptides, the glutamate and cysteine of GSH are joined by a γ-linkage, which confers resistance to hydrolysis by peptidases (Figure 4.18). Synthesis of GSH is carried out in two sequential steps (Figure 4.19). The first of these is catalysis by γ-glutamylcysteine ligase (GCL), which results in formation of the γ-linkage between glutamate and cysteine. The reaction requires ATP and is the rate-limiting step in GSH synthesis. GCL is a dimeric protein composed of a catalytic subunit that provides ligase activity and ATP hydrolysis and a regulatory subunit that lowers the K_m for glutamate.[200] The second step of GSH synthesis, addition of the glycine residue, is catalysis by GSH synthetase, another ATP-dependent enzyme. Regulation of GSH synthesis is mediated by GCL, which is sensitive to cellular redox status and GSH concentration. GCL is also regulated at the transcriptional level in conjunction with GSTs. Buthionine *S*-sulfoxime is a potent inhibitor of GCL that has been used extensively to study the biological and toxicological roles of GSH. Cysteine, which is the rate-limiting substrate for GSH synthesis, may come from several sources, including cystine, methionine, and the recycling of GSH itself, as depicted in Figure 4.19.[201]

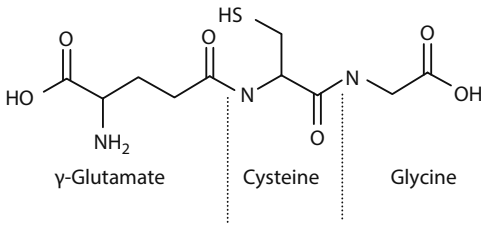


FIGURE 4.18 Structure of glutathione. Note the unusual γ configuration of the linkage between glutamate and cysteine.

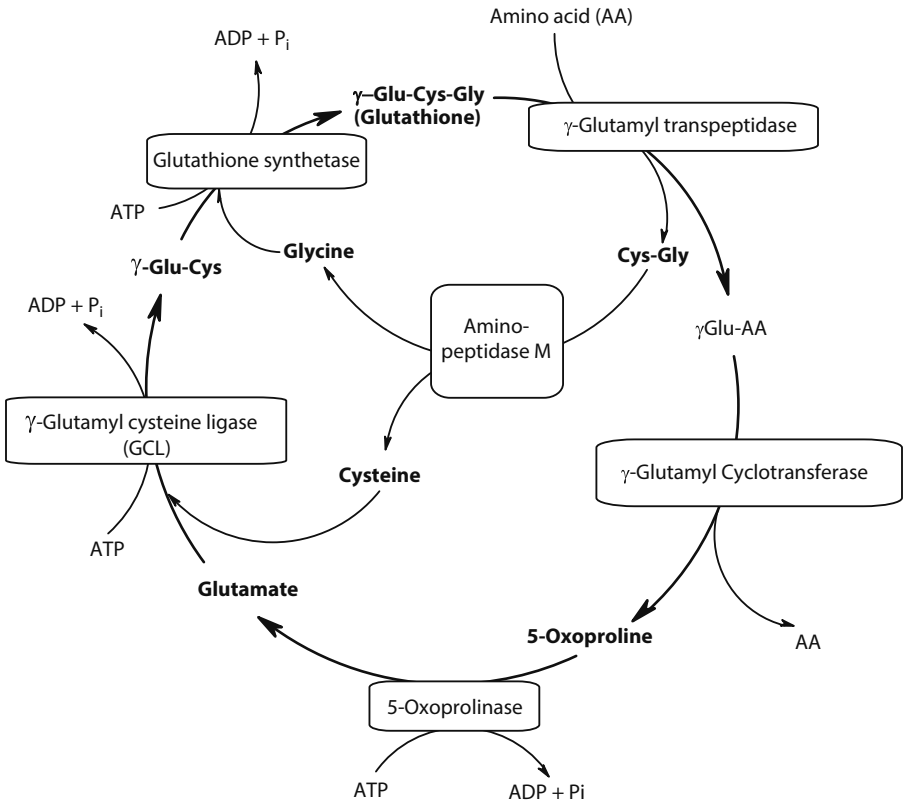


FIGURE 4.19 The γ-glutamyl cycle: biosynthesis of glutathione. Cysteine for glutathione synthesis may come from other sources, including cystine and methionine.

Organization, Structure, and Localization of GSTs

The mammalian GST superfamily is composed of three major families of proteins, which are expressed in the cytosol, mitochondria, and endoplasmic reticulum.[202] The cytosolic and mitochondrial proteins are dimeric, while the microsomal forms may exist as monomers, trimers, or higher-order aggregates.[202,203] The current nomenclature is based on amino acid sequence similarity and subunit composition. Enzymes within a class share >40% sequence similarity (generally, ~70%), while different classes have <25% sequence similarity. In humans, 11 major classes of GSTs have been identified. The cytosolic GSTs make up the largest family and include the alpha (α), mu (μ), pi (π), sigma (σ), theta (θ), zeta (ζ), and omega (ω) classes. The mitochondrial isoform, only a single member of which has been identified, is designated as the kappa (κ) class. The microsomal GSTs are designated as the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEGs), to reflect their predominant role in eicosanoid synthesis.

Cytosolic GSTs

The cytosolic GSTs are the major isoforms involved in xenobiotic metabolism, comprising over 95% of total cellular GSTs. Cytosolic GSTs are dimeric proteins with subunit molecular weights of approximately 22–26 kDa. Seven classes of cytosolic GSTs have been identified in humans, with varying numbers of distinct subunits in each class, each of which is encoded by a separate gene. The α and μ classes each have five subunits, the θ and ω classes each have two subunits, and only a single subunit has been identified for each of the π, σ, and ζ classes. Subunits from the α and μ classes can form heterodimers with other members of their class, but other classes of cytosolic GSTs exist only as homodimers. Cytosolic GSTs are named using an uppercase letter to designate class (A, M, P, S, T, Z, or O), followed by Arabic numerals to designate subunit composition[206]; for example, GSTA1-1 is an α-class homodimer of subunit 1. Previous GST nomenclature systems were based on either apparent substrate selectivity or elution order from chromatography columns,[204] and these designations are found in the older GST literature.

Like the cytochromes P450, the cytosolic GSTs exhibit broad and overlapping substrate specificities. The substrates for all isoforms are hydrophobic compounds possessing an electrophilic center. In addition to conjugation of xenobiotics, cytosolic GSTs play a role in metabolism of endogenous compounds, including amino acids, steroid hormones, and eicosanoids.[202,203,205] Cytosolic GSTs are also capable of binding chemicals on the enzyme surface. This binding may or may not inhibit the catalytic activity of the enzyme, but it prevents the xenobiotic from interacting with other critical cellular sites, such as proteins and nucleic acids. Some GSTs had previously been termed ligandins because of this property.[206,207]

Mitochondrial GSTs

Mitochondrial GSH constitutes approximately 10%–15% of the total cellular GSH pool, and several isoforms of GST have been identified in mitochondria. In rodents and humans, GST kappa (GSTK1-1) is a homodimeric mitochondrial GST containing a putative N-terminal mitochondrial targeting sequence that is responsible for its specific localization in mitochondria.[208] Structurally, mitochondrial GST is more similar to bacterial GSH-dependent isomerases and disulfide-forming oxidoreductases than it is to the mammalian cytosolic GSTs.[203] In rodents, GSTK is expressed at high concentrations in liver, kidney, stomach, and heart, but in humans, the tissue expression of this enzyme is ubiquitous. GSTK has high activity for 1-chloro-2,4-dinitrobenzene (CDNB) and cumene hydroperoxide, as well as eicosanoid peroxides such as (S)-15-hydroperoxy-5,8,11,13-eicosatetraenoic acid. Several other isoforms of GST are also found in mitochondria, including GSTA4-4, for which 4-hydroxynonenol is a preferred substrate.[209] Mitochondrial GSTs are thought to mediate diverse biological activity including signal transduction, and response to oxidative stress and dysfunction of mitochondrial GSTs has been associated with ageing and with various disease states such as diabetes and ischemia–reperfusion injury.[208] In addition to mitochondria, GSTK has also been found associated with peroxisomes, where it may play a role in fatty acid β-oxidation.[211] Its role in the metabolism of environmental chemicals and drugs remains to be elucidated.

Microsomal GSTs

Microsomal GSTs, currently referred to as MAPEGS, are membrane-bound enzymes involved primarily in eicosanoid synthesis. MAPEGs are divided into four subgroups, of which three (I, II, and IV) are expressed in mammals. MAPEGS exist as homotrimers, in which each monomer binds one molecule of GSH.[212] Interestingly, only one of the three GSH molecules is present as the catalytically active thiolate form.[210] MGST1, MGST2, and MGST3 are all capable of conjugating a variety of xenobiotic electrophiles, and the latter two isoforms also function as leukotriene C4 synthetases. For example, MGST1 has been shown to catalyze regioselective and stereoselective conjugation of fluoroalkenes,[211] though no specific xenobiotic substrates have been identified to date. MAPEGS also play a role in protection against oxidative stress and are upregulated following oxidative insult or covalent modification by electrophiles.[212,213] Recently, MGST1 has been shown to be highly expressed in a variety of different tumor cell types and, in some cases, correlates with poor response to chemotherapy, suggesting a potential role for this isoform in drug resistance.[214] Although MAPEGS are clearly capable of detoxication of xenobiotics, their quantitative contribution to xenobiotic metabolism is unclear.

Biochemistry of Glutathione S-Transferases

GSTs involved in xenobiotic metabolism catalyze the conjugation of GSH with substrates bearing an electrophilic atom.[215] The catalytic mechanism of GSTs involves stabilization of the S-deproteinated form of GSH (GS–). In the cytosolic classes of GST, this is accomplished by donation of a hydrogen bond to the glutathione cysteine sulfur, which effectively lowers the pK_a of the thiol to between 6 and 7.5. Thus, in the enzyme-bound state, the thiolate ion is the predominant form of GSH at physiological pH. The hydrogen bond may be provided by either tyrosine or serine, depending on the GST class. In the α-class enzymes, the thiolate receives additional stabilization from electrostatic interactions with an active site arginine residue, and similar stabilization by arginine is observed with MAPEGs.[213]

The substrate specificities of GSTs are broad and overlapping. The general structural characteristics of substrates for the cytosolic enzymes are that (1) they are relatively hydrophobic, (2) they possess an electrophilic center, and (3) they will react nonenzymatically with GSH at some measurable rate. The substrate selectivity of these enzymes is sensitive to small changes in the primary structure of the enzymes; for example, a GST of the π-class contains a tyrosine at a site important for selectivity (Tyr108), whereas a transferase of the α-class contains a valine. When the tyrosine of the π-class enzyme is replaced with a valine, its substrate selectively is changed toward that of the α-class. Similarly, by engineering minor modifications in GSTT1-substrate selectivity of this isoform can induce significant changes in the substrate-activity profile.[216,217]

Selective inhibitors of GST have been sought both as research tools and as potential therapeutic agents. Some commonly used inhibitors include ethacrynic acid, triphenyltinchloride, bromosulfophthalein, cibacron blue, and hematin.[218] Considering the broad substrate specificity of GSTs, it should not be surprising that the development of isoform-selective GST inhibitors has been challenging. A variety of chemotypes has been evaluated as selective GST inhibitors. These include GS–R conjugates (e.g., *S*-octylglutathione), GSH peptide analogs (e.g., substitution of SO_2NH for CONH in the γ-glu–cys linkage), nonpeptide GSH analogs, bivalent inhibitors that interact with both subunits of the dimeric protein simultaneously, and ligandin-type inhibitors.[219] These efforts have met with varying degrees of success, although truly isoform-selective inhibitors remain elusive.

Although not highly efficient in its reactions (relatively high K_m for the xenobiotic), GSTs are capable of catalyzing or reacting with a number of reactive chemical functional groups. Any lack of efficiency is made up for by the high cellular concentration of GSH and GST. Liver GSH concentrations are high (up to 10 mM), and GSTs can represent as much as 10% of the total hepatocellular proteins[220]; however, it is possible for GSH conjugation to become capacity limited at high doses of xenobiotics. GSH utilization can outstrip its synthesis, resulting in depletion, decreased conjugation, and increased toxicity.

Reactions of Glutathione S-Transferases

Some xenobiotics contain sufficiently electrophilic groups to react directly with GSH, whereas others must first undergo

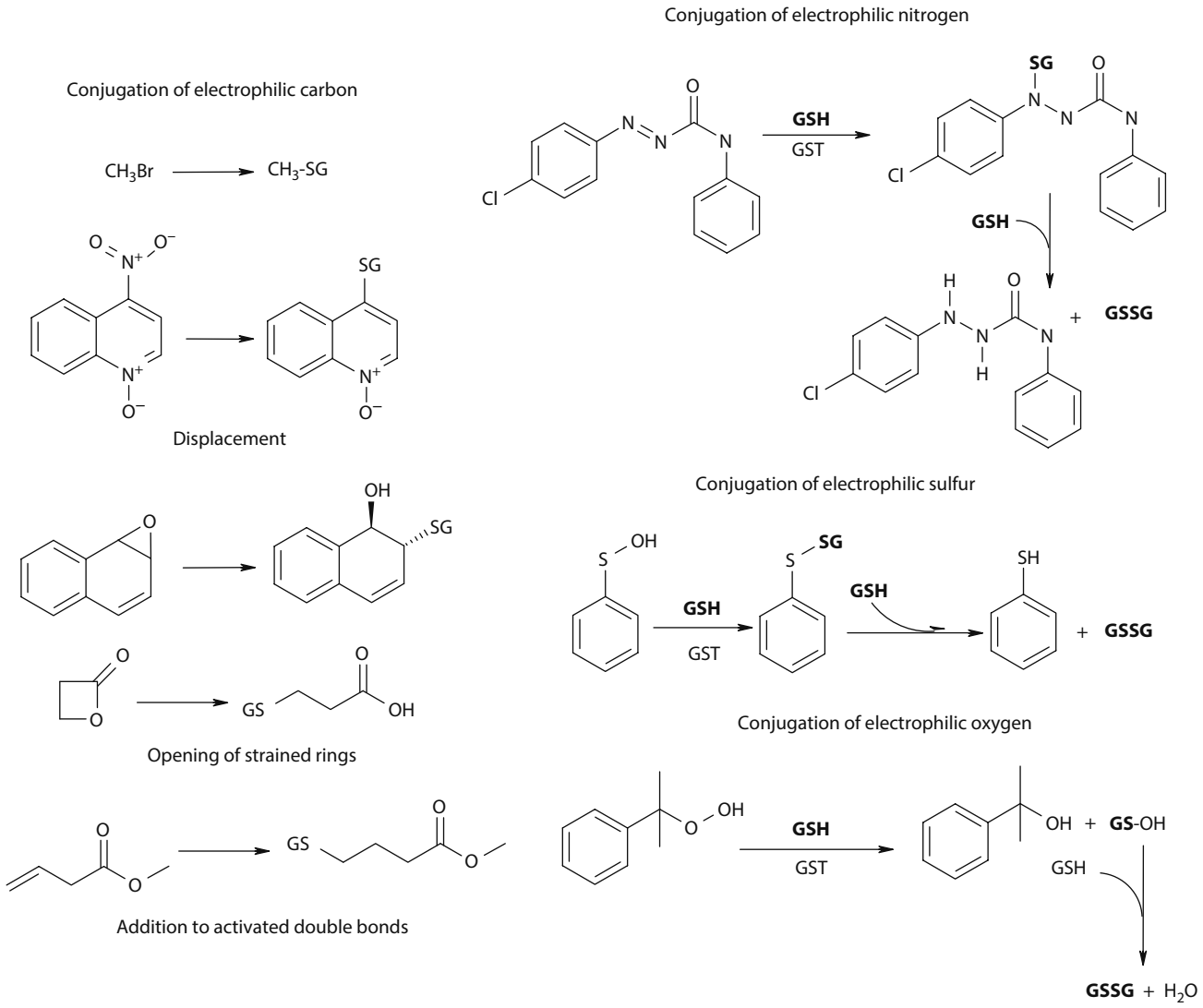


FIGURE 4.20 Examples of the reaction types catalyzed by GSTs.

phase I metabolism. Most xenobiotics react with GSH through the catalytic activity of the GSTs. GST reactions fall into four broad categories, as depicted in Figure 4.20: reaction with (1) electrophilic carbon, (2) nitrogen, (3) sulfur, or (4) oxygen.[221–223] For electrophilic heteroatoms, reaction with GSH results in formal reduction of the heteroatom with concomitant oxidation of GSH to its disulfide form (GSSG). The reactions types catalyzed by GST have been reviewed recently by Zimniak.[224]

Reaction with Electrophilic Carbon

The reactions of GSH with electrophilic carbon can be divided into three types: (1) displacement reactions, (2) opening of strained rings, and (3) addition to activated double bonds:

- Displacement of functional groups such as halides, sulfates, sulfonates, phosphates, and nitro groups from saturated or unsaturated carbon atoms. When carbon is bonded to an electronegative atom or group of atoms, electron density is drawn away from the carbon nucleus, creating an electrophilic center. Many electron-withdrawing groups make good leaving groups for nucleophilic substitution reactions. Such reactions occur for alkyl (sp^3-hybridized), olefinic, and aromatic (sp^2-hybridized) carbons. Displacement of the leaving group is facilitated if the saturated carbon atom bearing the leaving group is allylic or benzylic. Displacement of halide or nitro groups on aromatic rings can occur via an addition–elimination mechanism if the aromatic ring contains additional electron-withdrawing groups of sufficient strength (Figure 4.21). In this case, the rate of formation of a carbanion intermediate of the aromatic ring governs the overall rate of the reaction. Functional groups that withdraw electrons from the ring system stabilize the carbanion and are considered good leaving groups. On the other hand, electron-donating substituents

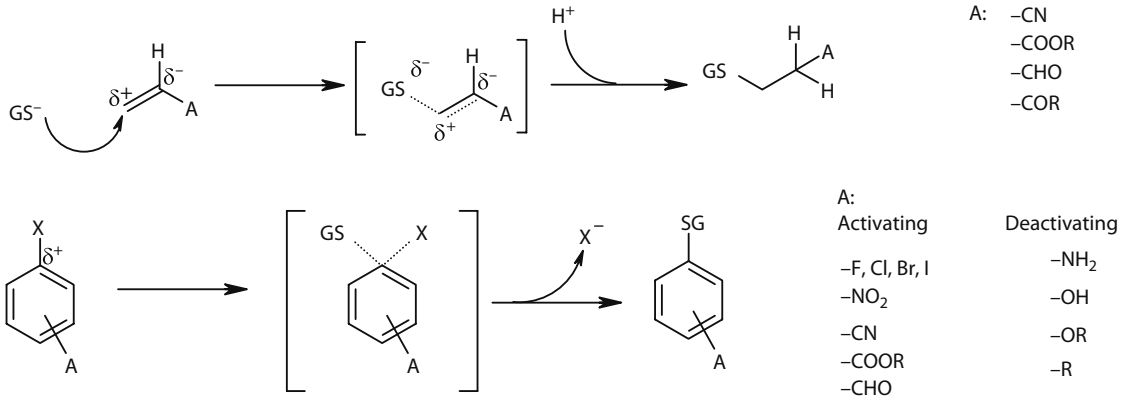


FIGURE 4.21 Putative reaction mechanisms for the GST-catalyzed Michael addition and aromatic substitution reactions. X represents a halogen atom, A represents the substituents listed to the right of each reaction, and R represents an alkyl group.

destabilize the anionic intermediate, deactivating the aromatic ring and making displacement of the leaving group by GSH less likely. A novel subclass of this reaction type that has received recent attention is transacylation with acylglucuronides and acyl-SCoA thioesters, which has been observed with a number of acidic drugs of toxicological interest.[225] Although this reaction is only weakly catalyzed by GSTs, the resulting conjugates may have significantly higher reactivity than the substrates, potentially contributing to toxicity of carboxylate-containing drugs.

- Opening of strained rings, such as epoxides and four-membered lactones. Ring systems with three or four members are highly strained, as the bond angles are forced from their minimum energy configuration (normally ~109° for sp^3-hybridized carbon). The presence of a heteroatom in such ring systems decreases electron density at the adjacent carbon and predisposes it to nucleophilic attack and ring opening. An example, shown in Figure 4.20, is conjugation of the 1,2-epoxide of naphthalene, resulting in a 1-naphthol conjugate of GSH. These reactions can be stereoselective; for example, phenanthrene-9,10-epoxide is converted exclusively to the 9S,10S-diasteromeric conjugate by rat GSTM1-1, while GSTM2-2 produces approximately equal amounts of each diastereomer.[226] Epoxide products of P450 are detoxified by this reaction and are an example of a phase II conjugation of a phase I-activated metabolite.
- Addition to activated double bonds via Michael addition. The presence of a carbonyl or cyano group adjacent to an olefinic group polarizes the electrons in the olefin double bond, creating an electron-deficient center on the β-carbon. The GSH thiolate anion will attack β-unsaturated xenobiotics due to this partial positive charge on the β-carbon, leading to 1,2-addition of GSH across the double bond, as shown in Figure 4.21.

Reaction with Electrophilic Nitrogen

GSH can react with electrophilic nitrogen atoms, such as diazenes.[227] The reaction is exemplified by the GSH-dependent reduction of the diazenecarboxamide JK-914.[228] As seen in Figure 4.20, the first step of the reaction is analogous to the Michael addition of GSH to polarized olefins. In the second step, the initial addition product reacts nonenzymatically with a second molecule of reduced GSH, resulting in the reduction of the diazenecarboxamide to a hydrazide with concomitant formation of oxidized GSH.

Reaction with Electrophilic Sulfur

Alkyl and aryl thiocyanates are substrates for GST-catalyzed conjugations, as shown in Figure 4.20. Products of this nucleophilic attack of the thiolate ion on the sulfur of the xenobiotic result in a mixed disulfide and hydrogen cyanide. The mixed disulfide can react nonenzymatically with another molecule of GSH to yield a thiol of the xenobiotic (RSH) and oxidized GSH (GSSG).

Reaction with Electrophilic Oxygen

Figure 4.20 illustrates how GSH reacts with organic hydroperoxides in a two-step sequence. The first step is catalysis by GST, which forms an alcohol or phenol and a glutathione sulfenic acid intermediate (G-SOH). Another GSH reacts nonenzymatically with the sulfenic acid to form oxidized GSH and water. An example of this reaction with endogenous hydroperoxides is the conversion of hydroperoxy-prostaglandin F_{2c} to prostaglandin F_{α}. Cumene hydroperoxide is metabolized as rapidly by purified GSTs as the classical transferase substrate probe CDNB. Denitrosation of trinitroglycerol is another example of a reaction with electrophilic oxygen.

Metabolic Fate of Glutathione Conjugates: Mercapturic Acid Formation

Mercapturic acids are N-acetylated, S-substituted, cysteine conjugates that arise from conjugation of a xenobiotic with GSH.[229] The GSH conjugates formed in the liver and other

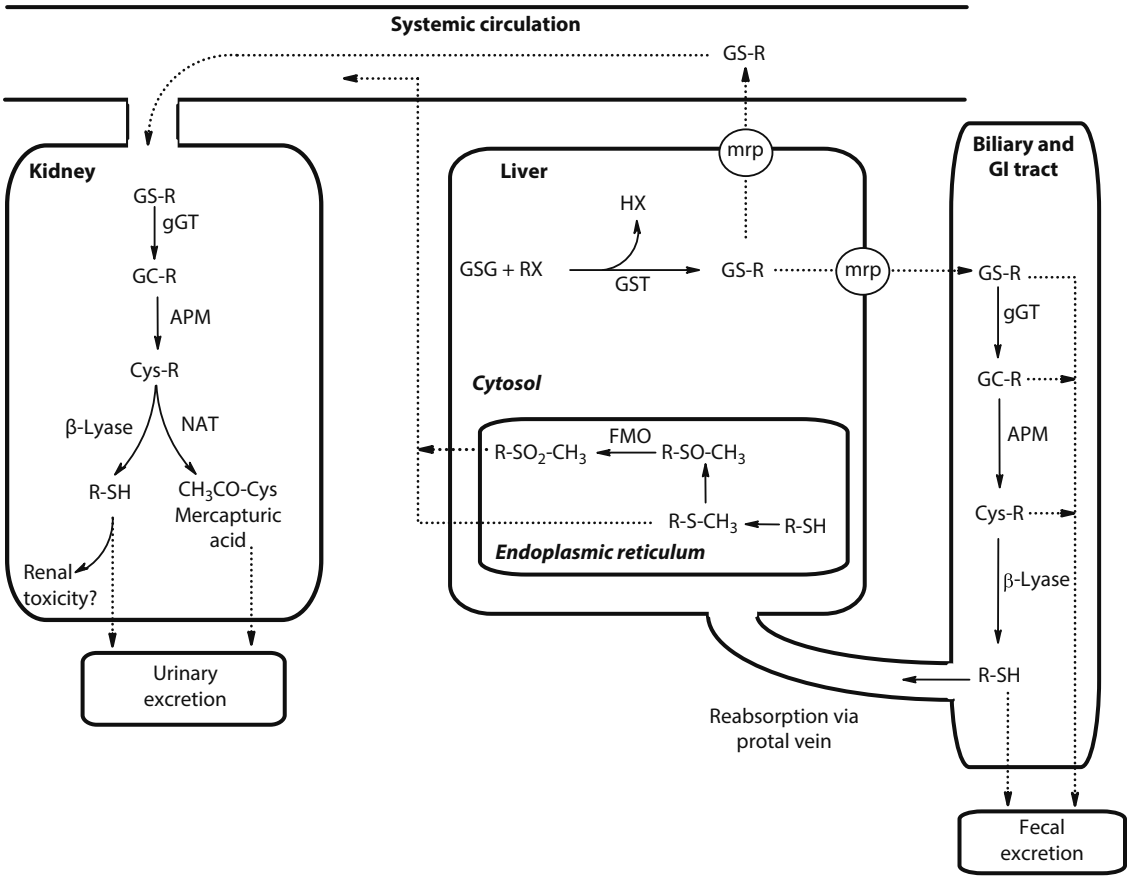


FIGURE 4.22 Summary of the metabolic fate of glutathione conjugates. GST, glutathione S-transferase; gGT, γ-glutamyltransferase; APM, aminopeptidase M; FMO, flavin-containing monooxygenases; NAT, N-acetyltransferase. Refer to text for details.

tissues are polar and partition into the aqueous phase of cells and blood. Because 25% of the blood flow passes through the kidney, GSH conjugates are transported to the kidney via systemic circulation. There, the GSH conjugate undergoes a series of reactions (shown in Figure 4.22) generally resulting in mercapturic acid formation; however, in some cases, bioactivation and nephrotoxicity are the outcomes.

The initial step in mercapturic acid synthesis is cleavage of glutamic acid from cysteine catalyzed by γ-glutamyltranspeptidase. This enzyme is embedded in the outer leaflet of the plasma membrane and is highly expressed in the brush border of the proximal tubules in the kidney.[230] Evidence that this enzyme is involved in GSH degradation comes from observations of pronounced glutathionemia and glutathionuria (high levels of GSH in the blood and urine, respectively) in patients who lack detectable γ-glutamyltranspeptidase. This enzyme not only hydrolyzes the GSH moiety but also transfers the γ-glutamyl group to a variety of amino acids and dipeptides and provides cysteine for de novo synthesis of GSH. Consistent with its role in regulating GSH concentration, gGT is upregulated in oxidative stress[231] and has been proposed as a biomarker for exposure to environmental pollutants.[232]

Next, the glycine group is cleaved from the resulting cysteinylglycine conjugate by aminopeptidase M, yielding the S-substituted cysteine conjugate of the xenobiotic. The cysteine conjugate is a substrate for N-acetyltransferases (NATs) that acetylate the free amino group of cysteine to yield the mercapturic acid, which is excreted in the urine. Alternatively, the cysteine conjugate can be cleaved by renal cysteine conjugate β-lyase, possibly resulting in bioactivation and nephrotoxicity. These two enzymes, γ-glutamyltransferase and aminopeptidase M, are also responsible for the normal turnover of GSH in mammalian cells previously shown in Figure 4.19.

Role of Glutathione S-Transferase in Detoxification

Free reactive electrophilic intermediates of xenobiotics can produce damage to important cellular constituents. Reduced GSH and the GSTs protect cells from this damage by capturing the reactive electrophiles before they can react at nucleophilic sites critical to cell viability.

The metabolism of acetaminophen, an analgesic that at high doses can produce hepatic necrosis, serves as an example of this protective system. A large body of work has shown that one of the principal ways in which acetaminophen produces its hepatotoxicity is via the reactive intermediate, *N*-acetyl-*p*-benzoquinoneimine. This intermediate is an electrophile that reacts readily with GSH and other tissue

nucleophiles. As long as the amount of glutathione present at the site of activation of acetaminophen is sufficient to bind the reactive intermediate, no toxicity ensues; however, as demonstrated in the classic study by Mitchell,[233] when glutathione is depleted by pretreatment with diethyl maleate, the benzoquinoneimine covalently binds to tissue proteins, resulting in tissue necrosis. Understanding of the role of glutathione in protection against acetaminophen-induced hepatotoxicity led to the introduction of *N*-acetylcysteine (Mucomyst®) as a standard antidote for acetaminophen poisoning. Mitchell[233] was among the first to propose that glutathione plays a fundamental role in protecting tissues against electrophilic attack by xenobiotics.

Since these early studies demonstrate the protective role of glutathione, numerous drugs and other chemicals have been shown to form conjugates with glutathione. Notable examples from the more recent pharmaceutical literature include troglitazone,[234] duloxetine,[235] and ticlopidine.[236] For a more thorough review of these reactions, see Chasseaud,[237] Koob and Dekant,[238] and Zimniak.[224]

Factors Affecting Metabolism

GSTs have been found in most species, including reptiles, birds, insects, amphibians, and plants. Factors that influence the availability of reduced glutathione drastically alter the effectiveness of GSTs. As was discussed previously, the toxicity of acetaminophen is modulated by the availability of reduced glutathione. Most xenobiotics that are highly reactive nonenzymatically with glutathione can deplete glutathione under appropriate conditions. Other mechanisms can also lower glutathione availability; for example, certain individuals have genetic defects in the γ-glutamyl cycle, resulting in low tissue concentrations of glutathione. These individuals generally are anemic due to the lack of glutathione and the resulting loss of protection from oxidative damage to erythrocytes.[239]

As discussed earlier, cysteine is the limiting factor for synthesis of glutathione via the cycle shown in Figure 4.19. Nutritional factors that limit sulfur amino acid availability decrease GST activity by reducing the availability of glutathione.[240] Methionine is an essential amino acid that can be used to synthesize cysteine and cystine via the transsulfuration pathway. Diets low in sulfur amino acids can decrease the availability of glutathione for conjugation with reactive intermediates of xenobiotics.

Regulation of Glutathione S-Transferases

GSTs are inducible by a wide variety of xenobiotics including phenolic antioxidants, PB, and planar aromatic hydrocarbons. Dietary ingredients, such as cruciferous vegetables, specific components of coffee, butylated hydroxyanisole (BHA), and organosulfur compounds of allium vegetables, can also induce GSTs. When cafestol and kahweol (diterpenes found in coffee) were administered to rats for up to 90 days, DNA adducts produced by AFB_1 were inhibited 50%. This appeared related to induction of GST and a decrease in P450 isozymes involved in the metabolic activation of aflatoxin.[241] Coffee consumption has been shown to increase salivary concentrations of the GSTs in humans.[242] Induction may be specific for one or more of the transferases and may be tissue specific.

Mechanisms of GST induction are complex and incompletely understood.[243] Induction of GSTs by antioxidants such as BHA occurs at the level of transcription and is controlled in large part by the ARE, also known as the electrophile response element (EpRE).[244,245] The major transcription factor that recognizes and binds to this response element is nuclear factor-E2-related factor 2 (Nrf2). Under basal conditions, this transcription factor is retained in the cytoplasm bound to the redox-sensitive protein Kelch-like ECH-associated protein 1 (Keap 1) and is targeted for proteasome degradation. Binding of Nrf2 to Keap 1 is controlled by at least three cysteine residues that act as redox and/or chemical sensors.[246,247] Oxidation or binding of electrophiles to these cysteines destabilizes the Keap 1/Nrf2 complex, leading to release of Nrf2 and its translocation to the nucleus, allowing interaction with the ARE. Nrf2 can form functional heterodimers with several binding partners, including Maf and c-Jun. In addition to the ARE, other regulatory elements play a role in regulation of GSTs and other phase II enzymes that respond to oxidative stress and electrophiles, including C/EBPβ and the AP-1 family of transcription factors. Some GST genes also contain an XRE in their 5′-flanking region, and this response element may play a role in GST induction by 3-methylcholanthrene and other planar aromatic compounds.[244] PB is thought to act through an AP-1 (Fos/Jun)-related mechanism[248] rather than through the CAR, which mediates CYP induction by PB. As mentioned earlier, cysteine, which catalyzes the rate-limiting step in glutathione synthesis, is also regulated by the Nrf2 and other transcription factors involved in GST induction.

Regulation of microsomal GST (MAPEG) expression has received little attention compared to cytosolic GSTs. Some isoforms, such as human MGST1 and mouse Mgst1, Mgst2, and Mgst3, are induced by oxidative stress, presumable through a Keap1/ARE-dependent mechanism.[249,250] Other microsomal isoforms such as FLAP, PGES-1, and LTC4 synthetase respond to proinflammatory mediators such as LPS, TNF-a, and IL-1b.[251–253]

Polymorphisms of Glutathione S-Transferases

GSTs exhibit polymorphic expression in humans. Among the most toxicologically relevant polymorphisms are those of the μ and θ class enzymes.[254,255] Both of these isoforms exhibit null genotypes resulting from homozygous deletion of the corresponding gene. Because these enzymes play a critical role in protecting the cell from cytotoxic and mutagenic damage, a number of population studies have been done to determine relationships between genotype and disease. Several studies have attempted to correlate lung cancer risk and transferase expression, with mixed results. A better correlation has been found between transferase genotype and diseases associated

with oxidative stress, especially for GSTM1 or GSTT1 polymorphism and colon cancer[256] and esophageal cancer.[257] No correlations were found between breast cancer and GSTM1 polymorphism.[258] The GSTM1*0 variant occurs at relatively high frequencies in Australians, Caucasians, and Africans[259] and has been associated with increased risk of cancers of the lung, colon, and bladder.[260] A fairly clear association of the GSTM1-null genotype with development of transitional cell carcinoma of the bladder has been observed, particularly among smokers and individuals with documented exposure to asbestos or chlorinated solvents.[254] A rapid metabolizer phenotype for GSTM has also been described in Arabian populations, resulting from duplication of the GSTM1 gene. High GSTM1 and GSTT1 expression has been positively associated with renal cell carcinoma and resistance to chemotherapy.[255] The GSTT1*0 genotype has been associated with an increased incidence of acute and chronic myelogenous leukemia. Polymorphic expression of other GST isoforms may also be of toxicological significance; for example, GSTO1-1 is the rate-limiting enzyme in the metabolism of inorganic arsenic, and variation of this enzyme may compromise an individual's ability to metabolize this toxic metal.[130] Similarly, GSTZ1-1 is involved in the detoxification of α-halocarboxylic acids such as dichloroacetic acid (DCA), and SNPs in the promoter region of this gene may impact DCA metabolism.[261] GSTP isoforms are overexpressed in a variety of human tumors, and this has led to their investigation as a chemotherapeutic target. GSTP1*A has been associated with the development of resistance to cisplatin and decreased response rate, while GSTP*B has been correlated with decreased cisplatin metabolism and increased chemotherapeutic response. In addition to their role in the detoxification of chemotherapeutic agents, GSTP1 may enhance tumor resistance by decreasing apoptosis via its inhibitory effect on the JNK–Jun signaling pathway.[262] Overall, it appears that some of the highest correlations between genotype and cancer susceptibility for GSTP1 are those where P450 genotype and transferase genotype are combined for analysis.[254] This again emphasizes the close relationship between metabolic activation by phase I enzymes and detoxification of reactive metabolites by phase II enzymes.

Species and Gender Differences

As mentioned earlier, GSTs have been found in most species investigated. Species differences in the expression, substrate specificity, and activity of these transferases can have a significant role in the toxicity of xenobiotics. For example, rats are susceptible to the potent hepatocarcinogen AFB_1, whereas mice are extremely resistant. This species difference results from the expression in mice of mGSTA3-3, which has a high activity toward the P450-generated activated metabolite of AFB_1 (the 8,9 epoxide). Although rats express a closely related transferase (rGSTA3-3), it has low activity toward the epoxide. These two transferases have equivalent activity toward a probe substrate (1-chloro-2,4-dinitrobeneze), but the rat form has 1000-fold less activity toward the AFB_1 epoxide compared to the mouse. This difference in activity between the transferases from the two species appears to be based on differences in as few as six critical amino acids.[263]

Hepatic GST activities are low in prepubertal male and female rats. As the rats reach sexual maturity between 30 and 50 days of age, glutathione-conjugating activity toward dichloronitrobenzene is two- to threefold higher in males than in females.[264] This difference in GST activity was not related to sex steroids but was dependent on pituitary secretions. Growth hormone may play a role in establishing GST activities,[265] as it does with P450. Although growth hormone is important in regulating adult levels of GST in the rat, it appears that other factors also play a role. The student of toxicology should be aware of the multifaceted way that xenobiotics can affect organisms; for example, monosodium glutamate, which produces lesions in the arcuate nucleus of the hypothalamus, can lower the GST activity in male rats. This, in turn, could increase their sensitivity to electrophilic chemicals.

Gender differences in the expression of GST have been suggested to be responsible for the higher susceptibility of female mice to the carcinogenicity of BP compared to males. Males express higher mGSTP1-1, mGSTA3-3, mGSTM1-1, and mGSTA4-4 compared to females. At higher doses of BP, this gender difference is lost, possibly due to the higher doses overcoming the protective role of the higher transferase activity in males.[266]

Some studies suggest that humans do not demonstrate gender differences in GSTs. No gender or age differences were seen in GSTM and GSTP activity in human lymphocytes, but an age-dependent decrease in glutathione was detected.[267]

Role of Glutathione S-Transferases in Metabolic Activation

Glutathione conjugation does not always produce an innocuous and readily excreted metabolite; for example, Elfarra and Anders[268] compiled a list of 1,2-dihaloalkanes and halogenated alkenes whose glutathione or cysteine conjugates were nephrotoxic. Glutathione reacts with these 1,2-dihaloalkanes via a GST-catalyzed reaction that yields sulfur mustards. An electrophilic episulfonium ion can be formed from the mustard when the second halogen atom is displaced by a cellular nucleophile (Figure 4.23). The episulfonium ion intermediate has been implicated in the toxicity of these chemicals. The major DNA adduct resulting from exposure to the carcinogen 1,2-dibromoethane was *S*-2-*N*^{7}-guanylethylglutathione.[269] This and other GSH-dependent bioactivation pathways for halogenated hydrocarbons have been extensively reviewed by Anders et al.,[270] Vamvakas and Anders,[271] and Anders.[272]

As shown in Figure 4.22, glutathione and cysteine conjugates (GSR and CySR, respectively) formed in the liver can be excreted in the bile. Glutathione conjugates can be hydrolyzed to cysteine conjugates by γ-glutamyltranspeptidase/aminopeptidase M, present in the bile duct epithelia, or by pancreatic peptidases in the small intestine. Cysteine conjugates originating from the bile and those formed by hydrolysis of glutathione conjugates are good substrates for microfloral

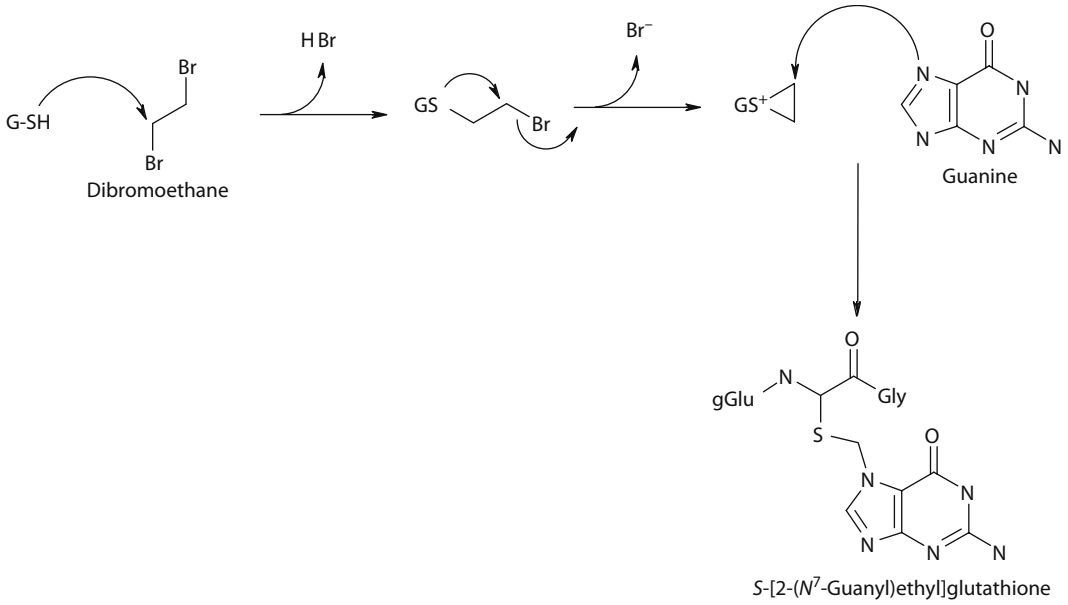


FIGURE 4.23 Bioactivation of 1,2-dibromoethane by conjugation with glutathione, resulting in formation of a DNA-reactive episulfonium ion.

β-lyase. β-Lyase, an enzyme found in liver, kidney, and intestinal microflora, cleaves thioether linkages in cysteine conjugates of xenobiotics.[273] The resulting thiol compounds are more hydrophobic than the conjugates and can be readily absorbed in the small intestine. These thiol metabolites return to the liver via the portal circulation and act as substrates for thiol S-methyltransferase that methylates the thiol group. Enterohepatic circulation of glutathione conjugates accounts for some of the unusual sulfur-containing metabolites that have been found in the urine of animals treated with xenobiotics, such as propachlor[276] and acetaminophen.[274] A portion of the glutathione-derived sulfur-containing metabolites formed in the small intestine is excreted in the feces.

Reactions of glutathione and cysteine conjugates of compounds shown in Figure 4.24 are believed to play a role in the nephrotoxicity of several xenobiotics. Cysteine conjugates are actively transported into renal tubular epithelia, where they may be bioactivated by renal β-lyase[275]; for example, the cysteine conjugate of trichloroethylene, *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), is a potent nephrotoxin and a β-lyase substrate. Inhibition of renal β-lyase with aminooxyacetic acid, an inhibitor of pyridoxyl phosphate-dependent enzymes, protected against DCVC-induced nephrotoxicity.[268] In contrast, the nephrotoxicity of hexachloro-1,3-butadiene was enhanced by the γ-glutamyltranspeptidase inhibitor acivicin, suggesting that the nephrotoxicity of this polyhaloalkene may not be mediated by its glutathione conjugate.[276,277]

Another glutathione-dependent bioactivation mechanism involves the reversible conjugation of isocyanates such as methyl isocyanate, the chemical responsible for the Bhopal disaster of 1984. Isocyanates are excellent substrates for glutathione conjugation, resulting in formation of an S-carbamoylated glutathione; however, the reaction is reversible, which can lead to regeneration of the free isocyanate.

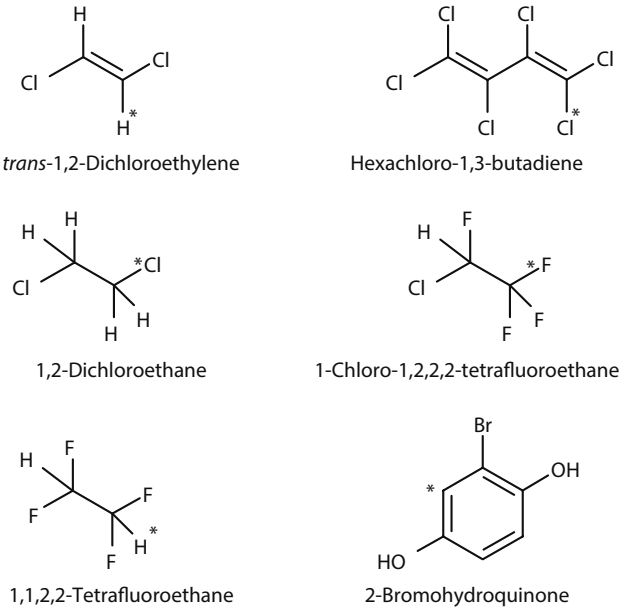


FIGURE 4.24 Representative halogenated compounds that form nephrotoxic glutathione conjugates. The site of reaction with glutathione is indicated by an asterisk (*).

Furthermore, both the glutathione and cysteine participate in transcarbamoylation reactions with tissue-free sulfhydryl groups and other nucleophiles[278]; thus, it has been suggested that glutathione conjugation may actually increase the toxicity of isocyanates by facilitating distribution and release of isocyanate within the body.[279]

In general, glutathione conjugate synthesis results in readily excreted polar metabolites; however, in some cases, the residence time of a glutathione conjugate in the body is prolonged. This can result in formation of metabolites that are

more reactive than the original parent xenobiotic or the glutathione conjugate. If these reactive metabolites interact with critical cellular sites, toxicity can ensue. For recent reviews, see Anders[276] and van Bladeren.[280]

Glutathione S-Transferases as Markers of Liver Damage

GSTs may be valuable as an adjunct to serum aminotransferases for detecting acute liver damage. In particular, GSTα is one of a series of novel candidate biomarkers currently under investigation for liver injury.[281] These transferases constitute as much as 5% of cytosolic protein in the hepatocyte and are uniformly distributed across the liver lobule, compared with aminotransferases, which are localize in the periportal region. Their plasma half-life is less than 60 min, compared to 48 h for the alanine aminotransferase. Selective use of these different characteristics between aminotransferases and GSTs may lead to more accurate diagnoses of hepatic damage produced by xenobiotics.[282] Recently, it has been suggested that the determination of GST should be included in toxicology studies, and validated methods for rats and dogs have been developed.[283]

Methylation

Methyl conjugation is an important pathway in the metabolism of many neurotransmitters, drugs, and xenobiotics. Methylation of endogenous substrates, such as histamine, amino acids, proteins, carbohydrates, and polyamines, is important in the regulation of normal cellular metabolism and accounts for the presence of this activity in mammalian cells. Only when a xenobiotic fits the requirements for the enzymes involved in these normal reactions does methylation become important in the metabolism of foreign compounds. Typical methylation reactions include O-, S-, and N-methylation.

Methylation can be achieved by two routes. First and foremost is the methyltransferase-catalyzed methylation that requires *S*-adenosylmethionine (SAM) as a cosubstrate. Most biological methylations require SAM as the methyl donor; however, some methyl transferases require SAM as a cosubstrate but vary in other requirements for optimal activity.[284] Reactions involving four of these SAM-dependent methyltransferases are shown in Figure 4.25. A secondary source of methylation is N^5-methyltetrahydrofolate (5-CH_3-THF)-catalyzed methylation. This methylation is important in the synthesis of nucleic acids; however, 5-CH_3-THF is 1000 times less reactive toward soft nucleophiles than SAM, suggesting that it plays a smaller role in xenobiotic metabolism.

The methylation of catechol oxygen atoms is catalyzed by catechol O-methyltransferase (COMT), which is most widely known as the enzyme catalyzing the methylation and deactivation of dopamine, other catecholamines, and catechol estrogens. Both cytosolic and membrane-bound forms exist and are encoded by a single gene but use two separate promoters.[285] Methylation of catechols inactivates their biological function and diverts them from secondary pathways that

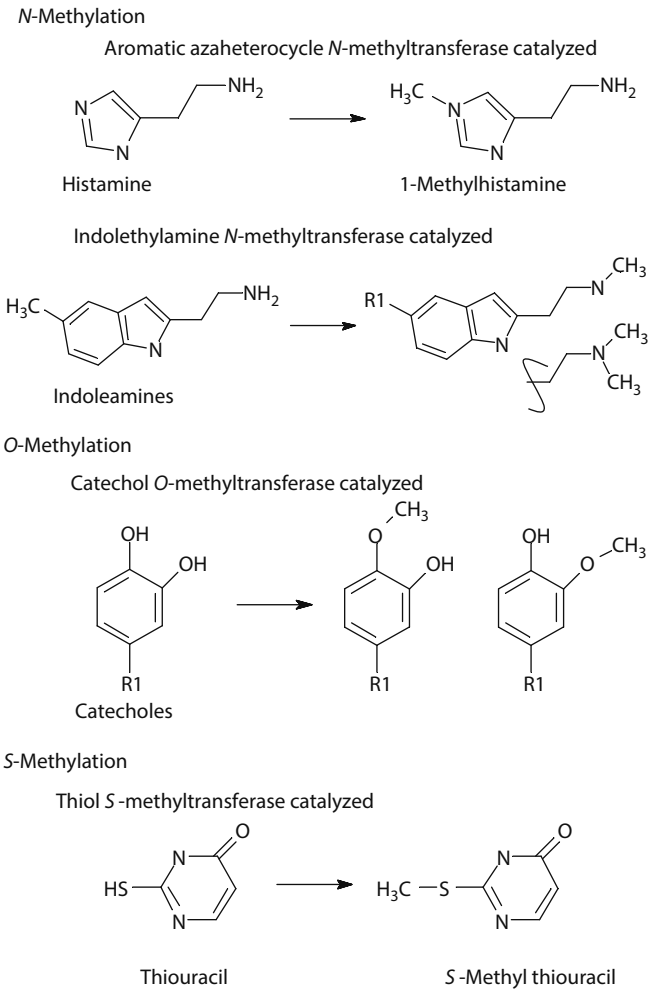


FIGURE 4.25 Methylation reactions.

lead to reactive semiquinone/quinone metabolites and oxyradicals, which are believed to damage catechol-containing neurons and vascular endothelial cells. SAM availability as a cofactor is critical to COMT activity. SAM provides the methyl group via COMT liberating *S*-adenosyl homocysteine (SAH). Because SAH can have an inhibitory effect on SAM, nutritional states that impair the removal of SAH and resynthesis of SAM can have negative consequences on SAM homeostasis and SAM-dependent methylation pathways. Thus, nutritional and biochemical deficiencies in B_6, B_{12}, and folate will disrupt SAM/SAH homeostasis.

Two enzymes catalyze nitrogen methylation: aromatic azaheterocycle N-methyltransferase and indolethylamine N-methyltransferase. N-Methylation of azaheterocycles typically leads to quaternary azaheterocycles. Examples where biotransformation has led to toxic products are more well known and include the biosynthesis of paraquat, the potent lung toxicant, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which, following oxidation via MAO, leads to the formation of 1-methyl-4-phenylpyridinium ions (MPP^+). MPP^+ is the active metabolite associated with Parkinsonian syndrome, which has been observed in humans after self-administration of designer drugs contaminated with MPT

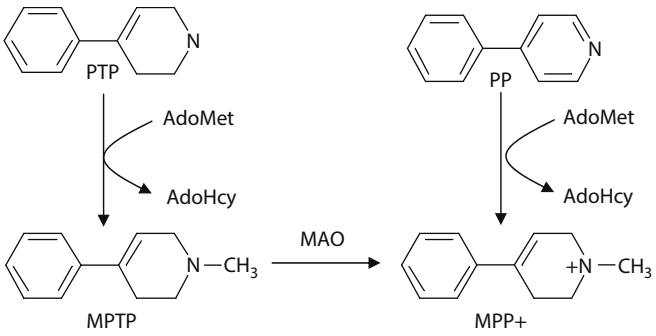


FIGURE 4.26 Metabolic conversion of 4-phenyl-1,2,3,6-tetrahydropyridine (PTP) and 4-phenylpyridine (PP) to the Parkinsonian neurotoxin 1-methyl-4-phenylpyridinium ion (MPP+): MPTP is oxidized by MAO in the brain to MPP+, which is selectively taken up into dopaminergic cells of the nigrostriatum via the dopaminergic reuptake transporter. Once in the pathway of the cells, MPP+ becomes concentrated in mitochondria, where it causes cell death by inhibiting mitochondrial respiration. (From Hoffman, J.L., *Adv. Pharmacol*., 27, 449, 1994. With permission.)

(Figure 4.26).[286] These N-methylation reactions form inhibitors of mitochondrial respiration.

The second pathway, catalyzed by indolethylamine N-methyltransferase, catalyzes the methylation of primary amines to secondary and tertiary amines. This enzyme N-methylates endogenous biogenic amines such as serotonin, tryptamine, tyramine, norepinephrine, and dopamine, as well as drugs and xenobiotics such as amphetamine, normorphine, and aniline.

Sulfur methylation occurs via thiol S-methyltransferase and is also dependent on SAM as the methyl donor. Substrates for S-methyltransferase reactions range from hydrogen sulfide to thiopurine. Hydrogen sulfide, produced by the anaerobic metabolic activity of gut microflora, is initially methylated by gut mucosal S-methyltransferase to yield methanthiol. Although a poorer substrate, methanthiol can be further methylated to dimethyl sulfide. S-Methyltransferase activity is a well-known detoxification pathway for the thiopurine drug 6-metcaptopurine and its prodrug azathioprine, the activity being known as thiopurine S-methyltransferase (TPMT). These cytotoxic drugs are effective against acute lymphoblastic leukemia and autoimmune diseases but have narrow therapeutic indexes, and toxic overdoses can be induced in patients with genetic variants of TPMT.[287] Through relatively simple measurements of TPMT activity in red blood cells, patient dose can be effectively modulated to match patient genotype and phenotype.

Amide Synthesis

Amide biosynthesis can take place via two principal routes:

- Conjugation of a carboxylic-acid-containing xenobiotic with the free amino group of an amino acid such as glycine
- Acetylation of a xenobiotic containing a primary amine ($-NH_2$)

Amino Acid Conjugation

Xenobiotics that contain a carboxylic acid moiety are susceptible to conjugation with endogenous amino acids. Xenobiotic conjugation occurs in hepatic mitochondria. The free carboxylic acid is activated by reaction with ATP followed by reaction with coenzyme A (CoA), as shown in reactions 1 and 2 of Figure 4.27. For example, the carboxylic acid of benzoic acid is activated to a thioester CoA intermediate that reacts with the primary amine of glycine to form the amide hippuric acid.

Glycine has historical significance in xenobiotic conjugation because it is one of the earliest reactions attributed to xenobiotic metabolism. Keller,[288] in 1842, administered benzoic acid to himself and then isolated and characterized the major metabolite, hippuric acid, a glycine conjugate. This reaction has been used as a liver function test in humans. The liver is the principal site of glycine conjugation. Other amino acids, such as taurine, can be used for conjugating aliphatic, aromatic, and heterocyclic carboxylic acids. Taurine conjugates of pioglitazone metabolites were identified in the bile of treated dogs.[289]

Acetylation

Acetylation, catalyzed by NATs, is the principal pathway of amide formation for primary aromatic amines, endogenous primary aliphatic amines, anutrient amino acids, hydrazines, hydrazides, and sulfonamides. NAT catalyzes the two-step transfer of an acetyl group from the donor (acetyl-CoA) to the aromatic amine. These enzymes are cytosolic, occur in many tissues, and are comprised of at least three families of isoenzymes. Acetyltransferases can also catalyze O-acetylation of xenobiotics, as has been shown especially for the acetylation of aryl hydroxylamines such as *N*-hydroxy-2-aminofluorene and 4-aminobiphenyl (Figure 4.28).

Some species, including humans, rabbits, and hamsters, express two independently regulated transferases, NAT1 and NAT2; other species, such as mouse, express three: NAT1, NAT2, and NAT3. NAT1 and NAT2 have been studied most extensively. They are structurally similar proteins with a cysteine residue at the active site, but they have different substrate specificities, although some overlap occurs. NAT1 is expressed in most tissues, whereas NAT2 is expressed only in the liver and gut. Polymorphisms in NATs are well described for the human population, and more than 26 different alleles have been reported.[290] NAT2 and more recently NAT1 have been shown to have several allelic variants resulting in fast and slow acetylator status, and simulated distributions of these alleles suggest sensitive populations.[291] Especially well described for the NAT2 enzyme, acetylator status can affect susceptibility to drugs such as dapsone and isoniazid in which slow acetylation leads to peripheral neuropathy.

Acetylation of aromatic amines is generally a detoxification pathway because the added acetyl group blocks further oxidation of the amide nitrogen; however, once oxidized, the resulting hydroxylamine can undergo O-acetylation. The resulting acetoxy ester is unstable, and heterolytic loss results in

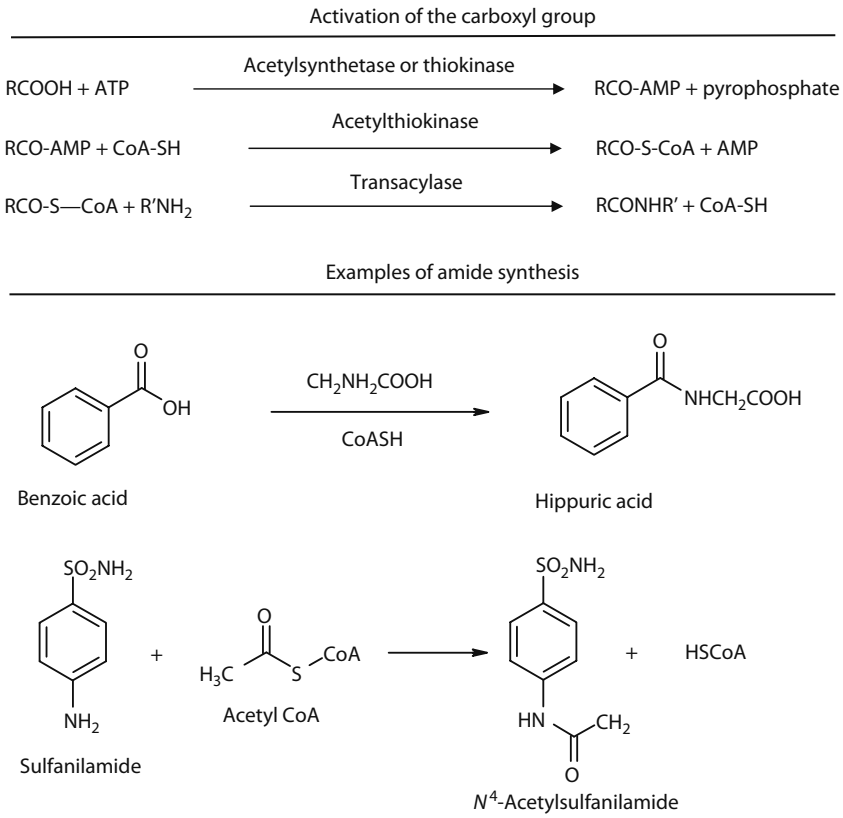


FIGURE 4.27 Series of reactions leading to amide formation from either a xenobiotic containing a carboxylic functional group (RCOOH) or a primary amine group (R′NH2).

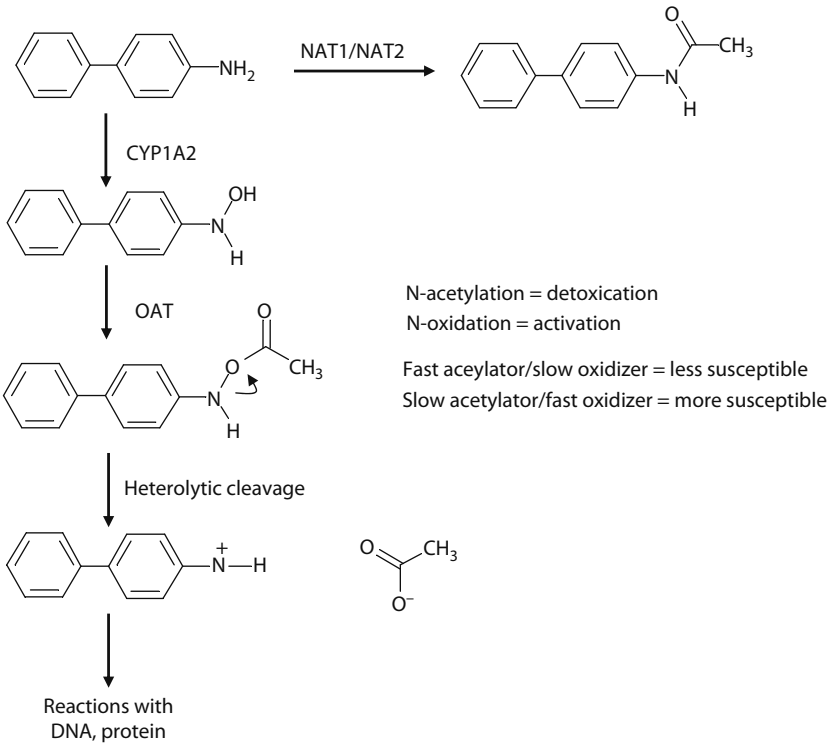


FIGURE 4.28 Metabolism of the bladder carcinogen 4-aminobiphenyl. NAT conjugation is a detoxification pathway that competes with CYP1A2 N-oxidation, which is the first step in metabolic activation that ultimately forms a highly reactive nitrenium ion. Some ethnic groups, such as those of Middle Eastern populations, have a high incidence of the slow acetylator phenotype, which, when coupled with a fast N-oxidation phenotype, results in a much higher risk of bladder cancer.

the formation of highly reactive nitrinium ions that form adducts with cellular DNA and consequently are mutagenic (Figure 4.28). It is frequently the balance between N-oxidation and N-acetylation that determines susceptibility to aromatic amine carcinogenesis. Dogs, which lack NAT activity, are highly susceptible to arylamine-induced carcinogenesis. Human susceptibility has been demonstrated for 4-aminobiphenyl in which N-oxidation and N-acetylation status is tightly associated with risk of bladder cancer among smokers. High N-oxidation status coupled with slow acetylator phenotype yields the highest bladder cancer risk among smokers.

Mercapturic acid formation in the kidney is an example of acetylation that has been presented and represents one of the unusual circumstances in which aliphatic amines undergo N-acetylation. In this reaction, the primary amine group of the cysteine conjugate of the xenobiotic is acetylated to form the mercapturic acid. This is an exception to the rule that aliphatic primary amines generally are not good substrates for the NATs.

HYDROLYSIS

Many xenobiotics and their phase I metabolites contain a carboxyl ester, an amide bond, or an epoxide that masks hydrophilic functional groups, such as alcohols, carboxylic acids, and amines. The rate at which an organism can hydrolyze these bonds and unmask these function groups can influence their toxicity. In fact, pesticides and therapeutic drugs have been synthesized with intent to modulate the bioavailability of the active species by affecting the rate of hydrolysis of the parent compound.

Hydrolysis normally competes with other detoxification reactions, but esterases are in very high content in many tissues, especially liver, and their affinity is low enough such that esterase-/amidase-mediated hydrolysis typically predominates. An example of competition is demonstrated by the metabolism of vinyl acetate (Figure 4.29). This molecule

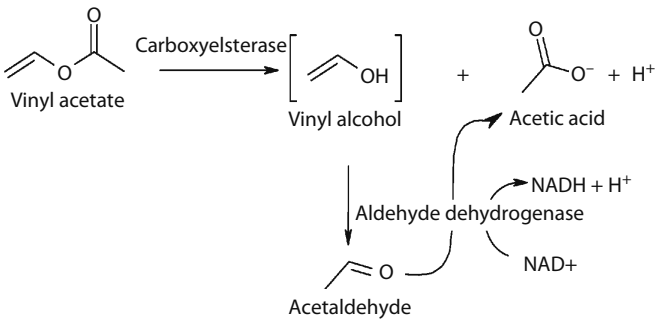


FIGURE 4.29 Carboxylesterase-mediated hydrolysis of vinyl acetate. Esterase hydrolysis is so efficient that cytochrome P450-mediated epoxidation of the double bond does not occur. Vinyl alcohol is an unstable intermediate and readily rearranges to form acetaldehyde, which undergoes further oxidation to acetic acid. The potential toxicity of acidic metabolites is often overlooked, but in the case of vinyl acetate, the acetic acid generated contributes substantially to the mechanism of toxicity.

contains both a double bond and an ester group and therefore would be expected to be a substrate for both epoxidation and carboxylesterase-mediated hydrolysis; however, the metabolism of this compound via the carboxylesterase pathway is so efficient that no epoxide is formed. Hydrolysis products such as alcohols, amines, or thiols and carboxylic acids are typically further metabolized.

Epoxide Hydrolase

Organisms may be exposed to epoxides in the environment or they may be produced during the oxidative metabolism of specific xenobiotics from their environment. Epoxides generally are reactive electrophilic compounds due to the highly strained oxirane ring. Excess strain energy can be released by ring opening in the presence of nucleophiles, which may result in covalent modification of the macromolecule. Modification of DNA results in a biochemical lesion that may be the precursor to a number of pathological lesions, including cancer. Reaction of the epoxide with cellular nucleophiles, such as proteins, could also lead to other mechanisms producing acute or subchronic toxicity.

The chemical reactivity and, consequently, the biological activity of epoxides are influenced by the constituents attached to the oxirane ring carbons. Asymmetric substitution and electron-withdrawing substituents near the oxirane ring tend to destabilize the epoxide and enhance its reactivity. Epoxides that hydrolyze in water are among the most reactive and electrophilic but may not be toxic when hydrolyzed. Alternatively, if they are generated close to or have a long enough half-life to reach and react with critical cellular macromolecules, epoxides can have both cytotoxic and genotoxic consequences. An example of a relatively stable epoxide is styrene-7,8-oxide, which is the substrate typically used as a generic assay for epoxide hydrolase activity (Figure 4.30).

A major route for biodisposition of epoxides is hydration catalyzed by epoxide hydrolase to vicinal (from the Latin vicinalis, neighboring) dihydrodiols. The membrane-bound and cytosolic forms were first characterized in 1973 and 1976, respectively. They are now known to comprise a large and heterogeneous group of enzymes. Their structure, function, and mechanism have been reviewed recently.[295] This microsomal enzyme catalyzes the biotransformation of a diverse group of arene oxides and aliphatic epoxides (Figure 4.30). In most cases, this enzymatic pathway results in less reactive diol metabolites that are more readily excreted from the organism, either as the diol or as a glucuronide or sulfate conjugate of the diol.

Epoxide hydrolases occur as membrane-bound proteins located in the endoplasmic reticulum and as a soluble enzyme in the cytosol of most mammalian cells. The membrane-bound microsomal form has broad substrate specificity, while the soluble, or cytosolic, form has a higher affinity for nonbulky trans-substituted oxiranes.[293,294] Humans demonstrate considerable variation in epoxide

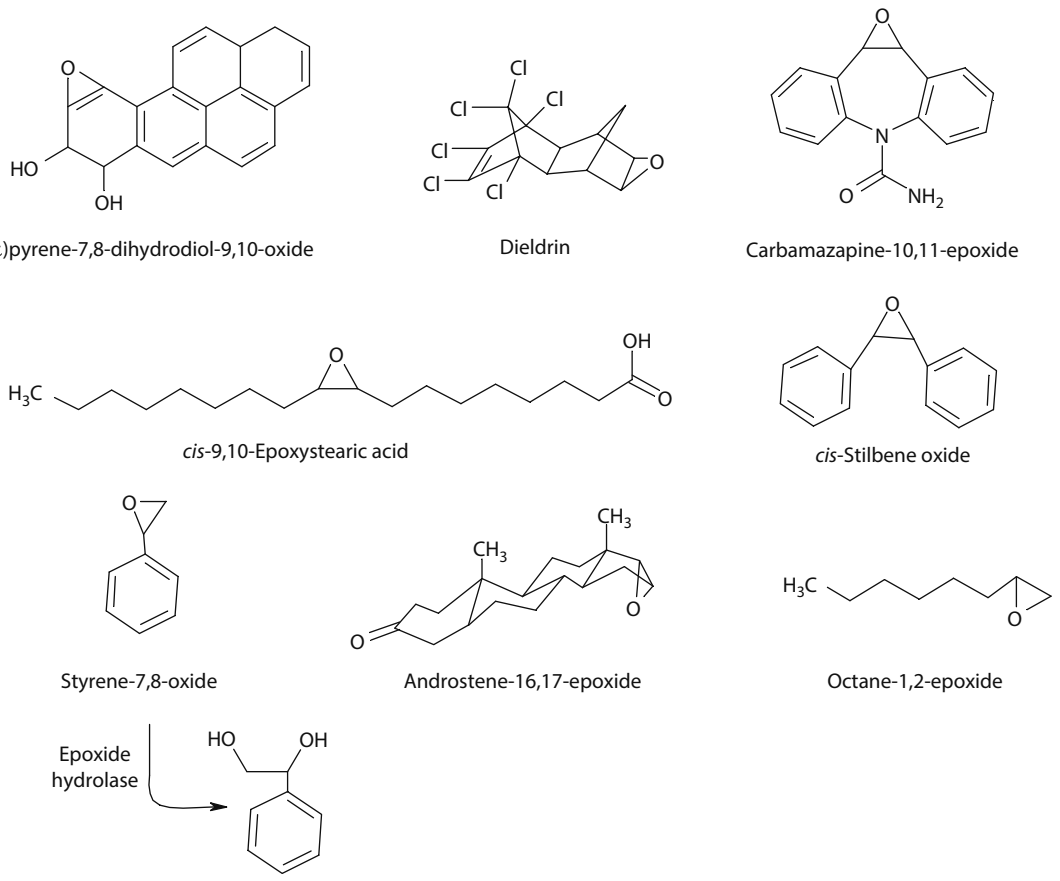


FIGURE 4.30 Structural diversity of epoxides that are substrates for epoxide hydrolase. Benzo(*a*)pyrene-7,8-dihydrodiol-9,10-oxide illustrates a potent mutagen, which is first oxidized to the 7,8-epoxide, followed by epoxide hydrolase hydrolysis of the epoxide and then secondary oxidation at the 9,10 position. The dihydrodiol imparts a steric hindrance toward the 9,10 epoxide, leaving the epoxide relatively, although not completely, resistant to hydrolysis. (Adapted from Arand, M. et al., *Drug Metab. Rev.*, 25, 365, 2003.)

hydrolase activity. These human polymorphisms may not result in significantly altered enzyme activity or posttranscriptional regulation,[295] although more work is required in this area.

Epoxide hydrolase has been found in a variety of tissues, including liver, kidney, lung, skin, intestine, colon, testis, ovary, spleen, thymus, heart, and brain. The activity of liver microsomal enzyme is relatively low in newborn rats and increases during neonatal development until adult males have about twice the activity of females. This sexual dimorphism is remarkably similar to that seen in the rat for P450. In contrast, the renal epoxide hydrolase of male and female rats does not demonstrate age-dependent changes or gender differences. Human hepatic microsomal epoxide hydrolase activities increase during gestation, but no gender difference in humans has been observed.[296]

The activity of this enzyme is induced by the classical inducers of cytochromes P450. Although trans-stilbene oxide has been shown to be an inducer of epoxide hydrolase, no specific inducer of epoxide hydrolase has been reported. Two widely used inhibitors of epoxide hydrolase are trichloropropane oxide and cyclohexene oxide.

An immunologically distinct epoxide hydrolase has also been identified in the cytosol of some species. This enzyme may play a role in the hydrolysis of more water-soluble epoxides that partition out of the endoplasmic reticulum. Epoxide hydrolases may compete with glutathione transferases for cytosolic epoxides; however, epoxide hydrolase has a higher affinity for many epoxide substrates than glutathione transferase and is therefore generally a more efficient detoxification mechanism.

Esterases and Amidases

Hydrolysis of xenobiotics containing ester linkages and amide bonds is catalyzed by a group of enzymes with broad substrate specificity. A new nomenclature system for five gene families of mammalian carboxylesterase designates the human genes as CES and the mouse and rat genes as Ces and each is followed by a family number.[297] The reactions carried out by this diverse group of enzymes are illustrated

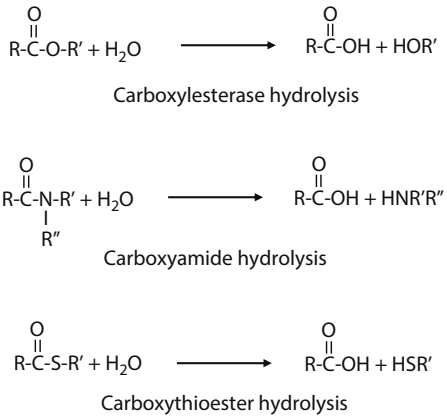


FIGURE 4.31 Reactions catalyzed by esterases and amidases.

in Figure 4.31. The specificity of carboxylesterases depends on the nature of the R groups rather than on the atom (O, N, or S) adjacent to the carbonyl carbon.[298] The esterases have been broadly grouped into three categories based on their reactivity with organophosphorous compounds.[299] Those esterases preferring carboxylesters with aryl groups in the R position and that can use organophosphate esters as substrates are classified as A-esterases (Table 4.7). Those esterases preferring esters with alkyl groups in the R position and that are inhibited by organophosphate esters are classified as B-esterases. Another group of esterases that prefer acetate esters and do not interact with organophosphates are referred to as C-esterases. The mechanism of organophosphate and carbamate insecticide toxicity is inhibition of acetylcholinesterase, a B-type esterase. Organophosphate insecticides, such as malathion, target serine hydrolases (B-esterases) and are detoxified in mammals by A-esterase hydrolysis.[300] Many insects have lower levels of A-esterases than mammals. The selective toxicity of malathion in birds and insects can be explained by the low activity of A-esterases compared to mammals.[301]

Carboxylesterases are widely distributed in the body including tissues lining the major portals of entry (i.e., skin, gastric mucosa, and respiratory tract). Liver has the highest capacity for esterase hydrolysis, but on a tissue-weight basis, other tissues, such as the olfactory mucosa, contain comparable levels.[302] Hydrolytic activity at these sites can be used to improve drug bioavailability by designing ester-containing prodrugs that are more lipid soluble than their alcohol or carboxylic acid analogs and therefore are more readily absorbed; however, carboxylesterase activity at the portal of entry can also result in metabolic activation when the hydrolysis products are toxic. Such is the case for vinyl acetate, a volatile organic monomer that, when inhaled, is absorbed and metabolized by carboxylesterases within the nasal cavity mucosal lining.[303]

MICROFLORAL METABOLISM

Because the majority of microbes that colonize various surfaces of the mammalian body reside in the intestinal tract, most of this discussion will center around intestinal microflora metabolism. The intestinal microflora can alter xenobiotic bioavailability by metabolizing the parent compound to a metabolite that may be absorbed to a greater or lesser extent. Intestinal microflora can also metabolize products of xenobiotic biotransformation that are secreted into the

TABLE 4.7
Classification of Esterases by How They Interact with Organophosphates and Substrate Specificity

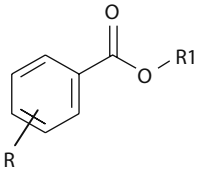
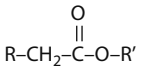
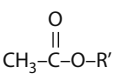
Esterase	Interaction with Organophosphates	Substrates	Examples
A-Esterases (arylesterases)	Substrates	 Aromatic esters	Organophosphate and carbamate insecticides
B-Esterases	Inhibitors	 Aliphatic esters	Acetylcholine, acrylate esters Succinylcholine, propanidid
C-Esterases (acetylesterases)	No interaction	 Acetate esters	*p*-Nitrophenyl acetate, *n*-propylchloroacetate

TABLE 4.8
Types of Metabolic Reactions Carried out by Intestinal Bacteria

Reaction	Representative Substrate
Hydrolysis	
Glucuronides	Estradiol-3-glucuronide
Glycosides	Cycasin
Sulfamides	Cyclamate, amygdalin
Amides	Methotrexate
Esters	Acetyldigoxin
Nitrates	Pentaerythritol trinitrate
Dehydroxylation	
C-Hydroxy groups	Bile acids
N-Hydroxy groups	*N*-Hydroxyfluorenylacetamide
Decarboxylation	Amino acids
N-Demethylation	Biochanin A
Deamination	Amino acids
Dehydrogenase	Cholesterol, bile acids
Dehalogenation	DDT
Reduction	
Nitro groups	*p*-Nitrobenzoic acid
Double bonds	Unsaturated fatty acids
Azo groups	Food dyes
Aldehydes	Benzaldehydes
Alcohols	Benzyl alcohols
N-Oxides	4-Nitroquinoline-1-oxide
Other reactions	
Nitrosamine formation	Dimethylnitrosamine
Aromatization	Quinic acid
Acetylation	Histamine
Esterification	Gallic acid

intestine directly from the blood or via the bile, saliva, or swallowing respiratory tract mucus. Metabolism of secreted metabolites is a common mechanism by which microflora influence xenobiotic toxicity. The hallmark of metabolism by organisms colonizing the intestinal tract of mammals is reduction (Table 4.8).

Xenobiotic Biotransformation by Microbes Colonizing Mammals

The intestinal tract of mammals contains a variety of microorganisms. The majority of mammals have a gradient of microflora that increases in numbers and species diversity from the upper to the lower gastrointestinal tract. Most research on microflora metabolism has focused on microorganisms that colonize the large intestine of humans, as most of the research in toxicology is directed toward understanding the toxicity of chemicals in humans. In vivo and in vitro models have been developed for studying human colonic flora.[304] Since diet affects intestinal microflora, diet has an important influence on microbial metabolism of xenobiotics.

Examples of Xenobiotics Whose Toxicity Is Dependent on Microflora Metabolism

Nitroaromatics

The toxicity of many nitroaromatic compounds is dependent on microflora metabolism. One of the most studied nitroaromatics is 2,6-dinitrotoluene (DNT), which is hepatocarcinogenic in male rats.[305] DNT is metabolized to the 2,6-dinitrobenzylalcohol glucuronide conjugate that is preferentially excreted in the bile of male rats (Figure 4.32).[306] The glucuronide conjugate is hydrolyzed by gut microflora β-glucuronidase, and one or both of the nitro groups are reduced by microflora nitroreductase to a reduced aglycone. The resulting aminobenzyl alcohol is relatively nonpolar and reabsorbed in the intestine, where it returns to the liver via the portal circulation. In the liver, the aglycone is activated to the putative proximate carcinogen by N-hydroxylation of the amine functional group followed by sulfation of the N-hydroxy group.[307] Evidence that intestinal microflora were required for the activation of DNT was provided by studies indicating that the genotoxicity of DNT in hepatocytes isolated from rats treated with DNT was dependent on the presence of bacteria in the intestinal tract.[308] Rats raised in a germfree environment showed minimal levels of genotoxicity. Additional evidence emphasizing the role of microflora in the metabolic activation of DNT was the observation that DNT was not genotoxic when tested in vitro in isolated hepatocytes.[308] These results indicated that liver metabolism was not sufficient to activate the molecule to the ultimate carcinogen. The genotoxicity of DNT to liver cells only occurred when the compound was administered to the animal and was allowed to undergo enterohepatic circulation involving intestinal microflora. Dietary treatments that increased the microbial metabolic capacity of the rat's large intestine also increased the covalent binding of DNT-derived radioactivity to hepatic macromolecules.[309]

Cyclamate

The sodium and calcium salts of cyclamic acid (cyclohexylsulfamic acid) were used as an artificial sweetening agent until 1969 in the United States, when it was removed from the market because a metabolite (cyclohexylamine) was suspected of being a bladder carcinogen. Most of the hydrolysis of cyclamate to cyclohexylamine takes place in the gut by the microflora. Cyclohexylamine is more lipophilic than the parent acid and is readily absorbed from the intestine and excreted in the urine. Minor urinary metabolites include cyclohexanol and trans-cyclohexane-1,2-diol.

Although only trace amounts of the cyclohexylamine could be detected in human-administered cyclamate, chronic exposure to the acid increased the capacity to produce this metabolite.[312] It was found that certain individuals possessed a greater capacity to metabolize cyclamate to cyclohexylamine; these individuals were called converters. Thus, cyclamate is a good example of how prior exposure to a xenobiotic can alter the disposition of the xenobiotic. For additional

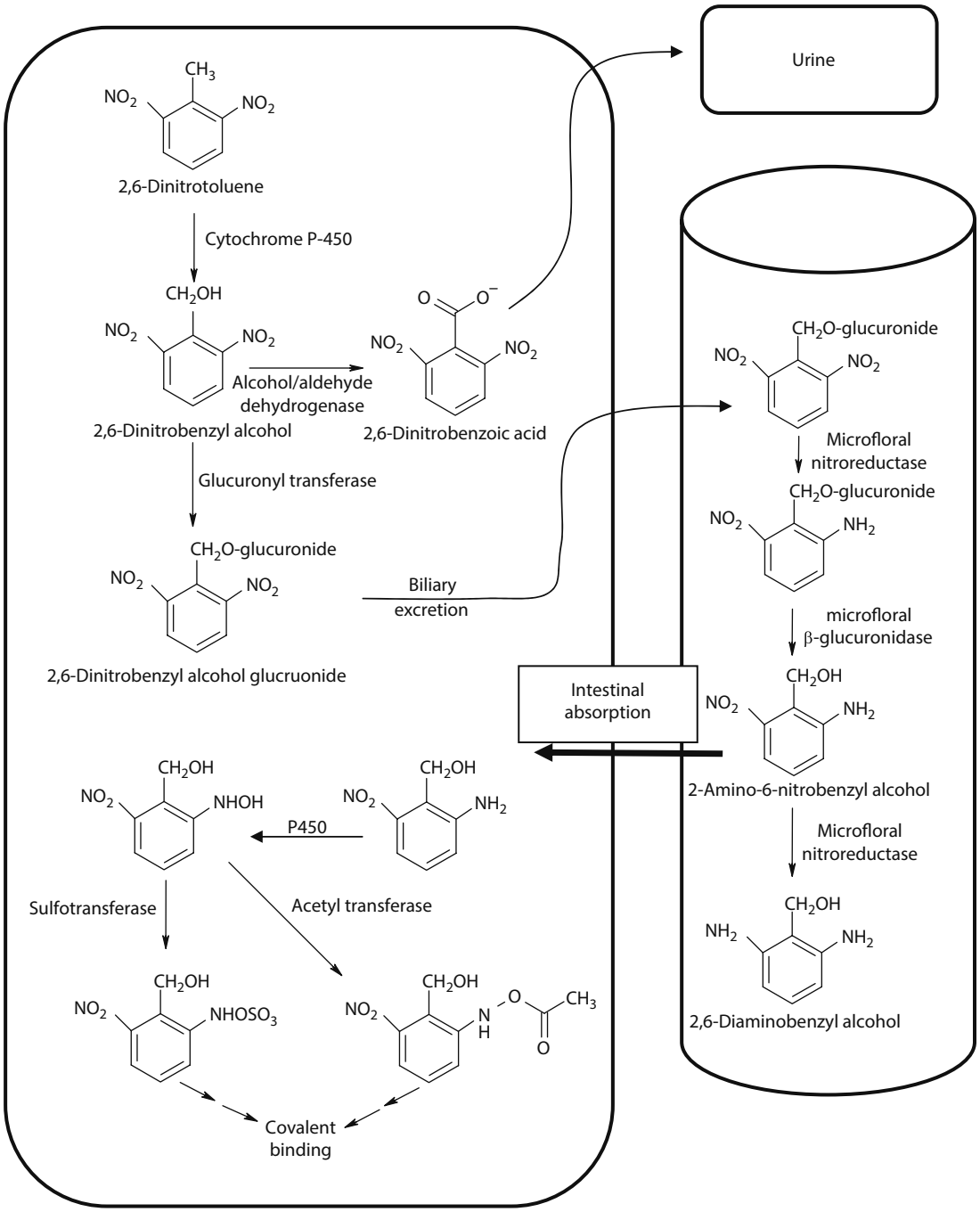


FIGURE 4.32 Putative route of disposition of DNT.

reading on intestinal microflora xenobiotic metabolism, see Goldman[310] and Scheline.[39]

TRANSPORTERS

More than 400 human uptake and efflux membrane transporters have been identified and reported. Considerable progress has been made, particularly over the last decade, regarding characterization of drug–transporter interactions, understanding potential clinical significance, and clarification of regulatory implications.[311–315] Drug transporters recognize a structurally diverse range of substrates including drugs, metabolites, and endogenous compounds. Transporters are expressed in many types of endothelial and epithelial cells where they facilitate the absorption, elimination, and distribution of drugs into various organs.[316–318] The process by which transporters function to facilitate the uptake and efflux of substrates ultimately affects overall tissue concentrations, which in turn affects rates of drug elimination via metabolism and excretion. Thus, the interaction

of a drug with membrane transporters can potentially lead to alterations in exposure that result in toxicity, or in certain instances, therapeutic failure.[319,320]

In general, efflux and uptake transporters are categorized as either ATP-binding cassette (ABC) or solute carrier (SLC) transporters. The expression, function, and relevance of ABC and SLC transporters with respect to xenobiotic transport and potential DDIs have been extensively reviewed.[144,311–315,321–324]

ABC Transporters

ABC transporters are transmembrane proteins that utilize the energy of ATP hydrolysis to translocate various substrates across cell membranes, a process that can occur against a considerable concentration gradient.[311,312,314,322,325] ABC transporters extrude a wide variety of substrates from cells, including exogenous compounds such as drugs, drug conjugates, and metabolites and endogenous compounds such as lipids, sterols, and bilirubin. Interactions involving efflux transporters have been implicated in tumor resistance, cystic fibrosis, bacterial multidrug resistance, and a range of other inherited human diseases. ABC transporters considered important in the absorption and disposition of drugs and various endobiotics include P-gp (MDR1/P-gp), the breast cancer resistance protein (BCRP), the bile salt export protein (BSEP), and the multidrug-associated resistance proteins (MRP2, MRP3, MRP2).

SLC Transporters

SLC transporters are primarily unidirectional uptake transporters, although some SLC transporters function in a bidirectional manner.[144,311,313] SLC transporters include over 300 members organized into 47 families, including both facilitative and secondary active transporters. Solutes, or substrates, that are recognized and transported across membranes by SLC transporters include charged and uncharged organic molecules as well as inorganic ions. Uptake transporters considered important in the absorption and disposition of drugs and various endobiotics include the organic anion-transporting polypeptides (OATP1B1, OATP1B3, OATP1A2, and OATP2B1), organic cation transporters (OCT1 and OCT2), organic anion transporters (OAT1 and OAT3), the multidrug and toxicant extrusion proteins (MATE1 and MATE2-K), and the oligopeptide transporters (PEPT1 and PEPT2).

Determining the relative importance of the many uptake and efflux transporters, as well as their influence on drug disposition, therapeutic efficacy and safety, and in particular DDIs, has been the focus of considerable research. Evolving from what was originally widely accepted as passive transport, it is now recognized that nearly all drugs and their metabolites enter cells to some extent, and in many cases to a significant extent, through the activity of membrane transporters.[315,321,326] However, many challenges exist for scientists in the field of drug transporters. For example, few selective substrates and inhibitors have been identified for clinical use; relatively sparse conclusive clinical data are available describing transporter-mediated DDIs; and substrates that interact with one uptake or efflux transporter typically interact with multiple uptake and/or efflux transporters.[315,335] As such, extrapolation of in vitro data to predict in vivo outcome remains quite challenging, and designing static or dynamic models incorporating transporter interactions is still a developing field.

Prediction of drug interactions has traditionally focused on the evaluation of interactions involving drug-metabolizing enzymes (DMEs) such as CYP450s; however, more recent studies describe interactions of drugs with transporters as being mechanistically responsible for clinical DDIs.[318,327–330] Many DDIs previously thought to occur via interaction of a substrate and inhibitor with DMEs have been reevaluated and are now ascribed to interactions involving DMEs and transporters with drugs that are substrates and/or inhibitors. Thus, the interplay of transporters with DMEs is a critical concern when investigating the potential impact of transporters on drug absorption and elimination.[315,331–333]

Tools Used to Study Transporter Interactions

Characterization of a drug or experimental compound as a potential substrate, inhibitor, or modulator[336] of membrane transporters is important from a safety perspective. The Caco-2 cell line, which is derived from a human colorectal adenocarcinoma, is one of the most commonly used in vitro models to evaluate the interaction of drugs with ABC transporters such as P-gp, MRP2, and BCRP.[334–338] Similarly, human embryonic kidney (HEK293) or Chinese hamster ovary (CHO) cells expressing SLC transporters are often used to study transporter-mediated uptake.[339] Interactions between drugs or compounds in development and transporters are routinely investigated by researchers in the pharmaceutical industry. As recommended by the International Transporter Consortium (ITC), these studies typically include OATP1B1, OATP1B3, OATP2B1, OAT1, OAT3, and OCT2.[315,323]

Various other in vitro systems can be used to study drug–transporter interactions, including but not limited to primary cultured proximal tubule cells, rat brush-border membrane vesicles, and more recently, human kidney slices.[340] Drug–transporter interactions, including the potential interplay between uptake transporters, efflux transporters, and DMEs, can be studied using isolated hepatocyte systems. The sandwich-cultured hepatocyte format is a well-characterized system, which allows for the estimation of biliary clearance and can maintain hepatic phenotype and transport activity for up to 10 days in the absence of serum.[341] This model promotes establishment of cell polarity, exhibits reasonable expression levels of both uptake and efflux transporters, and offers the opportunity to study the effect of nuclear receptor activation and cytotoxicity.[341,342] However, cultured hepatocyte systems have various drawbacks such as a decline in uptake and efflux transporter expression following several days in culture, which can potentially affect clearance and DDI predictions.[343,344]

In vivo and ex vivo models are also routinely used to study drug–transporter interactions. These include rat blood–brain barrier (BBB) penetration studies using P-gp and BCRP knockout models, isolated perfused rat lung, transporter knockout mice, and whole-body autoradiography. While an overestimation of the potential for changes in human brain levels based on the mouse data may occur,[320,345] use of these models can still provide valuable insights. Additionally, chemical knockout of P-gp using selective inhibitors can be used.[346] Even though rat knockout models are generally less advanced,[347] they provide an easier animal model for multisampling pharmacokinetic analysis when compared to the mouse.

Influence of Transporters on Drug Efficacy

Transporters can affect the disposition of many drugs, in particular the ability of a drug to reach tissues in which the pharmacologic target is located. For example, resistance to drugs used to treat diabetes mellitus can occur due to the function of ABC transporters expressed on target cells.[348] In cancer chemotherapy, drug efficacy can be compromised by an increase in efflux of chemotherapeutic agents out of targeted cells, leading to a reduction in intracellular chemotherapeutic concentrations. A well-established cause of multidrug resistance involves the increased expression of ABC transporters such as P-gp.[349] Overexpression of these efflux transporters, which is likely to occur in transformed cells, can eventually trigger drug insensitivity.[350] This phenomenon often compromises the efficacy of multiple chemotherapeutic agents and can include various drug classes and chemical structures.

The target organ for central nervous system (CNS) drugs is the brain; however, the presence of the BBB is a complicating factor in the design of efficacious CNS drugs. The BBB is composed of microvessel endothelial cells sealed by tight junctions that effectively restrict the entry of many drugs into the CNS. Additionally, efflux transporters such as P-gp, BCRP, and various MRPs are densely expressed on the BBB surface and effectively restrict the distribution of CNS drugs into the brain.[324,351] The primary target organ for many human hepatitis C (HCV) drugs is the liver. In vitro models can be instrumental in helping to determine the potential for a compound to undergo active, or transporter-mediated, absorption into hepatocytes. HCV infection has been shown to trigger an increase in the expression of MDR1, MRP1, and MRP3,[352] but also triggers a decrease in the expression of CYP1A2, CYP2E1, CYP3A4, NTCP, OATP1B1, MRP2, and OCT1.[353,354] Interestingly, the expression of transporters and DMEs is frequently altered during active HCV infection, other inflammatory diseases, and cancer, an effect that can be correlated with the elevated production of inflammatory cytokines.[355]

Influence of Transporters on Safety/Toxicology

The role of transporters in cellular toxicity is recognized and increasingly appreciated by researchers and clinicians, especially when transporter activity is compromised by alteration of function via xenobiotics, disease states, or genetic polymorphisms.[331] To date, most safety-related concerns involving transporters include DDIs. Transporter-mediated DDIs can involve transporters expressed in the intestine, liver, and kidney, which can affect not only the pharmacokinetic profile of a drug but also its pharmacodynamics.[323] Researchers are continuously developing a more advanced understanding of drug–transporter interactions that may result in an unwanted toxicological effect. Two examples of transporters that are known to be implicated in a toxicological response are outlined in the following: muscle toxicity/myopathy from an increase in statin exposure caused by interactions involving OATP1B1 and cholestasis caused by inhibition of bile salt transporters. As this field of study continues to mature, there will undoubtedly be a better understanding of how the interplay between transporters and DMEs can affect toxic response.

Statin-Induced Myopathy

Statins are a frequently prescribed class of drugs that are generally considered safe and well tolerated. However, an unexpected increase in the plasma exposure of statins, for example, simvastatin and pravastatin, has been associated with an increase in the risk of muscle toxicity, or myopathy, which can range from relatively mild to severe.[356,357] While statins differ with respect to their metabolism by DMEs, all statins are substrates of the liver uptake transporter OATP1B1, the product of the SLCO1B1 gene, which has been shown to be polymorphic. Individuals carrying the c.521TT genotype of SLCO1B1 are at significantly higher risk of statin-induced myopathy compared to individuals who do not possess this allelic variant.[321,356,357] Two haplotypes that demonstrate compromised uptake activity, SLCO1B1*5 and SLCO1B1*15, are quite prevalent in Caucasians (~15%–20%), somewhat less prevalent in Asians (~10%–15%), and are much less common in sub-Saharan Africans and African Americans (~2%).[358] Many drugs have been identified as inhibitors of OATP transporters as well; this inhibition can also result in an increase in the plasma exposure of statins and other drugs that are OATP substrates.[331] Thus, careful analysis of the pharmacogenomic influence of OATP1B1 and the potential for DDIs triggered by inhibition of OATPs is an important consideration, especially when evaluating the potential for toxicity of a drug that will be administered to patients taking statins or other drugs that are known substrates of OATP transporters. Interestingly, it has recently been reported that Rotor syndrome, which is a rare autosomal disorder causing conjugated hyperbilirubinemia and mild jaundice, is caused by deficiency of OATP1B1 and OATP1B3.[359] While Rotor syndrome is rare and hyperbilirubinemia can be caused by other factors such as polymorphisms or chemical inhibition of UGT1A1,[360] this finding provides insight into previously unexplored mechanisms of toxicity involving interactions with drug transporters.

Cholestasis

Bile flow is driven by the vectorial transport of bile acid across hepatocytes. Interference with this process by

inhibition of bile acid transporters located on the sinusoidal and canalicular hepatocellular membranes can trigger hepatotoxicity.[361,362] The sodium taurocholate-cotransporting polypeptide (NTCP) is a sodium-dependent transporter located on the sinusoidal hepatocellular membrane that facilitates the transport of bile acids into hepatocytes.[363,364] The bile salt export pump (BSEP) is an ABC transporter located on the canalicular hepatocellular membrane that pumps bile acids out of hepatocytes and into bile.[365,366] Maintaining proper functionality of bile salt transporters is vital, as evidenced by mutations in BSEP that have been shown to cause progressive familial intrahepatic cholestasis type II, which is fatal.[367] Certain drugs such as rifampicin, glibenclamide, and cyclosporine A have been reported to inhibit NTCP and BSEP, resulting in cholestasis.[361] Inhibition of BSEP as a contributor to cholestatic liver injury has been described for various drugs, for example, troglitazone, ketoconazole, lapatinib, nefazodone, and bosentan.[362,368] In vitro systems such as inverted membrane vesicles expressing BSEP,[362] or cultured cells expressing BSEP and NTCP,[361] are commonly used to evaluate the interaction of a compound with these transporters. However, it should be noted that in vitro inhibition of BSEP does not automatically implicate a test compound or drug as a causative agent in cholestatic liver disease in humans, as the therapeutic concentrations in plasma (and liver) need to be compared with the potency of BSEP inhibition.[368] Researchers in the pharmaceutical industry are currently exploring the value of using early determination of BSEP inhibition as a way in which to benchmark compounds for further development, in order to minimize the risk of liver injury later in clinical development.[362]

INTEGRATION OF METABOLIC PATHWAYS

From the sections presented earlier, it is clear that pathways of xenobiotic metabolism range from simple, such as carboxylesterases, to complex, such as the various pathways capable of introducing oxidations. Also clear is the fact that a xenobiotic is not necessarily subject to a single metabolic transformation; rather, xenobiotics are more typically metabolized and eliminated following multiple transformations. Thus, it is important that an integrated view of xenobiotic metabolism be considered when assessing the extent to which metabolism is a determinant of toxicity.

Consider the case of acetaminophen, a drug whose three primary metabolic transformations (oxidation, sulfonation, and glucuronidation) have been discussed in multiple sections earlier. It is the balance of the transformations, the predominance of one pathway over the other, and the factors that determine the predominant pathway that determine toxicity (Figure 4.33). Acetaminophen is eliminated metabolically in the liver primarily by sulfate conjugation of the phenyl hydroxyl group; however, competing with this high-affinity, low-capacity pathway is elimination via glucuronidation also of the hydroxyl group. At high doses of acetaminophen, PAPS pools become depleted and rate limiting, and the secondary pathway of glucuronidation predominates. Glucuronidation thus provides a backup route of elimination. Saturation of these two elimination pathways allows more acetaminophen to flow through the oxidation pathways.

In liver, acetaminophen is oxidized predominantly by CYP2E1 to yield the *N*-acetyl-*p*-benzoquinoneimine. This pathway of metabolic activation forms reactive species that bind to critical macromolecules in the liver and is believed responsible for hepatotoxicity. Thus, inducers of CYP2E1, such as ethanol, increase hepatic capacity for CYP2E1-mediated oxidation and may enhance hepatotoxicity.

Acetaminophen has been associated with analgesic nephropathy. Acetaminophen can also be oxidized by prostaglandin synthase to a reactive metabolite capable of binding to macromolecules. The realization that a pathway other than the well-characterized P450 route was involved occurred through observations that acetaminophen was covalently bound in the inner medulla of the rabbit kidney, a site nearly devoid of P450 activity.[369] Covalent binding has been attributed to prostaglandin synthase-mediated oxidation in the inner medulla; however, acetaminophen-induced renal toxicity is largely limited to the renal cortex, which contains P450 activity. This is because prostaglandin synthase activity of the inner medulla is inhibited at high acetaminophen doses, but the P450 pathway of the cortex is not inhibited and is thus responsible for the cortical damage. This gradient of oxidation pathways across the kidney (with the cortex possessing higher P450 activity than prostaglandin synthase, the outer medulla being intermediate, and the inner medulla possessing far greater prostaglandin synthetase activity) results in certain xenobiotics being more toxic to one region of the kidney than another.[370] The example of acetaminophen highlights that many factors, including species, organ of metabolism, and dose, can influence the expression of xenobiotic-metabolism-mediated toxicity.

COMPUTATIONAL APPROACHES FOR PREDICTION OF BIOTRANSFORMATION

Currently, tens of thousands of chemical entities can be found in commerce and in the environment for which little or no toxicological or metabolism data are available, and chemical manufacturers and pharmaceutical companies continue to develop new compounds at a prodigious rate. Individual testing of such large numbers of chemicals and drugs is time-consuming, costly, resource intensive, and, in the case of whole-animal testing, ethically unsupportable. For these reasons, a great deal of interest has developed in computational (in silico) methodologies to predict chemical toxicity and, more recently, metabolism based on structure and physicochemical properties. The two basic computational approaches to the prediction of chemical metabolism are quantitative structure–activity relationships (QSARs) and expert systems. QSAR, as the name implies, seeks to quantitatively correlate physicochemical, electronic, quantum mechanical, spatial, and other descriptors of a molecule with rates of metabolism, generally focusing on a particular reaction at a particular site within a series of structurally related compounds. These

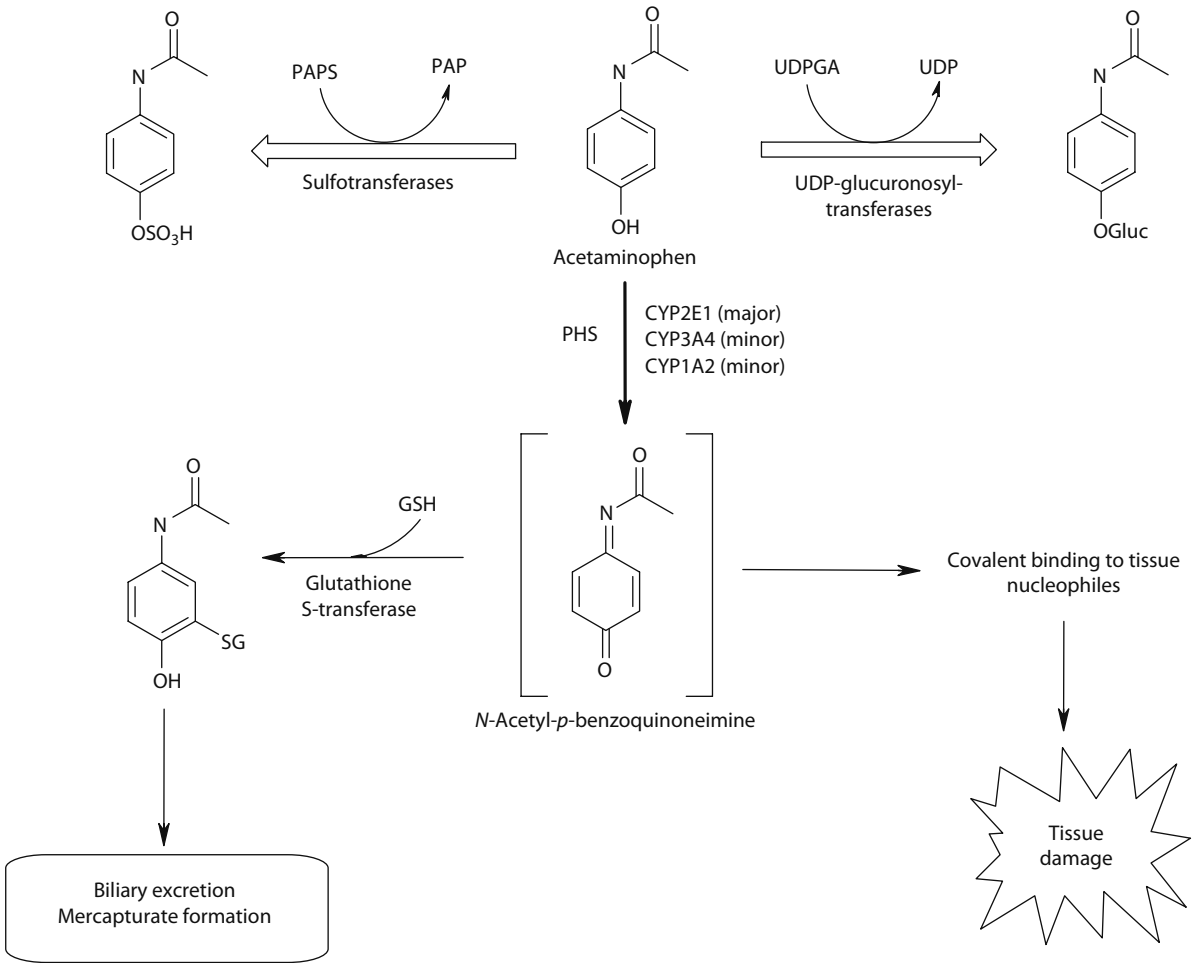


FIGURE 4.33 Integration of metabolic pathways: acetaminophen. Major detoxification pathways are indicated by open arrows and include sulfonation and glucuronidation. Bioactivation of acetaminophen involves oxidation to *N*-acetyl-*p*-benzoquinoneimine (NAPQI), and it may be catalyzed by several P450 isozymes as well as PHS. At therapeutic doses, conjugation pathways predominate and bioactivation is negligible. Following overdose, conjugation pathways become saturated and oxidation to the reactive intermediate NAPQI becomes significant. NAPQI may be detoxified by reaction with reduced GSH, either by a spontaneous or GST-catalyzed reaction and excreted as the corresponding mercapturate. At high exposure levels, GSH may become depleted, allowing NAPQI to react with tissue nucleophiles, resulting in toxicity.

techniques can be very useful in understanding the molecular and electronic determinants of substrate–enzyme interactions and in optimizing (or minimizing) the interaction of a compound with a particular enzyme.

Perhaps of greater interest to the toxicologist is the so-called expert system approach, which seeks to predict global biotransformation pathways based on chemical structure.[139] Expert systems are based on libraries of generalized metabolism rules distilled from large sets of experimental metabolism data. Each rule describes an individual metabolic transformation on a particular substructure or molecular fragment. Several metabolism expert systems are currently commercially available, including METEOR®,[371] TIMES®,[372] META®,[373] and MetabolExpert®.[374] These programs allow the user to draw structures using a graphical interface or to input structures in a variety of file formats. Most are capable of calculating some physicochemical parameters based on the query structure and use these as additional inputs for metabolite prediction. In general, expert system predictions are qualitative, but some programs, such as TIMES, also provide a quantitative estimate of metabolite production.

A major strength of rule-based expert systems is that they incorporate mechanistic insight into predictions and can be used to guide chemistry efforts in designing desirable biotransformation properties into new compounds. A downside to these systems is the potential to generate excessive numbers of unrealistic metabolites via multiple pathways, leading to a so-called combinatorial explosion.[375] To address this problem, most programs have cutoff filters based on properties such as molecular weight and lipophilicity, or they allow the user to constrain the analysis as to types of transformations predicted (phase I vs. phase II) or the total number of metabolites predicted in any given pathway. In addition to predicting the products of biotransformation, many of these programs also allow assessment of potential toxicity of metabolites, either through an integrated toxicity prediction

engine or through links to external companion programs. Newer approaches to combined prediction of metabolism and toxicity seek to integrate rule-based systems for metabolism prediction with biochemical pathway analysis and high-throughput biology techniques (omics technologies) in a systems biology approach.[376] Although still in its relative infancy, systems biology promises significant advances in our ability to understand and predict the interactions among biotransformation, biological activity, and toxicity of chemicals and drugs.

REGULATORY AND PRODUCT DEVELOPMENT ASPECTS OF XENOBIOTIC METABOLISM

International regulatory agencies require information about the metabolism of drugs and other chemicals that fall under their jurisdiction. The amount of data required will depend on the use of the chemical, its potential exposure to humans, and the potential role of metabolism in its efficacy and toxicity. Early information on the metabolism of drugs is becoming essential for the selection of drug candidates for further development. With the development of combinatorial synthetic chemistry and high-throughput pharmacological screening, the number of potential drug candidates that must be rapidly screened for their potential metabolism has increased dramatically. This is leading to the development of rapid methods to predict metabolism and potential DDIs. Data are needed from in vitro and in vivo animal models that will be used in safety assessments to allow the toxicologist to design appropriate studies during the preclinical phase of a safety assessment. Data are also needed concerning the potential human metabolism of the drug candidate to allow the toxicologist to extrapolate animal safety data to humans. In the recent past, it was difficult to obtain in vitro data from human tissues. With the current knowledge of the human P450 isozymes and the commercial availability of human hepatic preparations and cells that express human P450s, it is possible to obtain data concerning human metabolism of drug candidates, pesticides, and other chemicals.

The U.S. Food and Drug Administration (FDA) has recently released a guidance document concerning in vitro drug metabolism and DDI studies during the drug development process.[377] This document stresses the importance of obtaining information on the metabolism of a drug candidate during the early stages of development. This information is important in predicting potential individual differences based on polymorphic expression of xenobiotic metabolism enzymes and in predicting DDIs. The guidance document is based on the following general observations:

- The concentrations of a drug or its active metabolite circulating in the body determine the extent of its desirable and/or adverse effects.
- A major determinant of the concentration of a drug is clearance, and metabolism is a major determinant of clearance.
- Drugs that are not substantially metabolized may impact the metabolism of other drugs.
- Large differences in blood concentrations can occur because of polymorphic metabolism. DDIs can also produce large changes in the blood concentration of a drug.
- Major advances have been made in availability of human tissue and recombinant enzymes for in vitro studies of drug metabolism.

The guidance document suggests that the goals of in vitro metabolism and interaction studies should be to (1) identify major metabolic pathways and the specific isozymes involved and (2) explore and extrapolate the effects of the drug candidate on the metabolism of other drugs and the effect of other drugs on the metabolism of the candidate. To accomplish these goals, the FDA suggests starting from human hepatic microsomes, now commercially available, then moving to cell-based systems that express specific human P450s, which are also commercially available. They note that it is possible to move to hepatocytes and precision-cut liver slices but recognize the technical difficulties associated with these preparations.

A companion guidance document outlining recommendations for in vivo characterization of drug metabolism and potential DDIs was released shortly after the in vitro guidance document.[378] The conceptual basis for this document is similar to the in vitro guidance and stresses the importance of determining whether excretion or metabolism is the major mechanism of drug clearance, as well as the identification of major metabolic routes in the latter case. Further, the guidance lays out recommendations on study designs for the investigation of both inhibition and induction of metabolism of the drug of interest. These studies are useful in determining changes in dosage regimen for the target and interacting drugs during polypharmacy.

QUESTIONS

4.1 During the development of a new drug, it was decided that the introduction of a hydroxyl group onto the molecule would make the compound more water soluble, which offered the advantages of increasing gastric solubility and improving the pharmaceutics properties. An initial study of the plasma concentrations of the nonhydroxylated analog in rats had been completed in anticipation of beginning a subchronic toxicity study. When the plasma concentrations of the less lipophilic, hydroxylated drug were determined, it was found that the plasma concentrations were maintained for a longer period of time than with the nonhydroxylated analog. How would you explain this finding?

4.2 As a toxicologist, you have been asked to design a program to assess the potential hazard of a chemical. What type of information concerning its metabolism would you want to have before you design the hazard

assessment program? Based on the metabolism information you have requested, how would you choose the species to be used in the hazard assessment program?

4.3 A cancer chemotherapeutic drug has been shown effective in treating a specific type of cancer; however, the drug is also cytotoxic and produces severe side effects if it is not rapidly metabolized by cytochromes P450. It is important, therefore, not to treat a patient with doses of the drug that are too high for the patient's capacity to rapidly metabolize it to the less toxic product. What characteristics of the patient should be considered when attempting to choose a dose that will minimize the side effects?

4.4 A compound is functionalized by the P450 system and then forms sulfate and glucuronide conjugates and a mercapturic acid before being excreted. How may its metabolism be altered as the dose is increased from a no-observable-effect level (NOEL) to a dose that produces severe toxicity?

4.5 Many chemical carcinogens are metabolized by routes that represent detoxification and by other routes that represent metabolic activation. What are the various phenomena that may shift the balance between detoxification and metabolic activation?

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5 Toxicokinetics

A. Roberts and Andrew Gordon Renwick

CONTENTS

It has been recognized for many years[1] that the relationship between drug administration and therapeutic response can be subdivided into two aspects:

1. *Pharmacokinetics*, which relates to the movement of the drug within the body
2. *Pharmacodynamics*, which is concerned with the pharmacological effects once the drug is delivered to its site of action/receptor

The principles of *pharmacokinetics* apply to any environmental nonnutrient (xenobiotic), and many of the biological processes involved in the absorption, distribution, and elimination, such as the metabolizing enzymes, are nonspecific and shared by drugs and other low-molecular-weight organic molecules such as additives, pesticides, and contaminants. The term *toxicokinetics* is now widely accepted to describe *pharmacokinetic* processes for potentially toxic chemicals, which do not have a therapeutic effect. Toxicokinetics is the application of pharmacokinetic principles to animal toxicity studies and to human toxicity data, in order to provide information on internal exposure to the parent compound and its metabolites, and other aspects such as accumulation during chronic exposure.

The incorporation of toxicokinetic data from animal studies into risk assessment requires data from related in vivo studies in humans at appropriate doses, or from the results of in vitro data incorporated into a physiologically based pharmacokinetic (PBPK) model (see later and Chapter 6).

The understanding and interpretation of toxicological findings require information on two key areas: (1) delivery of the compound to its site of action (toxicokinetics) and (2) the mechanism of action and potency of the chemical at the site of action (toxicodynamics) (Figure 5.1). Toxicokinetic data may assist in understanding the dose–response relationship in animal toxicity studies and its relevance to humans, and allow the identification of potentially at-risk subgroups of the exposed human population.

The term *toxicokinetics* means the movement of a toxicant around the body and is primarily concerned with the relationship between the external dose, as usually measured in toxicity studies (e.g., mg kg^{-1} body weight), and a measure of the internal dose of the active compound delivered to the target for toxicity (Figure 5.1), such as the concentration in the general circulation or at the target for toxicity. The concentration in the general circulation is influenced by absorption, distribution, and elimination processes, as shown in Figure 5.2, and frequently, the compound has to pass many lipid and metabolic barriers prior to reaching the site of toxicity. Knowledge of the concentrations of the parent compound and any metabolites in plasma and tissues, allied to the rate of change on further dosing or cessation of administration, allows logical selection of the animal species most appropriate for toxicity testing and extrapolation of any toxicity observed in animals to the likely risk for humans.[2–14]

Such information may be derived by various methods and approaches including

1. Data from studies in which the chemical contains a radiolabel, usually ^{3}H, ^{14}C, or ^{35}S
2. Chemical-specific data using a sensitive analytical method

Radiolabeled studies are valuable for following the fate of the chemical skeleton as it moves from the site of administration into the blood, is distributed to the tissues, and finally is eliminated from the body in air, urine, or bile. Measurement of total radiolabel is nonspecific and reflects both the chemical and its metabolites; this is both an advantage and a disadvantage. The advantage is that it

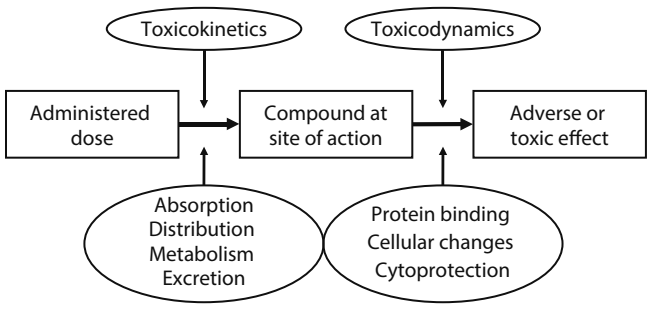


FIGURE 5.1 The relationship between in vivo response and toxicokinetics and toxicodynamics.

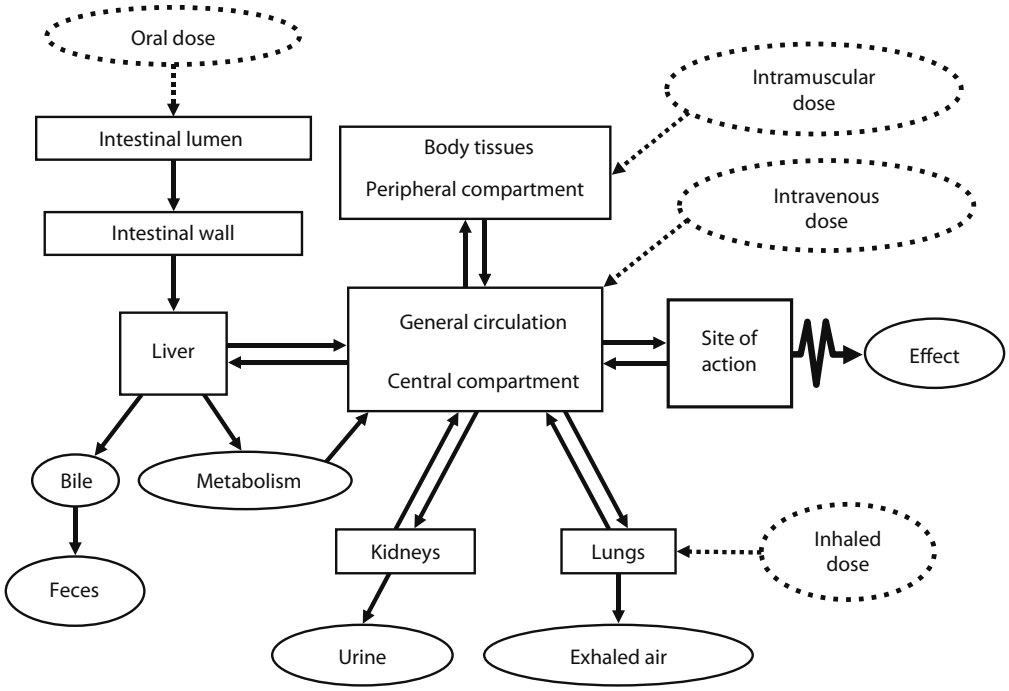


FIGURE 5.2 Toxicity in relation to pharmacokinetics. The chemical may be given orally or by injection or inhalation. The concentration at the target organ is in equilibrium with that in the systemic circulation, which is itself in equilibrium with a large number of other physiological processes, which can increase or decrease that concentration. The transfer from one tissue to another usually involves transfer across a lipid membrane and frequently entails entering a tissue with high elimination capacity such as the liver or kidneys. The parent chemical is eliminated from the body when it is converted into a different structure by metabolism or is eliminated in the urine, bile, or expired air.

allows quantitative balance studies to be performed, for example, to determine how much of the dose is absorbed, which organs accumulate the compound and/or its metabolites, the pathways of metabolism, and the routes of excretion. The disadvantage is that radiolabeled *ADME* (absorption, distribution, metabolism, and excretion) studies do not allow an assessment of how much of the chemical is absorbed intact and how much is distributed around the body as the parent chemical or as metabolites. Combining radiolabeled ADME studies with chromatographic separation methods is useful to define the overall fate of the chemical in the body and identify the main chemical species (parent compound and/or metabolites) that are present in the circulation and delivered to the site of toxicity. The development of automated analytical techniques of high sensitivity and specificity (such as HPLC, LC-MS, and LC-MS-MS) has allowed toxicokinetics to provide useful information on in vivo absorption, distribution, and elimination in laboratory animals and the magnitude and the duration of exposure of targets for toxicity. The key to interpreting plasma concentration–time curves was the development of suitable mathematical models to derive rates of absorption, metabolism, and excretion. Problems of accumulation on repeated dosing and saturation of elimination are particularly pertinent to high-dose animal toxicity studies, and information on these areas can be obtained only from suitably designed in vivo toxicokinetic studies.

Toxicokinetic data have the potential to define

1. The internal exposure (internal dose) in animals based on plasma or blood concentrations of the parent compound or its active metabolite in relation to the dose given to the animals
2. The relationship between plasma or blood concentrations and those at the site of toxicity
3. The information allowing quantitative interspecies comparisons derived from appropriate blood/plasma data after the administration of tracer doses to human volunteers

This chapter concentrates on the derivation and interpretation of chemical-specific toxicokinetic data based on measurements of the concentrations in plasma and urine. Toxicokinetic data are also useful in extrapolating between species and across different routes of exposure or administration, as well as from single doses to chronic administration. Chemical-specific toxicokinetic measurements are essential if the results of in vitro toxicity tests are to be interpreted logically, because they can define the upper limit of biologically plausible in vivo concentrations.

BIOLOGICAL PRINCIPLES

Certain general principles governing the disposition of therapeutic drugs are applicable to nearly all low-molecular-weight organic compounds. The processes involved in absorption,

distribution, and elimination of foreign compounds were outlined in previous editions of this book, and only essential concepts are now presented under the discussion of mathematical principles.

MATHEMATICAL PRINCIPLES

In order to describe adequately the changes in blood or plasma concentrations of foreign compounds, it is necessary to assign a suitable mathematical model that accurately describes the shape of the concentration–time curve. However, certain aspects are model independent and are considered first, because they are usually constituent parts of the various mathematical models. In recent years, there has been a trend away from multicompartmental mathematical analysis, which offers little apart from mathematical predictability, toward physiologically more relevant model-independent concepts such as clearance.[15] Physiologically related parameters such as clearance and bioavailability represent an intermediate level of information between mathematical multicompartment models and full PBPK models.

Model-Independent Considerations

Biochemical and physiological processes are usually either zero-order or first-order reactions. In zero-order reactions, the rate of change in concentration with time occurs at a fixed amount per unit of time, that is,

$$\frac{dC}{dt} = k$$

where
- C is the concentration
- t is the time
- k is a constant with units of amount per time, for example, micrograms per minute

Zero-order reactions are particularly important at high concentrations, when enzymes are working at maximum rate and an increase in C cannot result in an increase in rate. This situation produces nonlinear or saturation kinetics, which can assume considerable importance in toxicity studies and is discussed later.

In first-order reactions, the rate of change in concentration is proportional to the concentration of the chemical available for the reaction, that is,

$$\frac{dC}{dt} = kC$$

where k is a constant that represents a proportional change with time and has units of time^{-1}, for example, min^{-1}.

Most kinetic processes (e.g., diffusion, carrier-mediated uptake, metabolism, and excretion) are first-order reactions at low concentrations. *Most of the following equations given make this assumption*.

First-order reactions can be described by equations that include exponential functions. In many cases, the entry of a foreign compound into the body or into a tissue follows an exponential increase, which may be described mathematically by

$$\text{Uptake} = 1 - e^{-kt} \tag{5.1}$$

where the uptake is the concentration present at time t divided by the final concentration when all the compound has entered the body or tissue. This equation assumes that there is no elimination process. The elimination of a compound (by a first-order process) once it has entered the body or tissue may be described by an exponential with a negative slope

$$C = C_0 \cdot e^{-kt} \tag{5.2}$$

where
- C is the concentration present at time t
- C_0 is the initial concentration

In Equations 5.1 and 5.2, k is the rate constant for that process.

Exponential equations of the type given in Equation 5.2 may be solved as

$$\ln C = \ln C_0 - kt$$

or using $\log_{10}$

$$\log C = \log C_0 - \frac{kt}{2.303}$$

These are equations of the generalized form

$$y = A + Bx$$

where
- x and y are variables
- A and B are constants

In such cases, a plot of x against y gives a straight-line graph with a slope of B and an intercept of A. Thus, for toxicokinetics, a graph of ln C against time gives a slope of $-k$ and an intercept of ln C_0. Because the units of k are time^{-1}, which is a difficult unit to visualize, the rates of a first-order processes are usually described by their half-life or $t_{½}$ (see later).

Usually, the equation necessary to describe the kinetics of a compound in the body requires the use of at least two exponential rate terms. This is illustrated in Figure 5.3 in which two phases are seen in the plasma concentration–time curve

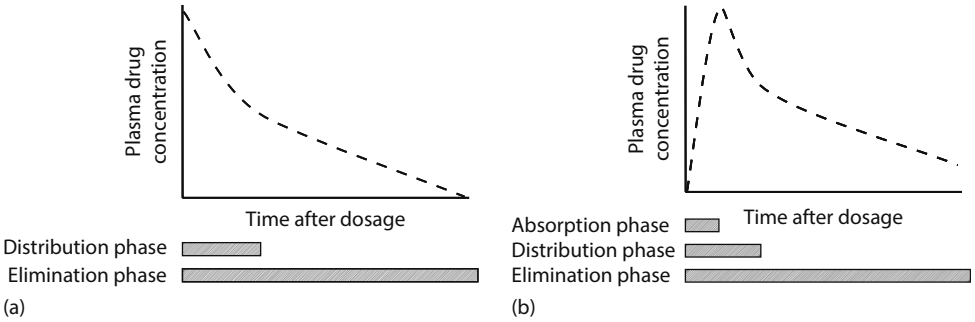


FIGURE 5.3 The plasma concentration–time profile of a chemical following intravenous and oral dosage. The concentration is on a logarithmic scale. Rapid processes, such as absorption and distribution, do not significantly affect later time points that are determined largely by the slowest process (elimination in the diagrams shown). (a) Intravenous administration and (b) oral administration—rapid absorption.

after an intravenous dose and three different phases after an oral dose. Each phase requires a different rate constant, and the early time points in the concentration–time curve are influenced by all rates. Eventually, the influence of the component with the higher rate (the faster component) becomes negligible, while the slower component (with the smaller rate constant) still affects the concentration. Thus the terminal phase of the concentration–time curve is determined by the slower process, that is, the one with the smaller rate constant, and the earlier phase by the sum of both processes. This process allows both rate constants to be determined by the procedure known as the method of residuals or stripping (see later).

Absorption

Absorption describes the processes involved in the transfer of a chemical from the site of administration into the systemic blood circulation. Absorption from the gut is of greatest importance because most toxicity studies are performed using oral administration. Absorption may involve first-order or zero-order processes or a mixture of the two. The extent of absorption can be determined by the comparison of concentrations in plasma or urine after oral and intravenous administration. The fraction absorbed may be low due to either poor uptake from the site of administration or metabolism between the site of administration and the systemic circulation (first-pass metabolism).

Tissue Extraction

The rate constant for tissue uptake may depend on either the tissue blood flow (*perfusion limited uptake*), where k is related to the tissue flow rate, or the rate of diffusion through membranes (*diffusion-limited uptake*) where k is related to the diffusion rate constant and is not readily measurable. In general, diffusion rate limitation applies to highly water-soluble compounds, whereas perfusion rate limitation applies to the entry of lipid-soluble compounds into slowly perfused tissues, such as adipose tissue. The extent of tissue uptake depends on the relative affinities of the blood and the different tissues.[16,17]

Plasma Protein Binding

Protein binding is an equilibrium reaction:

$$P^r + C_f \leftrightarrow C_b$$

where

P^r is the free protein
C_f is the unbound or free chemical
C_b is the chemical–protein complex

The fraction unbound α is given by

$$\alpha = \frac{C_f}{C_f + C_b} = \frac{C_f}{C} \tag{5.3}$$

where C is total plasma concentration. The binding of organic compounds to albumin can involve two or more binding sites. It should be appreciated that the extent of plasma protein binding is not incorporated into most pharmacokinetic analyses because the plasma kinetic parameters are calculated using the total concentrations ($C_f + C_b$). If the free nonbound concentration of the chemical is used to calculate kinetic parameters, this has to be specified in the description of the parameters; in the absence of such a description, it is assumed that the total plasma concentration has been used. Because plasma protein binding is a saturable process (see later), in vitro binding studies should be performed over a range of concentrations.

Clearance

There are two main mechanisms by which the circulating levels of a foreign compound may be reduced: metabolism and excretion. Metabolism is a major source of both species differences and human variability,[18–22] and its toxicological consequences are discussed in an earlier chapter (Chapter 4).

Clearance (CL) is defined as the ratio

$$CL = \frac{\text{Rate of elimination}}{\text{Plasma concentration}}$$

and may be regarded as the volume of plasma or blood that is cleared of compound in unit time by the route under

consideration. The units are volume time^{-1}, for example, frequently mL min^{-1} because if rate is μg min^{-1} and plasma concentration is μg mL^{-1}, the plasma clearance will be mL min^{-1}.

Renal Clearance

The renal clearance (CL_R) is given by

$$CL_R = \frac{\text{Rate of elimination in urine}}{\text{Plasma concentration}} = \frac{C_u \times F_u}{C} \qquad (5.4)$$

where

C_u is the urine concentration
F_u is the urine flow (volume in unit time)
C is the plasma concentration at the midpoint of the urine collection period

The concentration in urine is dependent on three main variables.

Glomerular Filtration

The glomerular membrane has pores of 70–80 A, and all molecules smaller than about 20,000 Da are filtered. Thus proteins and protein-bound compounds remain in the plasma.

The rate of removal in the glomerulus is given by

$$\text{GFR} \times C_f = \text{GFR} \times C \times \alpha \qquad (5.5)$$

where

GFR is the glomerular filtration rate
C_f is the unbound concentration in plasma
C is the total plasma concentration
α is the fraction unbound

Compounds that are extensively bound to plasma proteins show limited elimination by glomerular filtration. The chemical–protein complex does not dissociate in the glomerulus, but releases more unbound compound when the plasma is diluted by water reabsorbed in the distal parts of the renal tubule.

The glomerular filtration rate is about 130 mL min^{-1} in men and 120 mL min^{-1} in women, or approximately 2 mL min^{-1} kg^{-1}, which is lower than that of the Wistar rat (3.4 mL min^{-1} kg^{-1}).[23]

Reabsorption

Reabsorption from the renal tubule back into the blood is variable and dependent on the lipid solubility of the compound, the pH of the urine, the pKa of the chemical, and the extent of concentration of the urine due to water reabsorption. The pH of the urine can be altered appreciably by treatment with weak acids or bases, whereas the plasma shows little change because its pH is buffered by the high protein content. It is therefore possible to affect the pH partitioning of foreign compounds between renal tubule contents and plasma, and this possibility should be considered when preparing dose solutions.

Tubular Secretion

A number of different transporters, including OAT—transporters for organic acids, OCT—transporters for bases, peptide transporters, and nonspecific transporters (members of the MRP family), have been identified on either the basolateral or apical membranes of the renal tubule or on both.[24–26] They show relatively low substrate specificities and differences between species and sexes. The extent of their involvement for a particular compound is dependent on the affinity between the compound and the carrier protein. The transporters give active saturable processes, and saturation of secretion causes a dose-dependent decrease in elimination at high plasma concentrations.

All three processes described earlier can alter, simultaneously and independently, and the overall renal clearance may be regarded as a composite expression:

$$\text{Renal excretion} = \text{Glomerular filtration} - \text{Reabsorption} + \text{Tubular secretion}$$

$$\text{Rate of excretion} = \text{GFR } C\alpha - \text{Rate of reabsorption} + \text{Rate of tubular secretion}$$

The values of GFR, C, and α can be determined experimentally. Measurement of inulin clearance (or creatinine clearance in humans) determines the GFR, because this compound does not undergo significant reabsorption, tubular secretion, or protein binding. The extent of reabsorption and secretion of a compound may be inferred by comparison of its renal clearance with the value of GFR × α. If $CL_R < \text{GFR} \times \alpha$, then reabsorption must be occurring and is greater than any secretion (which may or may not be present); if $CL_R = \text{GFR} \times \alpha$, then reabsorption, which may or may not be present, is negated by an equal rate of secretion; and if $CL_R > \text{GFR} \times \alpha$, then tubular secretion must be occurring and is greater than any reabsorption (which may or may not be present).

The mathematical implications of the renal elimination process have been the subject of a number of reviews.[27–29]

Hepatic Clearance

The clearance of a compound by the liver may be regarded as dependent on the rate of delivery to the organ (blood flow) and the efficiency of removal from the blood.

The uptake and metabolism of nutrients, hormones, and absorbed chemicals are a primary function of the liver. Hepatocytes and hepatic sinusoids show a number of features that facilitate these processes, including fenestrae in the endothelium, which allow even large molecules to leave the circulation and enter the space of Disse, a fluid collagen containing matrix in the space of Disse, a brush-border on the hepatocytes that greatly increases the surface area for absorption, active uptake transporters for some chemicals, and very high enzyme activity (intrinsic clearance) within the hepatocytes.

If the internal cellular metabolic clearance (CL_{int}) is high, then the hepatic clearance approximates to the hepatic blood flow and becomes dependent on the hepatic blood flow (which is constant under normal physiological conditions). If CL_{int} is low, then the hepatic clearance is relatively constant and independent of blood flow and is a first-order rate constant.

The relationship between internal cellular metabolic clearance and enzyme kinetics is given by

$$CL_{\text{int}} = \frac{V_{max}}{K_m + C_f} \tag{5.6}$$

where

V_{max} and K_m are Michaelis–Menten constants for the enzyme metabolizing the chemical
C_f is the hepatic venous concentration of unbound chemical

If C_f is low and is much less than K_m for the enzyme (i.e., well below saturation levels and a first-order reaction), this term may be ignored and

$$CL_{int} = \frac{V_{max}}{K_m} = \text{Constant}$$

When the value of C_f approaches or exceeds K_m, the substrate concentration is sufficient to saturate the enzyme, and the kinetics become zero order and nonlinear in relation to dose. This situation is a distinct possibility in high-dose toxicity testing and is discussed later in more detail.

The concepts given are important for the use of V_{max} and K_m values in interspecies comparisons. Species differences in clearance will relate to differences in V_{max}/K_m for low-clearance metabolized compounds, but will relate to liver blood flow for high-clearance compounds (irrespective of differences in V_{max}/K_m). This possible source of error in extrapolation across species is avoided when both the enzyme activity and organ blood flows are part of a PBPK model.

Biliary Clearance

The bile is an important route allowing foreign compounds to move from the general circulation into the gut.[30] Organic cation transporters on the sinusoidal membrane transfer large polar cations into the hepatocyte and from the hepatocyte in the bile.[31,32] Species differences exist in the molecular weight threshold for significant biliary excretion, which has been estimated as 325 ± 50 Da in the rat, 440 ± 50 Da in the guinea pig, 475 ± 50 Da in the rabbit, and about 500 in humans.

The clearance via the bile CL_B is given, by analogy with renal clearance, as

$$CL_B = \frac{\text{Rate of elimination in bile}}{C} = \frac{C_B \times F_B}{C} \tag{5.7}$$

where

C_B is the concentration in bile
F_B is the volume of bile in unit time (bile flow)
C is the plasma concentration

Plasma Clearance

Plasma clearance (CL) may be defined as

$$CL = \frac{\text{Rate of elimination from plasma}}{C}$$

The plasma clearance is the sum of the various contributory clearance processes:

$$CL = CL_R + CL_H + CL_B + \text{etc.}$$

Plasma clearance, which is one of the most valuable toxicokinetic constants, is determined from the plasma concentration–time curve and is discussed in detail later.

Physiologically Based Pharmacokinetic Models

In recent years, disposition models have been developed, which are based on the principles discussed earlier, that is, organ blood flow, tissue extraction, and rates of metabolism and excretion. These models are derived from the physiology of the test animal and are discussed in detail in the chapter by Clewell and colleagues in this volume (Chapter 6). PBPK models have been applied successfully to a number of compounds and have been particularly successful for organic solvents, for example, benzene.[33] This approach represents a powerful method, capable of dealing with saturation of metabolism[6,34,35] and valuable for the extrapolation of animal data to humans.[6,34–42] However, its ability to predict concentrations is dependent on the precision of the parameter estimates used and the model chosen.[43] Therefore, PBPK modeling should be considered as one of three possible approaches to the analysis and interpretation of toxicokinetic data:

1. Simple physiologically related concepts, such as bioavailability and clearance (this chapter)
2. Compartmental analysis, which gives mathematical precision but is difficult to relate directly to metabolic or physiological processes (this chapter)
3. PBPK modeling, which can be used to predict target organ concentrations (Chapter 6)

Compartmental Analysis: Modeling

In order to describe plasma concentration–time curves mathematically, an appropriate predictive model has to be fitted to the data. The correlation between the actual data and the plasma or blood concentration–time curve generated using the model shows the suitability of the model in describing the experimental results.

Considering the data presented in Figure 5.4, it is apparent that the same model cannot describe the properties of both compounds, although in both cases, the initial and final plasma concentrations were the same. The differences in the plasma concentration–time profiles originate in the number of rates at which the compound may leave and enter the plasma. When the tissues show instantaneous equilibration with plasma, there is a simple exponential decrease in plasma concentrations that results from the elimination process(s) (Figure 5.4a). Alternatively, the compound may leave the plasma to enter *other tissues* at measurable rates, as well as

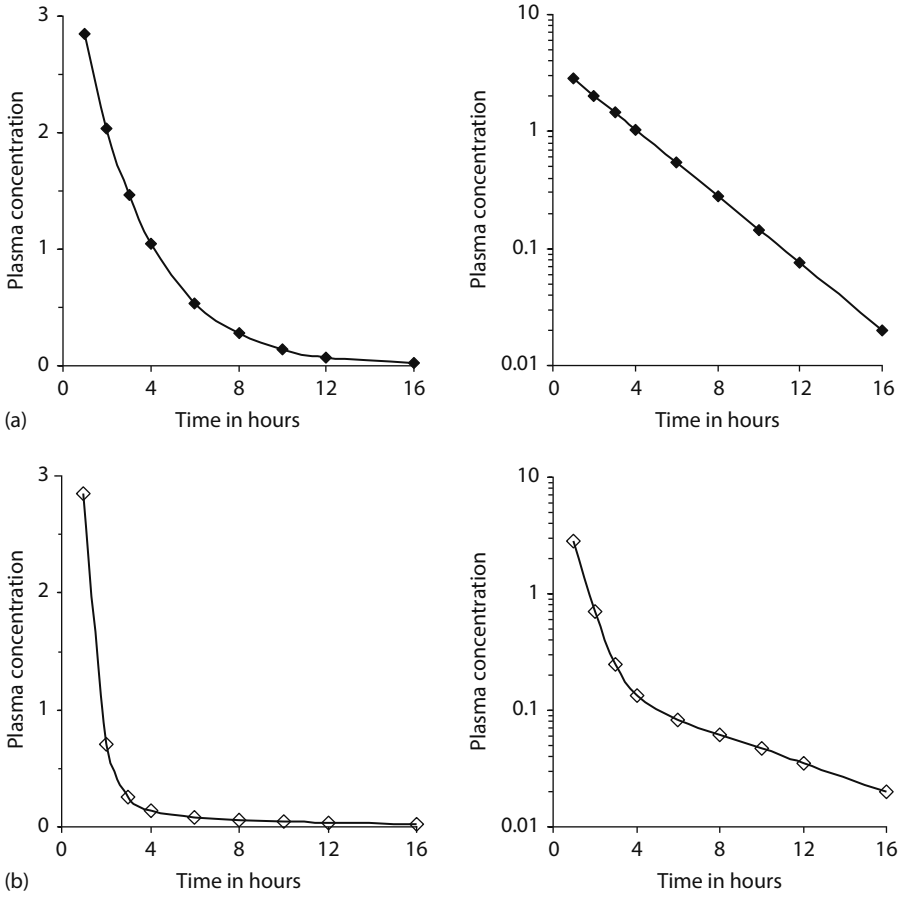


FIGURE 5.4 Plasma concentration–time data for two compounds with the same initial and final measured concentrations but with different distribution characteristics. The results are plotted in linear and semilogarithmic forms.

undergoing elimination from the plasma (Figure 5.4b). Under such circumstances, the *other tissues* may be adequately described mathematically by a second exponential term. It is important to realize that these *other tissues* share only one criterion, that is, their associated rate of uptake and transfer back into plasma, and biologically diverse tissues may be part of the same mathematical compartment. In some cases, two or more additional compartments are required, and elimination may occur from compartments other than the central compartment (plasma plus tissues that reach equilibrium before the first plasma measurement). Multicompartment analysis can be extremely complex,[44] but in many cases, knowledge of the best model for fitting to the data is not necessary.

Because the aim of this chapter is to provide an introduction to toxicokinetics (i.e., samples needed, data handling, and the type of information that can be obtained), only simple models are discussed in detail. The two models discussed show widespread applicability, and an understanding of the principles underlying these simple models is essential, if the data generated by computer analysis using more complex models are to have any meaning.

Texts recommended for further reading include those by Gibaldi and Perrier,[45] a classic text, which is a mathematical approach that is well explained and illustrated using actual experimental data; Rowland and Tozer,[46] a well-written readable text with many excellent illustrations and study problems at the end of each section; and Wagner,[47] an approach similar to that of Gibaldi and Perrier[45] but with a useful biological introductory chapter and expanded sections on dosage regimen calculations, pharmacological response, and automated pharmacokinetic analysis. An additional useful book is that by Gabrielsson and Weiner,[48] which gives a clear account of different models in relation to the use of WinNonlin and provides excellent explanations of the basics of data fitting.

One-Compartment Open Model

Intravenous Bolus Dose

The compound is dissolved in and evenly distributed within a single compartment of volume V (Figure 5.5). Elimination of the compound, by both excretion and metabolism, is by first-order processes, and changes in plasma concentration are reflected in similar and simultaneous decreases in the tissue concentrations, because all tissues represent part of the single compartment. In Figure 5.5, V is the volume of distribution, k_{ex} is the excretion rate constant, and k_m is the metabolism rate constant. In mathematical terms, such a system may be described adequately by a simple first-order

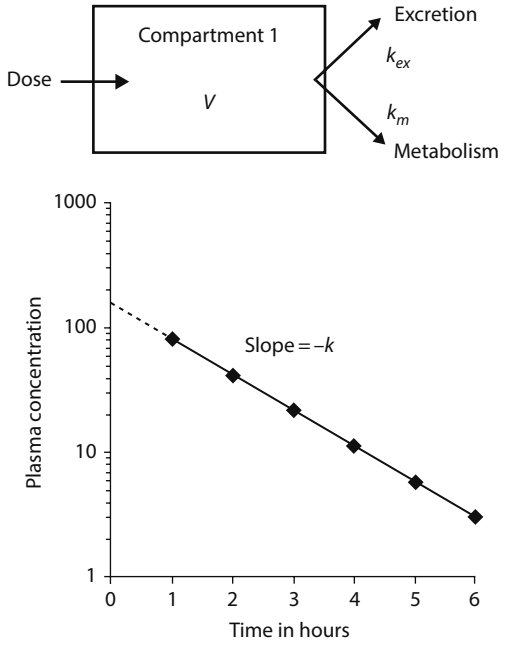


FIGURE 5.5 Plasma concentration–time curve after a bolus intravenous dose for a one-compartment system. The concentrations have been shown on a logarithmic scale for illustrative purposes; the slope $-k$ would be calculated by plotting the natural logarithms of the concentrations against time.

equation, where the rate of removal of a compound from the body (e.g., in milligrams per hour) is proportional to the body load (e.g., in milligrams):

$$\frac{dAb}{dt} = -kAb \tag{5.8}$$

where

Ab is the amount of compound in the body
k is the elimination rate constant (k equals $[k_{ex} + k_m]$)

A solution to this equation to give the amount remaining in the body at time t after injection is given by

$$Ab = Ab_0 e^{-kt} \tag{5.9}$$

where

Ab is the amount of compound at time t
Ab_0 is the amount at time zero

For a one-compartment model, the concentration in the plasma (C) may be related to Ab by the *apparent volume of distribution* (V). This volume may be regarded as the volume of plasma in which the body burden (body load or Ab) would have to be dissolved, in order to give the plasma concentration measured:

$$C = \frac{Ab}{V} \tag{5.10}$$

where

C is the plasma concentration
V is the apparent volume of distribution

Thus, Equation 5.10 may be rewritten in its more usual form

$$C = C_0 e^{-kt} \tag{5.11}$$

where

C is the plasma concentration at time t
C_0 is the concentration at time zero

For such a system, we can define the following parameters.

Apparent Volume of Distribution

The apparent volume of distribution (V) is the volume of plasma into which the dose *appears* to have been dissolved to give the initial plasma concentration, C_0, that is,

$$V = \frac{Ab}{C} = \frac{\text{Dose}}{C_0} \tag{5.12}$$

The units are usually liters, milliliters, liters per kilogram, or milliliters per kilogram.

For a chemical that is lipid soluble or that readily binds to tissue components, the plasma concentration represents a small fraction of the total amount in the body, and thus the compound appears to have been dissolved in a large volume of plasma that may greatly exceed the physiological volume of plasma. In practice, such compounds usually require a model with at least two compartments to describe the concentration–time curve (see later).

Elimination Rate Constant

The elimination rate constant (k) represents the fractional loss of compound from the body per unit time, that is,

$$k = \frac{\text{Amount of chemical eliminated in unit time}}{\text{Amount of chemical in the body}} = \frac{(dAb/dt)}{Ab}$$

Equation 5.11 may be rewritten as

either $\ln C = \ln C_0 - kt$

or $\log C = \log C_0 - \dfrac{kt}{2.303}$

Thus a graph of $\ln C$ against time (illustrated using a logarithmic scale in Figure 5.5) has a slope of $-k$ and an intercept of $\ln C_0$. The units of k are time^{-1} (e.g., h^{-1} or min^{-1}). If the elimination rate constant is 0.4 h^{-1}, it means that 40% of the body load is removed each hour. The value of k is the summation of component elimination rate constants (e.g., k_{ex}, k_m).

Elimination Half-Life

The elimination half-life ($t_{1/2}$) is the time taken for the amount in the body (Ab) or the plasma concentration (Ab/V) to decrease to one-half. Thus, after one half-life, C in Equation 5.11 equals $C_0/2$, that is,

$$\frac{C_0}{2} = C_0 e^{-kt_{1/2}} \quad \text{or} \quad \frac{1}{2} = e^{-kt_{1/2}}$$

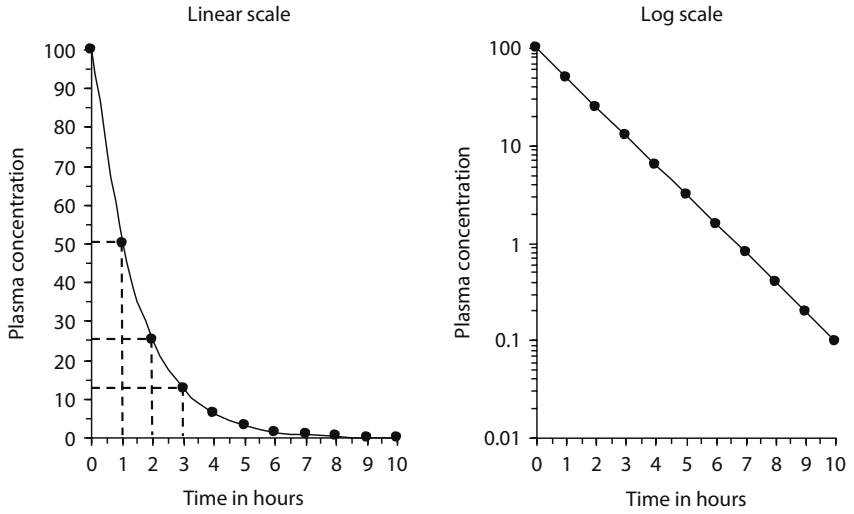


FIGURE 5.6 The elimination of a chemical with a half-life of 1 h from a one-compartment model, plotted on linear and semilogarithmic scales.

Therefore, $\ln 0.5 = -kt_{½}$ or $-0.693 = -kt_{½}$

$$t_{1/2} = \frac{0.693}{k} \tag{5.13}$$

where the units are time (e.g., hours or minutes).

For first-order reactions, the half-life is independent of dose, body burden, and plasma concentration (Figure 5.6).

Plasma Clearance

Plasma clearance (*CL*) relates the amount of chemical eliminated in unit time to the plasma concentration and may be regarded as *the volume of blood that is cleared of chemical in unit time*. *CL* is a constant for first-order reactions. In many respects, *CL* is a better reflection of the inherent capacity of the tissues to eliminate the compound than is the half-life or elimination rate constant:

$$CL = \frac{\text{Rate of elimination from the body}}{\text{Plasma concentration}}$$
$$CL = \frac{\left(dAb/dt\right)}{C} \tag{5.14}$$

Substituting from Equation 5.8,

$$CL = \frac{kAb}{C}$$

The amount in the body at any time (*Ab*) is given by Equation 5.10; therefore,

$$CL = \frac{kCV}{C} = kV \tag{5.15}$$

where the units are in L h^{-1}, L min^{-1}, mL h^{-1}, or mL min^{-1}. Rearranging Equations 5.15 and 5.13,

$$k = \frac{CL}{V} \quad \text{and} \quad t_{1/2} = \frac{0.693V}{CL} \tag{5.16}$$

This equation shows clearly that the elimination rate constant (*k*) is derived from two independent variables, each of which can be related to physiological processes: the *clearance*, which reflects the capacity of the organs of elimination to remove the compound from the plasma, and the *apparent volume of distribution*, which reflects the proportion of the total body burden that is circulated to the organs of elimination. Plasma clearance may depend on the rate of the active process(es) in the organs of elimination or on the plasma flow to the principal organ(s) of elimination.

Clearance may be obtained without knowing the value of *V*. Rearranging Equation 5.14,

$$\frac{\mathrm{d}Ab}{\mathrm{d}t} = CL \times C$$

or in time d*t*, the amount lost $\mathrm{d}Ab = CL \times C \times \mathrm{d}t$. Integrating between time = 0 and infinity (∞), the total dose will have been eliminated, so that $\mathrm{d}Ab = dose$

$$\text{Dose} = CL \int_0^\infty C\mathrm{d}t$$
$$\text{Dose} = CL \times \text{AUC} \tag{5.17}$$
$$CL = \frac{\text{Dose}}{\text{AUC}}$$

where AUC is the area under the plasma concentration–time curve extrapolated to infinity. For Equation 5.17 to be valid, the dose has to be fully available to the organs of elimination (i.e., an intravenous dose), and the AUC has to be extrapolated to infinity.

This relationship can also be used to calculate *V*; substituting *CL* from Equation 5.16 into Equation 5.17,

$$V = \frac{\text{Dose}}{\text{AUC} \times k} \tag{5.18}$$

The importance of Equations 5.17 and 5.18 is that both the clearance and the apparent volume of distribution can be derived from infusion administration, where the determination of V using Equation 5.12 is not possible. These equations may also be applied to oral administration, providing that allowance is made for incomplete absorption of the dose (see later). This method of calculating CL is also applicable to multicompartment linear systems with elimination from the central compartment.

Information Obtainable from Urinary Data

Renal clearance can be calculated using Equation 5.4. Equation 5.4 can be rearranged as

$$\text{Rate of urinary excretion} = CL_R \times C$$

where

CL_R is the renal clearance
C is the concentration in plasma

Thus from Equation 5.15,

$$CL_R \times C = k_R \times V \times C = k_R \times Ab$$

where k_R is the renal excretion rate constant.

However, from Equation 5.9, Ab at any time = Dose $\times e^{-kt}$. Therefore,

$$\text{Rate of urinary excretion} = k_R \times \text{Dose} \times e^{-kt} \quad (5.19)$$

or

$$\ln(\text{rate of urinary excretion}) = (\ln k_R \times \text{Dose}) - kt \quad (5.20)$$

The significance of this is that a plot of the natural logarithm of the rate of urinary excretion (amount excreted per time interval) against time gives a straight line with a slope of $-k$, and an intercept of ($\ln k_R \times$Dose), and the decrease in the amount appearing in the urine mirrors the overall decrease in the plasma concentration. It is not possible to obtain CL or V without sampling the central (blood) compartment.

This approach is subject to considerable errors in the measurement of the excretion rate at different times after dosage due to factors such as incomplete bladder emptying. To overcome this problem, the rate constant can be derived more reliably from the amount remaining to be excreted, using the *sigma-minus* method. This method is based on the following equation, which is derived from integration between time zero and infinity of Equation 5.19:

$$A_{ex} = \frac{k_R \times \text{Dose}}{k}\left[1 - e^{-kt}\right] \quad (5.21)$$

where A_{ex} is the total amount excreted up to time t. At infinite time, $[1 - e^{-kt}]$ equals unity, so that

$$A_{ex}^{\infty} = \frac{k_R \times \text{Dose}}{k}$$

where A_{ex}^{∞} is the cumulative total amount excreted in urine up to time infinity. Substituting back into Equation 5.21,

$$A_{ex} = A_{ex}^{\infty}\left[1 - e^{-kt}\right]$$

or

$$A_{ex}^{\infty} - A_{ex} = A_{ex}^{\infty} e^{-kt} \quad (5.22)$$

The left-hand side of Equation 5.22 is equivalent to the amount finally excreted minus the amount excreted up to that time (ΔA_{ex}). Taking natural logarithms,

$$\ln \Delta A_{ex} = \ln A_{ex}^{\infty} - kt \quad (5.23)$$

A plot of $\ln \Delta A_{ex}$ against time gives a straight line of slope $-k$.

By analogy with Equation 5.17, CL_R may be calculated from the total amount excreted and the plasma AUC:

$$CL_R = \frac{A_{ex}}{\text{AUC}}$$

where A_{ex} and AUC refer to the same time interval.

Combining this with Equation 5.22 gives the following relationships:

$$\text{AUC}_0^{\infty} = \frac{\text{Dose}}{CL} = \frac{A_{ex}^{\infty}}{CL_R} \quad \text{or} \quad CL_R = CL \times \frac{A_{ex}^{\infty}}{\text{Dose}}$$

where AUC_0^{∞} is the AUC from zero to infinity after an intravenous dose.

In other words, renal clearance (CL_R) equals plasma clearance (CL) multiplied by the fraction of the dose eliminated unchanged in urine.

Constant Intravenous Infusion

During infusion, the plasma concentration (C) increases to reach a plateau or steady-state concentration (C_{ss}), at which time the rate of infusion equals the rate of elimination (Figure 5.7). The extent of accumulation to steady-state is given by analogy with Equation 5.1.

$$\frac{C}{C_{ss}} = \left(1 - e^{-kt}\right)$$

or

$$C = C_{ss}(1 - e^{-kt}) \quad (5.24)$$

The various kinetic parameters may be derived from the plasma concentration–time curve for infusion.

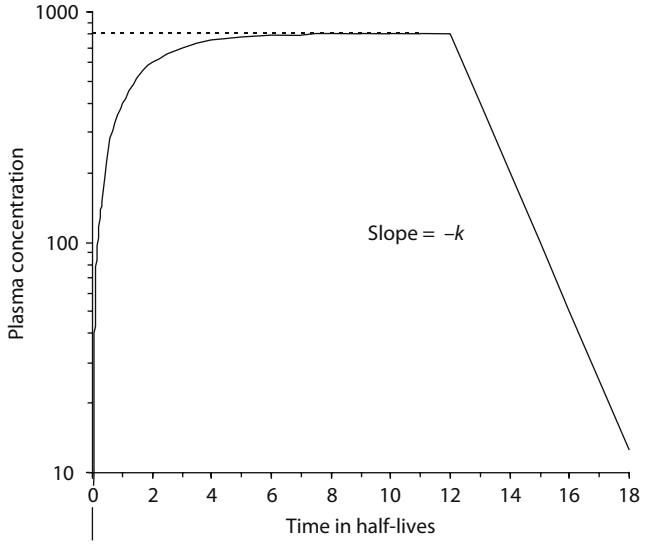


FIGURE 5.7 Plasma concentration–time curve for constant intravenous infusion into a single-compartment system. The foreign compound was infused at a constant rate from time = 0 to time = 12 half-lives when the infusion was stopped. The concentrations have been shown on a logarithmic scale for illustrative purposes; the slope $-k$ would be calculated by plotting the natural logarithms of the concentrations against time or $-k/2.303$ by plotting the logarithms to the base 10 of the concentrations against time.

Increase to Plateau

Rearranging Equation 5.24,

$$C_{ss} - C = C_{ss}e^{-kt}$$

Therefore, a plot of the natural logarithm of $(C_{ss} - C)$ against time gives a straight line with a slope equal to $-k$. The time taken to reach the plateau is therefore similar to the time taken to eliminate the compound after infusion or about 97% of the final steady-state level within five times the elimination half-life.

Steady-State Plasma Concentration: C_{ss}

At steady-state, the rate of infusion (R) equals the rate of elimination:

$$R = CL \times C_{ss}$$

or

$$CL = \frac{R}{C_{ss}} = V \times K$$

Decrease at End of Infusion

The slope of the decrease at the end of the infusion equals $-k$ because on cessation of entry, $C = C_0e^{-kt}$. The same slope would be obtained if the infusion was stopped at any stage during the infusion.

Area under the Curve

Both CL and V may be derived using Equations 5.17 and 5.18.

Oral Administration

Absorption frequently obeys first-order kinetics, but may involve a lag time due to delayed gastric emptying. The various pharmacokinetic parameters are related by the equation

$$C = \frac{F \times \text{Dose} \times k_a\left(e^{-kt} - e^{-kat}\right)}{V\left(k_a - k\right)} \tag{5.25}$$

where

F is the fraction of the dose absorbed (bioavailability)
k_a is the absorption rate constant

Increase to Peak

The increase to peak is determined by the more rapid of the two processes, absorption and elimination; for lipid-soluble compounds, absorption is usually more rapid than elimination. Measurement of the absorption rate constant must allow for the excretion occurring throughout the postdosing period, which can be done using the method of residuals (see Gibaldi and Perrier[45] for the mathematical basis of this method). The method is illustrated in Figure 5.8. In cases where elimination is more rapid than absorption, the rate of increase will be determined by the elimination rate constant (as is the case for a constant intravenous infusion—see earlier text). Thus the value of k_a can be assigned to the increase to peak only after demonstration that the value of k for the decrease is similar to that seen after intravenous dosing.

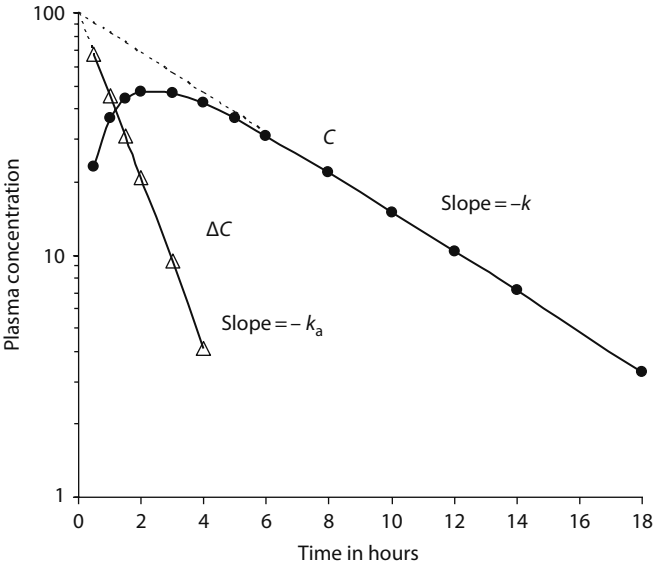


FIGURE 5.8 Use of the method of residuals to calculate the absorption rate constant for a one-compartment system. The dose was given at time 0, and plasma levels (C) were measured at intervals. The linear terminal phase was extrapolated to yield the values corresponding to the measurement times. The difference values (C extrapolated $-$ C measured) are plotted (ΔC) to yield slopes $-k_a$ or $-k$ (see text on flip-flop kinetics and Figure 5.9). The concentrations have been shown on a logarithmic scale for illustrative purposes; the slopes $-k$ and $-k_a$ would be calculated by plotting the natural logarithms of the concentrations against time.

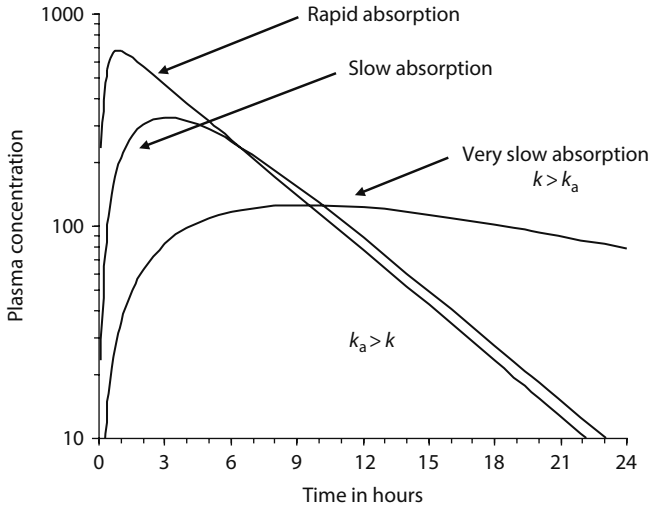


FIGURE 5.9 The effect of absorption rate on the shape of the plasma concentration–time curve.

Peak Plasma Concentration

The peak plasma concentration is determined by the dose, the bioavailability, the apparent volume of distribution (V), and the relative rates of k_a and k. The peak concentration may be more important toxicologically than the average internal exposure, especially for acute effects.

Decrease after the Peak

The decrease after the peak concentration is determined by the slower of the two processes (absorption or elimination), but it is usually elimination. Very polar compounds show slow absorption and rapid elimination, and the postpeak decrease is equivalent to $-k_a$, a situation described as *flip-flop* kinetics (see Figure 5.9). Flip-flop kinetics are very common after topical (dermal) application.

Area under the Curve

Both CL and V may be derived using Equations 5.17 and 5.18, providing that the dose used in the calculation is adjusted for the fraction absorbed (F), that is,

$$CL = \frac{\text{Dose}_{\text{oral}} \times F}{\text{AUC}_{\text{oral}}}$$

It is common to see CL_{oral} calculated as oral dose/AUC_{oral} in the absence of any information on F. Such a term is meaningless because what is calculated is CL/F, which is dependent on two physiological processes that are often unrelated. Intravenous data are necessary to relate a nonlinear change in AUC at high oral doses to either altered CL or F.

The value of F is determined by comparison of the plasma concentration–time curves after oral and intravenous dosing. Because of the different shapes after oral and intravenous administration (Figure 5.10), comparisons at a single time point are not valid. Instead, the overall systemic exposures, as indicated by the AUCs extrapolated to infinity (Figure 5.10), after oral and intravenous dosage are compared:

$$CL = \frac{\text{Dose}_{\text{oral}} \times F}{\text{AUC}_{\text{oral}}} = \frac{\text{Dose}_{\text{iv}}}{\text{AUC}_{\text{iv}}}$$
$$F = \frac{\text{Dose}_{\text{iv}} \times \text{AUC}_{\text{oral}}}{\text{AUC}_{\text{iv}} \times \text{Dose}_{\text{oral}}} \quad (5.26)$$

These relationships are valid only if the AUC/dose ratio is constant. If this ratio is dose-dependent, then the value of either F or CL must change with an increase in dose, suggesting saturation of absorption or elimination (see later).

An alternative method to estimate the fraction of the dose absorbed as the parent compound (F) may also be

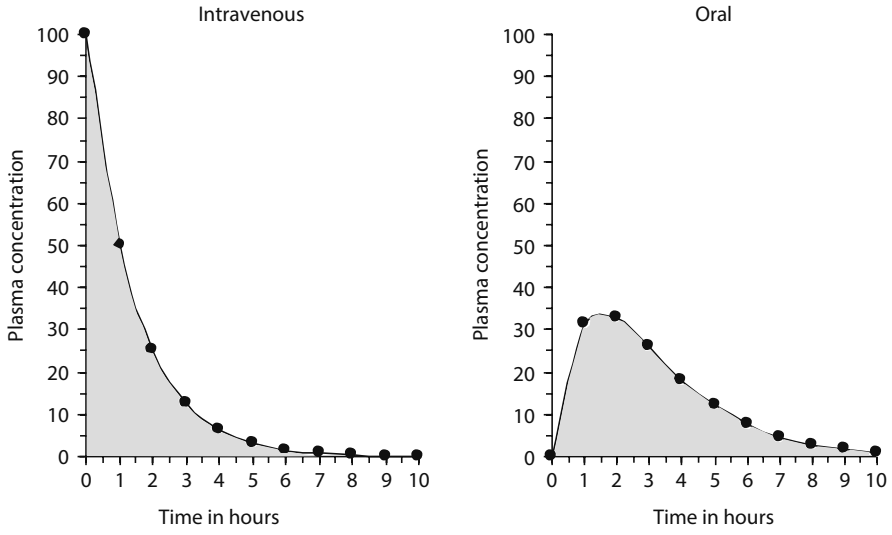


FIGURE 5.10 The shape of the plasma concentration–time curve after intravenous and oral administration. The shaded area is the AUC, which is used to calculate the bioavailability (F).

derived from the cumulative urinary excretion as the parent compound:

$$F = \frac{A^{\infty}_{ex\ \text{oral}}}{A^{\infty}_{ex\ \text{iv}}} \times \frac{\text{Dose}_{\text{iv}}}{\text{Dose}_{\text{oral}}} \tag{5.27}$$

Metabolite Kinetics

As discussed elsewhere, the biotransformation of xenobiotics usually results in detoxication, but is frequently associated with the formation of a toxic metabolite. Measurement of the rate of metabolism in vivo can provide much useful information on detoxication and/or bioactivation processes. In most cases, the rate of metabolite formation is governed by in vivo enzyme kinetics; enzyme reactions are first order only over a limited substrate concentration range. Saturation of metabolism is discussed in more detail later, and the following analysis relates to metabolite formation under first-order reaction conditions and when *CL* depends on enzyme activity rather than liver blood flow.

The one-compartment model for the parent compound (Figure 5.5) can be extended by a second compartment from the metabolism arrow with characteristics for the metabolite of V^m and an overall elimination rate constant of k^m. The concentration of the metabolite is given by

$$C^m = \frac{k_m \text{Dose}\left(e^{-kmt} - e^{-kt}\right)}{V^m\left(k - k^m\right)} \tag{5.28}$$

where C^m is the plasma concentration of the metabolite at time = t.

The elimination rate of the metabolite (k^m) is often greater than the overall elimination rate of the parent compound (k). In such cases, the term e^{-kmt} approaches zero before e^{-kt}, and thus at late time points, Equation 5.28 may be rewritten and solved omitting e^{-km} when it becomes

$$\ln C^m = \ln \frac{k_m \times \text{Dose}}{V^m\left(k^m - k\right)} - kt \tag{5.29}$$

Thus, a plot of the natural logarithm of the plasma concentration of the metabolite against time has a terminal slope similar to that of the parent compound (i.e., $-k$). In this case, the metabolite is present only as long as the parent compound remains in the body, and the ratio of metabolite:compound remains constant during the elimination phase.

In those cases where the elimination rate of the metabolite (k^m) is less than that of the parent compound (k), the term e^{-kt} approaches zero before e^{-kmt}, and thus Equation 5.28 may be written as

$$\ln C^m = \ln \frac{k_m \times \text{Dose}}{V^m\left(k - k^m\right)} - k^m t \tag{5.30}$$

and a plot of the natural logarithm of the plasma concentration of the metabolite against time has a slope of $-k^m$. In this case, the ratio metabolite/compound increases during the elimination phase. Such cases are of particular interest to toxicologists because on repeated exposure, the concentrations of metabolite increase more than the parent compound and may exceed those of the parent compound.

The overall elimination rate constants may also be derived from urinary metabolite levels as described earlier for the parent compound, although again the derived rate may be either k or k^m. However, if metabolite kinetics are based solely on urinary excretion data, the formation of more lipid-soluble metabolites may be missed because negligible amounts of such metabolites would be excreted in the urine.

Two-Compartment Open Model

Mathematically and physiologically, it is often more appropriate to regard the body as representing a simple two-compartment open system in which the distribution to certain peripheral tissues is not an instantaneous process. In such a system, the chemical initially enters a central compartment (the plasma and those tissues in which distribution is instantaneous) and is subsequently distributed to a second peripheral compartment. Elimination normally occurs from the central compartment, so that chemical in the peripheral compartment must transfer back to the central compartment in order to be eliminated. In Figure 5.11, k_{12} and k_{21} are the rate constants for transfer from compartments 1 to 2 and from 2 to 1, respectively, and k_{10} is the elimination rate from the central compartment. The relationships between these parameters and measurable rate constants (α and β) are outlined later.

Intravenous Bolus Dose

After a single intravenous bolus dose into a two-compartment system, the plasma concentration (C) at time t may be described by

$$C = Ae^{-\alpha t} + Be^{-\beta t} \tag{5.31}$$

where

- A and B may be regarded as analogous to C_0 for each compartment, and $A + B = C_0$
- α and β correspond to hybrid rate constants, each influenced by the individual distribution, redistribution, and elimination rate constants, that is, k_{12}, k_{21}, and k_{10}

The shape of a typical plasma concentration–time curve following a bolus intravenous dose is illustrated in Figure 5.11. As outlined for the determination of the absorption rate constant (see earlier), the method of residuals or line stripping is used to separate α and β. At later time points, $Ae^{-\alpha t}$ approaches zero, and the data are described by $C = Be^{-\beta t}$. At early time points, the difference between the actual C values and the concentrations derived by back-extrapolation of the $Be^{-\beta t}$ line are due to the contribution from $Ae^{-\alpha t}$. The values of A and α may be similarly derived by calculated linear regression analysis of the residuals or ΔC (C actual − C extrapolation).

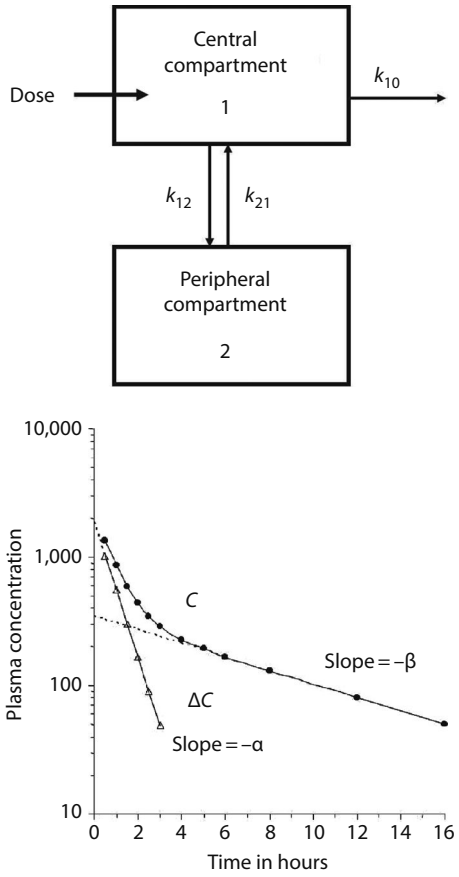


FIGURE 5.11 Plasma concentration–time curve for two-compartment system. The concentrations have been shown on a logarithmic scale for illustrative purposes; the slopes α and β would be calculated by plotting the natural logarithms of the concentrations against time and by fitting the two exponential terms simultaneously or using the method of residuals.

The rate constants α and β are composite rate constants, from which it is possible to derive k_{12}, k_{21}, and k_{10} using the following equations (see Ref. 45 for derivations):

$$C_0 = A + B$$

$$\alpha + \beta = k_{12} + k_{21} + k_{10}$$

$$V_1 = \frac{\text{Dose}}{A + B}$$

where V_1 is the volume of the central compartment, and

$$k_{21} = \frac{A\beta + B\alpha}{A + B}$$

$$k_{10} = \frac{\alpha\beta}{k_{21}}$$

$$k_{12} = \alpha + \beta - k_{21} - k_{10}$$

It is important to note that k_{10} and β do not relate to the same process, because k_{10} refers to the elimination from the central compartment, whereas β refers to the overall elimination from the body (and is slower due to transfer out of tissues as well as elimination from the central compartment).

As with the one-compartment system, an intravenous bolus allows derivation of most pertinent pharmacokinetic parameters:

1. A, B, α, and β may be derived from plasma data (see earlier).
2. k_{10}, k_{12}, k_{21}, and V_1 may be derived by manipulation of α, β, etc. (see earlier).
3. α, β, k_{10}, k_{12}, and k_{21} may be derived from urine by plotting either the excretion rate against time or by the sigma-minus method, where $\ln\left(A_{ex}^{\infty} - A_{ex}\right)$ (see Equation 5.22) is plotted against time.
4. The renal elimination constant, k_R, may be derived also from the renal clearance

$$CL_R = \frac{C_u \times F_u}{C}$$

and V_1 because $CL_R = k_R V_1$.

5. The concentration in the peripheral compartment (C_2) may be calculated from the following equation (which is analogous to Equation 5.25 for absorption into a single compartment):

$$C_2 = \frac{\text{Dose} \times k_{12}\left(e^{-\beta t} - e^{-\alpha t}\right)}{V_2(\alpha - \beta)} \qquad (5.32)$$

where V_2 is the volume of the peripheral or deep compartment. During the terminal phase of the concentration–time curve, $e^{-\alpha t}$ approaches zero, and therefore Equation 5.32 may be simplified as

$$C_2 = \frac{\text{Dose} \times k_{12} \times e^{-\beta t}}{V_2(\alpha - \beta)}$$

Therefore, a graph of $\ln C_2$ against time has a slope of −β. Thus the terminal rate of decrease in the peripheral compartment of a two-compartment system is identical to the decrease in the central compartment. In absolute terms, the calculation of C_2 is not particularly valuable, because the peripheral tissues comprising the deep compartment are not homogeneous, and the compound may not show a uniform distribution. If the concentration in the target organ is measured, then subsequent concentrations may be calculated using β defined from the central compartment.

A further useful kinetic parameter (V_β), which relates the total amount of chemical in the body to the plasma concentration, is given by the equation

$$V_\beta = \frac{\text{Dose}}{\text{AUC} \times \beta}$$

Just as β is a hybrid term reflecting the overall elimination from the body, so is V_β a composite but valuable function.

Constant Intravenous Infusion

The shape of the plasma concentration–time curve on intravenous infusion into a two-compartment open system is similar to that given in Figure 5.7, but with a biphasic increase at the start of the infusion and a biphasic decrease at the end. The kinetic parameters may be derived from the graph similarly to the one-compartment model, as follows.

Increase to Plateau

The increase to plateau follows a complex exponential function with 90% and 99% of the steady-state concentration being reached after, respectively, four and seven times the terminal (β-phase) half-life.

Plateau Level (Css)

At steady-state, the rate of infusion (R) equals the rate of elimination. Therefore,

$$\frac{R}{C_{ss}} = CL = V_1 \times k_{10} = V_\beta \times \beta$$

Decrease after Plateau

The decrease after plateau follows the equation:

$$C = A^* e^{-\alpha t^*} + B^* e^{-\beta t^*}$$

where

- A^* and B^* are the intercepts by back-extrapolation to the end of the infusion of the α and β slopes (determined as described for Figure 5.11)
- t* is the time since cessation of infusion

In many cases, a biexponential decrease is not seen in postinfusion data, because distribution will have occurred throughout the infusion.

Area under the Curve

The AUC can be used to derive the plasma clearance using Equation 5.17.

Oral Administration

Assuming first-order absorption into compartment 1, the plasma concentration at time t is given by

$$C = A^{\ddagger} e^{-\alpha t} + B^{\ddagger} e^{-\beta t} + C^{\ddagger} e^{-k_a t}$$

Graphical analysis by a semilogarithmic plot of ln C against time may reveal three separate phases from which α, β, and k_a should be measurable using the method of residuals. However, in practice, the value of k_a is frequently similar to α, and compounds that require a two-compartment model after an intravenous bolus dose appear to fit first-order absorption into a one-compartment model following oral dosing.[49,50] Thus detailed analysis is not possible without reference to intravenous data to determine which rate constant refers to the absorption rate.[45,47] The absorption rate is likely to be of greatest importance in acute toxicity studies, whereas the bioavailability (F) may be more significant in chronic studies; the latter may be measured using model-independent equations (Equation 5.26 or 5.27).

Metabolite Kinetics

Frequently, both the parent compound and the metabolites each require a two-compartment open model. The resulting equation requires four exponential terms, but often the concentration–time curve for the metabolite appears to be a simple biexponential decrease. The slow terminal phase of the metabolite is given by either β for the parent compound or the terminal rate for the metabolite (see earlier), and the faster rate is a composite of the other three rate constants.

Multiple Oral Dosing: Chronic Administration

On multiple dosing or continuous intake, the plasma levels increase over a period of four to five times the terminal half-life to establish a plateau concentration, similar to that seen with intravenous infusion (Figure 5.7). The average plateau (steady-state) level is subject to variations around a mean because material is eliminated between doses. In oral toxicity studies, these doses may represent either repeated single gavage doses or the feeding habits of the animals if the test compound is incorporated into the diet and fed *ad libitum*. On cessation of chronic intake, the rate of decrease in blood levels is usually but not always similar to that seen after a single dose.

One-Compartment Open Model

The average plateau level is given (by analogy with intravenous infusion) as

$$C_{ss\ mean} = \frac{\text{Dose} \times F}{V \times k \times T} \tag{5.33}$$

where

- F is the fraction absorbed
- T is the dose interval
- k is the elimination rate constant

However, it is important to realize that this equation includes the *elimination* rate constant k, which may or may not be the terminal rate observed following oral administration (flip-flop kinetics).

An alternative and more useful form of this equation can be derived from the fact that at steady-state, the rate of input ($F \times \text{dose}/T$) is balanced by the rate of elimination ($C_{ss\ mean} \times CL$); therefore,

$$C_{ss\ mean} = \frac{\text{Dose} \times F}{T \times CL}$$

The fluctuations around the mean plateau level depend on the dosing interval in relation to the terminal elimination rate. Thus compounds with a short half-life (2–3 h) show large fluctuations, as more of the chemical is eliminated between each dose, and with single daily dosing, the plasma levels

will approach zero prior to each dose. Inter-dose fluctuations may be reduced and blunted by slow absorption. The equations relating to these processes were summarized in the previous edition of this book.

$C_{ss\ mean}$ may be related to the AUC_{oral} for a single dose since

$$AUC_{oral} = \frac{Dose \times F}{V \times k} = \frac{Dose \times F}{CL}$$

and therefore

$$C_{ss\ mean} = \frac{AUC_{oral}}{T} \tag{5.34}$$

It is important to realize that this relationship assumes that the AUC is directly proportional to the dose and that *CL* does not alter during chronic administration. If a compound can induce its own metabolism on chronic treatment, then *CL* increases over the first few days of the study so that the steady-state concentrations and body burden are lower than would have been predicted from the AUC, *CL*, or *CL/F* measured after a single dose. Induction of cytochrome P450 (CYP) isoenzymes is a well-recognized phenomenon that can have toxicological implications in humans[51] and could also affect the outcome of animal toxicity studies.[52] Changes in *CL* may be assessed by comparison of $AUC_{0-\infty}$ for a single dose, with the AUC for a dose interval at steady-state (AUC_{0-T}). If AUC_{0-T} (chronic) < $AUC_{0-\infty}$ (single), this indicates either induction of metabolism or a decrease in bioavailability; conversely, if AUC_{0-T} (chronic) > $AUC_{0-\infty}$ (single), then inhibition or saturation of metabolism and/or an increase in bioavailability is indicated.

The extent of accumulation on repeated intake may be measured by the average amount in the body at steady-state ($Ab_{ss\ mean}$) divided by the amount in the body after a single dose (*Ab*), that is,

$$\text{Extent of accumulation} = \frac{Ab_{ss\ mean}}{Ab} = \frac{Ab_{ss\ mean}}{Dose \times F}$$

The amount in the body at steady-state is given by Equation 5.33:

$$Ab_{ss\ mean} = VC_{ss\ mean} = \frac{F \times Dose}{k \times T}$$

Therefore,

$$\text{Extent of accumulation} = \frac{1}{k \times T} = \frac{1}{0.693/t_{1/2} \times T} = \frac{1.44 \times t_{1/2}}{T}$$

Two-Compartment Open Model

The equations giving the plasma concentration at time *t* at steady-state into a two-compartment system with first-order absorption are considerably more complex than those for the one-compartment system. However, the simplified equation (Equation 5.33) applies in the form

$$C_{ss\ mean} = \frac{Dose \times F}{V_1 \times k_{10} \times T} = \frac{Dose \times F}{V_B \times \beta \times T}$$

and the value of $C_{ss\,mean}$ may still be derived from Equation 5.34:

$$C_{ss\ mean} = \frac{AUC_{oral}}{T} = \frac{Dose \times F}{CL \times T}$$

In addition, the relationship between the AUC between $t = 0$ and $t = \infty$ for a single dose and the AUC for a dose interval at steady-state applies providing that neither *CL* or *F* changes on chronic intake (see earlier text); a difference between these AUC estimates indicates changes in *CL* or *F* during chronic treatment.

Statistical Moment Analysis

In recent years, both clinical pharmacokinetic and animal toxicokinetic studies have moved away from compartmental models, because they involve multiple variables, which require numerous properly timed blood samples to characterize them adequately. Also, curve fitting is dependent on the terminal slope, which is frequently measured using plasma concentrations that approach the limit of detection of the assay method, that is, the weakest data. In contrast, terms such as clearance are measured from dose and AUC, the latter being determined largely from the highest and most accurately measured concentrations. Such *time-averaged* parameters may be extended to *time-related* parameters by the use of statistical moment theory, which allows assessment of additional useful kinetic parameters such as *mean residence time* (MRT) and *mean absorption time* (MAT).

The plasma concentration–time curve may be regarded as a statistical distribution curve[53] for which the zero and first moments are the AUC and MRT, respectively:

$$AUC = \int_0^\infty C\,dt$$

$$MRT = \frac{AUMC}{AUC} \tag{5.35}$$

where AUMC is the area under the first moment of concentration–time curve. That is, $\int_0^\infty t \times C\,dt$.

The AUC and AUMC may be calculated using the trapezoid rule applied to the observed data.

The measured plasma concentrations and time points are used for AUC calculation with extrapolation to infinity calculated as C_{last}/β.

For AUMC calculation, the measured plasma concentrations (*C*) and time (*t*) are multiplied to give *Ct* values for

each time point, and these are used with the time points. The AUMC for two consecutive time points is given by $[(C_1 \times t_1 + C_2 \times t_2)/2] \times (t_2 - t_1)$, and the total AUMC for the period of observation is the sum of the calculated AUMC segments. The AUMC from the last data point to infinity has to be calculated as

$$\frac{t_{\text{last}} \times C_{\text{last}}}{\beta} + \frac{C_{\text{last}}}{\beta^2}$$

Intravenous Administration

Following an intravenous bolus dose, the MRT can be calculated by Equation 5.35. The *apparent volume of distribution at steady-state* (V_{ss})[54] may be calculated as

$$V_{\text{ss}} = CL \times \text{MRT} = \frac{\text{Dose}}{\text{AUC}} \times \frac{\text{AUMC}}{\text{AUC}} = \frac{\text{Dose} \times \text{AUMC}}{\text{AUC}^2} \quad (5.36)$$

The MRT can be calculated from the AUMC following an intravenous infusion using the equation:

$$\text{MRT}_{\text{infusion}} = \text{MRT} + \frac{\text{T}}{2} \quad (5.37)$$

where

$\text{MRT}_{\text{infusion}}$ is calculated from the AUMC and AUC by Equation 5.35 from the infusion data
T is the infusion time

The V_{ss} cannot be calculated from infusion data.

In the same way that CL may be related to V by the rate constant k (Equation 5.15), so CL may be related to V_{ss} by the first-order rate constant k_{ss}.[45,53,54]

$$CL = k_{\text{ss}} V_{\text{ss}} = \frac{V_{\text{ss}}}{\text{MRT}}$$

The half-life derived from k_{ss} ($0.693/k_{\text{ss}}$ or $0.693 \times \text{MRT}$) is a composite half-life for compounds showing a distribution phase and may be regarded as the *effective* half-life and represents a useful kinetic parameter, particularly for a single dose.

Oral Administration

Absorption from the gut may involve more than one first-order rate or a zero-order component, or both. A major strength of the statistical moment approach is its ability to derive meaningful data following oral administration, because it is both more reliable and easier to use than most other methods[50,55] and does not rely on assumptions about the presence of a first-order or zero-order process. The most useful absorption parameter is the MAT, which is the difference between the MRTs following oral and intravenous dosing:

$$\text{MAT} = \text{MRT}_{\text{oral}} - \text{MRT}_{\text{iv}} \quad (5.38)$$

The statistical moment theory is a valuable technique for comparisons on the influence of dosage formulations on absorption.[50,55]

Dose-Dependent or Nonlinear Kinetics

Whereas simple diffusion obeys first-order kinetics at all concentrations, most other toxicokinetic processes involve an interaction between the foreign chemical and a specific site on a protein (examples being active transport across the gut, plasma and tissue protein binding, metabolism, and renal tubular secretion). Because of the limited availability of the protein, these processes have a finite capacity, and all of the specific sites on the protein may be occupied at high concentrations of chemical. Addition of further chemical cannot result in further interaction between the chemical and protein, and the concentration of free compound increases rapidly.

Depending on the nature of the protein–chemical interaction, there are a number of possible consequences, which are summarized in Table 5.1. This table represents a considerable simplification because the effect of saturation at one site may affect another protein–chemical interaction. Almost all of the processes listed in Table 5.1 may be described by a Michaelis–Menten equation of the type introduced in Equation 5.6, and

$$-\frac{\text{d}C}{\text{d}t} = \frac{V_{max} \times C}{K_m + C}$$

where

V_{max} is the theoretical maximum rate of the reaction
K_m is the Michaelis constant (which reflects the concentration giving 50% saturation of the protein)

At low concentrations, $C \ll K_m$, and $K_m + C$ approximates to K_m so that

$$-\frac{\text{d}C}{\text{d}t} = \frac{V_{max} \times C}{K_m}$$

and V_{max}/K_m is equivalent to a first-order rate constant k.

At higher concentrations, $C \gg K_m$, and $K_m + C$ approximates to C so that

$$-\frac{\text{d}C}{\text{d}t} = \frac{V_{max} \times C}{C} = V_{max}$$

and thus the elimination is a zero-order reaction.

The shape of the plasma concentration–time curve for a hypothetical compound showing saturation kinetics is given in Figure 5.12, which clearly shows that although low doses are indistinguishable from first-order elimination, the decrease at high plasma concentrations shows zero-order and then first-order reaction components.

It is important to note that the terminal slope and terminal half-life are derived from low plasma concentrations and do

TABLE 5.1
Consequences of Saturation of Chemical–Protein Interactions

Site	Interaction	Possible Consequences of Saturation at High Dose
Absorption	Active uptake	Reduced plasma levels and AUC after oral but not iv doses.
	First-pass metabolism	Increased plasma levels and AUC after oral but not iv doses.
Distribution	Plasma protein	Increased volume of distribution; increased glomerular filtration; increased hepatic clearance if extraction ratio is low.
	Tissue protein	Decreased volume of distribution; a graph of C_f/C against C will be nonlinear.
Metabolism	Metabolizing enzyme (saturation by substrate, depletion of cofactors, product inhibition)	Decreased clearance; AUC/dose ratio increases for parent compound, whereas AUC of metabolite/dose ratio may decrease for both oral and iv doses; enzymes with high K_m values may handle a larger proportion of the dose.
Excretion	Renal tubular secretion	Decreased renal clearance; AUC/dose ratio increases for oral and iv doses; nonrenal routes of elimination become of more importance; total excretion in urine per dose may decrease depending on the availability of other routes of elimination.
	Renal tubular reabsorption (rare)	Opposite of effects for saturation of renal tubular secretion.
	Biliary excretion	Decreased biliary clearance; decreased enterohepatic recirculation; renal route may become more important; AUC/dose ratio increases for oral and iv doses.

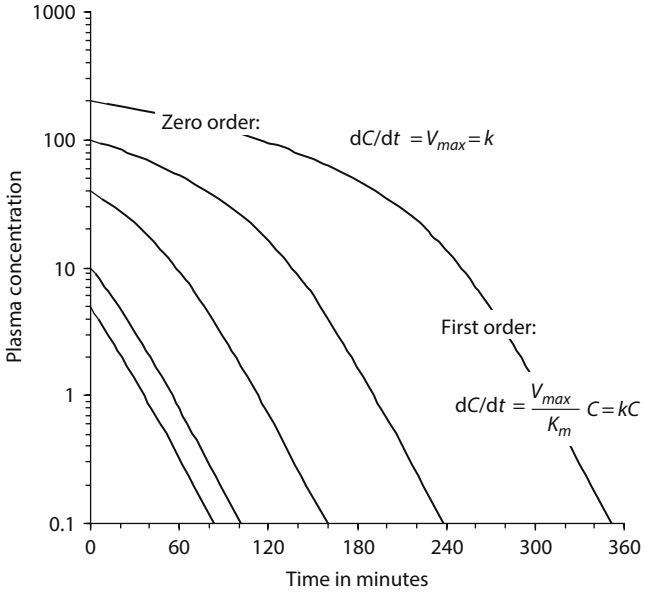


FIGURE 5.12 Plasma concentration–time curve for a compound showing saturation kinetics. The data were generated using an apparent V_{max} of 1 μg min^{-1} and a K_m of 20 μg mL^{-1} for initial concentrations of 5, 10, 40, 100, and 200 μg mL^{-1}. Data points were obtained using the equation $V_{max}(t - t_0) = C_0 - K_m \ln(C_0/C)$.

not provide evidence of dose-dependent kinetics. However, the plasma clearance, which is derived from AUC data and which reflects the capacity of the organs of elimination to remove the chemical from plasma, provides the best evidence of saturation.

An increased understanding of saturation kinetics can be obtained by the determination of K_m and V_{max} from in vivo data. The value of K_m that reflects the plasma concentration necessary to give 50% saturation of the active process is particularly useful for interpreting toxicity dose–response relationships. Derivation of these constants using the *Lineweaver–Burk*, *Hanes–Woolf*, and *Woolf–Augustinsson–Hofstee plots* was described in the previous edition of this book.

Of greatest importance for toxicology is the clear demonstration of saturation at high doses, an estimation of the plasma concentration above which first-order kinetics cease to apply, and the plasma concentrations present in animals showing overt toxicity. Simple methods for showing the absence of dose-dependent kinetics include graphs of *C*/dose against time that are superimposable, kinetic parameters derived by fitting the same model that have similar values at different doses, and the ratio of AUC/dose, which is constant.

A consequence of nonlinear kinetics is that the time to reach steady-state is also dose-dependent. This situation should be borne in mind when planning short-term studies.

PRACTICAL METHODS

It must be emphasized at the outset that the key to successful kinetic studies is the development of an assay of high specificity that measures the chemical without interference by its metabolites, and that is of sufficient sensitivity to define the terminal slope accurately (see later).

It is essential that the necessary legal and ethical approvals are obtained prior to any in vivo experiment in animals. This applies to noninvasive procedures, such as the incorporation of a chemical into an animal's feed, as well as invasive procedures such as the collection of blood samples. Under UK legislation, separate Home Office licenses are required for the premises, the individual and the procedure.

General information on techniques for blood sampling may be obtained from the texts by Waynforth[56] and Cocchetto and Bjornsson,[57] the latter providing 501 references and an extensive and invaluable literature review of methods for the collection of body fluids.

The methods for dosing, blood sampling, urine collection, etc., outlined later are largely related to the rat, as this is the species most commonly used in toxicological studies.

Administration Techniques

Oral Dosing

Rats, guinea pigs, and mice may be dosed by gavage using a syringe fitted with a suitable intubation needle: in rabbits, a polyethylene cannula is passed into the stomach while the jaws are held open by a gag.

Certain precautions should be taken to prevent artifacts. For example, if the chemical is given as a suspension, the apparent absorption rate may include a component due to dissolution of the chemical, which may be rate limiting. The ideal vehicle for dissolution is water or a small volume of a water-miscible solvent such as ethanol, propylene glycol (propane-l,2-diol), or dimethylsulfoxide, although for very lipid-soluble compounds, it may be necessary to give the dose in corn oil or as an emulsion. Excess acids or bases should not be used to dissolve the test compound, and the pH of the dose solution should be near pH 7.

The volume of water or solvent/water used to dissolve the chemical should be kept low in order to avoid artifacts. If dose-dependent absorption is suspected, the different doses should be given in the same volume of solution. The maximum volume of an aqueous solution that can be administered without the possibility of interference with absorption is approximately 5–10 mL kg^{-1}. Larger volumes may be given, although nonlinear kinetics seen under such circumstances may be due to solvent-induced alteration of intestinal function.

The use of water-immiscible solvents such as corn oil, which are sometimes used for gavage doses, should be avoided if possible, because mobilization from the vehicle may be rate limiting. However, such a vehicle would obviously be appropriate if it was the method of administration used in toxicity studies.

The rate of absorption can affect not only the time to maximum concentration and the maximum concentration, but also the total amount entering the systemic circulation, by saturating hepatic uptake and first-pass metabolism. An example of this is the hepatotoxicity of oral carbon tetrachloride, which is markedly higher after a bolus oral dose compared with gastric infusion.[58]

When toxicity studies are performed by mixing the compound into the animals' diet, it is important to measure the concentration–time curve over a 24-h period at steady-state using dietary administration, because both the peak concentration and the AUC may be different from data obtained from bolus gavage studies.

Nasal Administration

Methods have been described for assessing absorption from the nasal cavity based on plasma pharmacokinetics following intranasal and intravenous dosing and by in situ perfusion experiments.[59,60] A technique for inhalation with nose-only exposure has been described for studies in guinea pigs.[61]

Rectal Administration

Because a number of therapeutic compounds are given as suppositories, an indication of the bioavailability after rectal administration is sometimes required. Normally, toxicity studies and initial drug formulations of such compounds are performed by the oral route, and the rectal formulation comes late in development and marketing.

Inhalation

A major problem associated with determining the kinetics of inhalation concerns the measurement of the extent to which the chemical is absorbed across the lung, rather than passed back into the mouth to be swallowed, exhaled in the expired air, or absorbed across the nasal mucosa or skin. Comparison of the plasma AUC or the total urinary excretion of unchanged compound, after a period of inhalation with the same parameter after a known intravenous dose, can be used to determine the total dose entering via the lungs plus gut, etc.

A method used successfully by McKenna et al.[62] to obtain kinetic data involved a 6 h exposure to the vapor of [^{14}C] vinylidene chloride in rats, after which the animals were transferred to a metabolism cage. The body load at the time of removal was determined by the total recovery of radioactivity in the expired air, excreta, cage washings, and carcass. This method is appropriate for determining the total dose because the nonspecific measurement of ^{14}C includes parent compound and all metabolites. If the parent compound alone is measured, the inhalation data must be compared to intravenous data in order to measure the extent of exposure after inhalation.

The metabolism rate constants of inhaled 1,1-dichloroethylene have been determined by the measurement of the rate of removal of the compound from circulating air in a closed chamber system containing the experimental animal.[63] The air was recirculated, with oxygen added to maintain the concentration at 19%–21%, and the air was sampled at regular intervals and analyzed for unabsorbed 1,1-dichloroethylene by gas–liquid chromatography. The rate of removal showed two phases; a rapid initial phase and a slower second phase, which showed saturation kinetics and the constants K_m and V_{max} were derived in terms of the concentration of chemical in the chamber. This approach is interesting because the data are obtained by a noninvasive method, and the kinetic constants are derived in terms of vapor or gas concentrations, which are most appropriate when interpreting inhalation studies in relation to human exposure to volatile agents.

Percutaneous Absorption

The dermal absorption of vapors can be assessed in rats using a body-only chamber,[64] but there may be major species differences related to the presence of hair follicles and the barrier function of the stratum corneum. Shaving the hair from the backs of rats can provide a suitable site for in vivo absorption studies,[65] but this can change the permeability characteristics of the stratum corneum. In reality, in vitro data can provide a suitable model for extrapolation to humans.

Intravenous Injection

The bolus intravenous dose is the most important single technique for deriving toxicokinetic information. Aqueous or aqueous miscible solvents should be used, although the maximum dosage volume is about 2 mL kg^{-1} for aqueous and 1 mL kg^{-1} for solvent–aqueous mixtures. Ideally, the solution should be isotonic. The data processing assumes that the material is administered instantaneously at time zero and that 100% of the dose is intravenous, and none ends up in a perivascular site.

The tail and hind paw veins of rats are convenient for dosing, but neither is particularly easy to use or gives 100% intravascular dosing repeatedly and routinely without the necessary expertise. Cannulation of a vein, such as the external jugular or femoral vein, under anesthesia provides a more secure method of intravenous administration. The same cannula can be used for subsequent sampling, providing that the compound is known not to be adsorbed onto the cannula. The external jugular vein may also be used for intravenous dosing of guinea pigs. For rabbits, the vein running around the periphery of the ear lobe is of sufficient size and visibility to give reliable intravenous dosing.

Intravenous Infusion

For intravenous infusion studies, the dose must be given via an indwelling cannula. If the infusion period is prolonged, such that recovery from anesthesia is envisaged, the cannula can be run under the skin from the ventral surface of the neck and exteriorized on the dorsal surface behind the ears. If the cannula is then secured on the dorsal surface, the animal should be prevented from damaging it while being permitted a degree of movement. This method of exteriorization is also valuable as a method of long-term sampling.

The delivery of compound during intravenous infusion must be at a constant but low rate such that the animal is not subjected to excessive hemodilution. Methods include the use of a high-quality infusion pump and osmotically driven minipumps, which can be implanted into the animal, and deliver a constant rate as low as 0.5 μL h^{-1} for up to 2 weeks.[66]

Sampling Techniques

Blood (Plasma and Serum)

When considering the frequency, timing, and duration of blood sampling, it is important that an adequate number of samples are taken to define each section of the plasma concentration–time curve. A three-compartment system can be accurately analyzed by about 12 samples provided that they are correctly timed. It has been suggested that plasma samples be collected during the first four to five half-lives, during which time 93%–97% of the compound will have been eliminated. However, it is possible that such a restriction may mask a quantitatively minor distribution component, and as a general guideline, the plasma concentrations should be measured until the limit of detection of the analytical method is reached. Obviously, if the limit of detection allows analysis over a large number of half-lives, less frequent sampling is required during the slow terminal phase. The corollary is that a relatively insensitive analytical method may be incapable of yielding full pharmacokinetic data.

Using the methods described later, it is possible to withdraw a significant fraction of the total blood volume (64 mL kg^{-1} in the rat), thereby modifying the perfusion of the organs of elimination and corrupting the derived pharmacokinetic data. This problem can be avoided by taking the smallest samples consistent with accurate analysis and the minimum number of samples necessary to define adequately the various phases (i.e., smaller samples at early time points). As guidance, individual blood samples should be restricted to a maximum of about 0.5 mL kg^{-1} body weight, providing the total number of samples is small (i.e., less than 10).

Various methods have been used successfully to obtain small serial blood samples from anesthetized and conscious rats. Whole-blood samples, of approximately 100–200 μL, can be obtained from the tail vein, either by snipping the very end off the tail under local anesthesia or by making a small incision closer to the base of the tail. An advantage of this method is that usually only a single manipulation is necessary, since washing the tail vein with warm water will often remove the blood clot and reinstigate blood flow. A disadvantage is that the sampling site may be contaminated by urine and feces, although washing the tail may remove the polar metabolites excreted in urine and feces. Other published methods that have been used to obtain blood samples include clipping the toe nail into the vascular bed, multiple cardiac sampling, and rupture of the sinus membrane at the back of the orbit, although these produce considerably more trauma and, depending on national or local legislation, may have to be performed in nonrecovery animals (i.e., under terminal anesthesia).

Alternative and more reliable methods require the insertion, under anesthesia, of a cannula into an exposed vein, such as the external jugular vein. Such tubing can then be used for sampling and may remain patent for periods up to 2 months. The use of silicon tubing is preferred to polyethylene for long-term studies because it is more flexible for exteriorization on the dorsal surface and less apt to cause thrombosis. For long-term studies, the cannula is exteriorized such that the animal cannot damage the tubing.[57,67] Cannulation of both the external jugular vein and carotid artery under general anesthesia in nonrecovery animals can be used for both intravenous dosage (venous) and blood sampling (arterial). For experiments performed under anesthesia in other species, a major vein or artery (e.g., jugular, carotid, femoral) can be cannulated.

The orbital sinus is a reliable site for the collection of blood from the mouse while under terminal anesthesia. The marginal ear vein can be used for the rabbit without anesthesia.

Urine

Knowledge of the urinary excretion rate is necessary for calculating the overall renal clearance of a compound. The bladder causes variable slowing of the output, and for compounds with a short half-life, a method of overcoming sporadic

urination is necessary. Calculating results by the sigma-minus method (Equation 5.22), rather than using excretion rate data, reduces the importance of incomplete bladder emptying and the resultant scatter in the data. For a compound with a half-life of many hours, sufficiently frequent samples may be obtained from daily urine collections using a metabolism cage.[57]

In nonrecovery animals under anesthesia, the effect of the bladder may be overcome by cannulation of the urinary bladder via the urethra and allowing the urine to be expelled naturally or with the aid of gentle massage or by cannulation of both ureters directly.

Renal clearance studies may be performed either after single doses or during infusion at steady-state (when the clearance can be related to total clearance and plasma concentration). Insights into the extent of reabsorption and tubular secretion can be obtained by measuring the renal clearance of inulin given simultaneously (1–20 μCi of [^{14}C] inulin kg^{-1} or 50–100 μCi of [^{3}H]inulin kg^{-1}).

Bile

In rats, bile may be collected from a cannula inserted into the common bile duct under general anesthesia such that the tip is located at the point of bifurcation near the hilar region of the liver. Bile, which usually flows at a rate 0.5–1.0 mL h^{-1} in the rat, may be collected either by exteriorizing the cannula or by passing the tubing into a suitable container (sealed plastic sachet) placed subcutaneously. A problem with biliary excretion studies is that changes in bile composition occur if the bile salts are not allowed to recirculate. To minimize this, the biliary excretion can be measured soon after establishing the cannula or the bile can be recirculated back into the gastrointestinal tract while the animal recovers from surgery.[68]

In animal species that possess a gallbladder (i.e., guinea pig and rabbit), it is necessary to prevent this organ from delaying elimination by ligation around its base.

DATA HANDLING

Data on the concentrations of a chemical in plasma and urine can be analyzed graphically, but the availability of powerful personal computers means that such approaches are largely redundant. Computerized analysis by fitting multiexponential equations is the method of choice because data handling is optimized.

Computation

There are a number of suitable programs available for nonlinear least-squares regression analysis, which is the most appropriate method (e.g., BLIN, NONLIN, SIPHAR), and readers are referred to Gibaldi and Perrier,[45] Wagner,[47] and Gabrielsson and Weiner[48] for further details. Such programs automatically put the best fit line through the data. In the analysis of data by computer program, it is common to apply a suitable weight to each data point to ensure the most appropriate fit. The weights that can be applied to the concentration data include

1. All weights equal, which is applicable if the errors in measurement are a constant amount, for example, ±2 μg mL^{-1}
2. Weighted by 1/y, which is applicable if the errors of measurement are a constant proportion, for example, ±2%
3. Weighted by 1/y^2, which can be used to force the fit through the later time points at the expense of the early higher values.

The second option, 1/y, closely represents the accuracy of most assay procedures and is used most frequently.

It is important that errors arising from the choice of an inappropriate model or incorrect weighting should be assessed by either a graphical representation or analysis of the deviation between observed and calculated concentrations (error analysis). Another factor to consider is that although use of more complex models may give a closer fit to the data, the available data may not be adequate to estimate accurately the larger numbers of parameters.

USE AND INTERPRETATION OF IN VITRO DATA

In vitro data can provide important qualitative insights into the metabolic fate of the compound, but care must be taken in their quantitative incorporation into risk assessment.

There is a wide variety of in vitro systems of increasing cell integrity,[69,70] which can be used, for example, subcellular fractions (such as microsomes), cell homogenates, isolated cells and cell lines, and tissue slices. Each preparation has strengths and weaknesses, and these can be exploited to provide useful information.[71–73]

Strengths of In Vitro Systems

Microsomes comprise the smooth endoplasmic reticulum and its associated enzymes—cytochromes P450 and UDP-glucuronyl transferases. The rates of reaction in vitro are determined by the availability of appropriate cofactors, and it is possible to study oxidation by the addition of NADPH, and glucuronidation by addition of UDPGA. Addition of NADPH but not UDPGA allows the rates of P450-mediated oxidation to be studied directly, because part of the primary oxidation produced is not lost due to conjugation. An additional major value for such simple systems is that they can be used to generate metabolites for structural analysis.

More complex systems, such as isolated cells and tissue slices, provide a more comprehensive picture of the metabolic fate of the compound. All enzyme systems are present, including cytoplasmic and mitochondrial enzymes, and the cell architecture can affect cell uptake and intracellular distribution of the chemical. Perhaps the most integrated in vitro system is the isolated perfused rat liver, which can give excellent correlations with in vivo clearance.[74,75] In consequence,

these systems can provide information on the relative importance of alternative metabolic pathways.

A major advantage of in vitro systems is that they allow data to be generated on the potential metabolism in humans, without the need for in vivo exposure. This has been particularly valuable for carcinogens, where the generation of in vivo data would be unethical. Identification of the specific isoenzymes of cytochrome P450 is important in understanding the potential variability in metabolism within the human population. Such information can be generated by in vitro studies in three ways:

1. Comparisons of the rates of metabolism in stored (banked) liver preparations from individuals with characterized isoenzyme profiles
2. The use of isoenzyme-specific inhibitors or inducers (in cell-intact preparations)
3. The use of expression systems, in which the DNA for specific isoenzymes is incorporated into and expressed by a suitable host, such as a yeast or bacteria[76,77]

The generation of in vitro data using human tissues allows characterization of qualitative and quantitative species differences both by generating appropriate enzyme constants V_{max} and K_m. Such data represent critical components of PBPK models[78] and for the prediction of in vivo clearance.[79–83]

The outline given earlier should be sufficient to indicate the huge potential for in vitro studies and explain why these methods have been the basis for much of our understanding of pathways of xenobiotic metabolism.

Precautions with In Vitro Systems

A number of limitations need to be remembered when considering in vitro data:

1. Many studies give data on the extent of metabolism at a single high concentration in vitro and therefore represent V_{max}, which may be of limited relevance to in vivo concentrations. A full analysis of the enzyme kinetics is necessary to give both V_{max} and K_m.
2. Changes in enzyme expression occur in vitro, for example, isolated cell lines show a different complement of cytochrome P450 activities to those in the same cells at isolation.
3. Many human data are generated from stored liver samples obtained at postmortem. The in vitro enzyme activity could be affected by both in vivo aspects, such as drugs given in attempts at resuscitation, disease, etc., and ex vivo aspects, such as the period between death and freezing and storage.[84]
4. In vitro data may still be misleading, even if all of these aspects are optimal. An important example of this is when the clearance of compound is limited by organ blood flow rather than enzyme activity (see earlier). Under these conditions, both interspecies differences and interindividual variability will reflect organ blood flow, rather than V_{max} and K_m. A good example of this is furan[85] for which the rate of oxidation in vitro would greatly exceed delivery via the liver blood flow. This problem can be avoided if the in vitro data are incorporated into a PBPK model that will take into account organ blood flow, partitioning between blood and tissue, and enzyme kinetics.

The increasing use of in vitro test systems facilitates a quantitative analysis of the dose–toxicity curve and may provide information on mechanisms of action.[71,86,87] The logical interpretation of such data with respect to human risk requires information on

- The steady-state concentrations of the active chemical species in the target organ and plasma of the test animals during chronic toxicity testing
- The toxicokinetics of the chemical in the test animal at toxic doses
- The toxicokinetics in humans at the likely exposure level

USE AND INTERPRETATION OF IN VIVO TOXICOKINETIC DATA

There are three principal aims of in vivo toxicokinetic studies:

1. Toxicokinetics can provide an understanding of the physiological processes that are involved in the fate of the chemical in the body.
2. The relation between dose and toxicokinetics may be the key to either the establishment of appropriate dose levels for chronic studies or the interpretation of data from such studies.
3. Comparative toxicokinetics can reduce uncertainty involved in the extrapolation from animals to humans.

Toxicokinetic studies are important in compound development, and such information is considered necessary before proceeding with long-term and carcinogenicity tests.[2] If the kinetic evidence indicates tissue accumulation on prolonged dosing, saturation of elimination at subtoxic doses, the formation of chemically reactive metabolites, or that the main route of metabolism is via an enzyme that shows a major genetic polymorphism,[88,89] then chemical analogs without these problems may be selected for development.

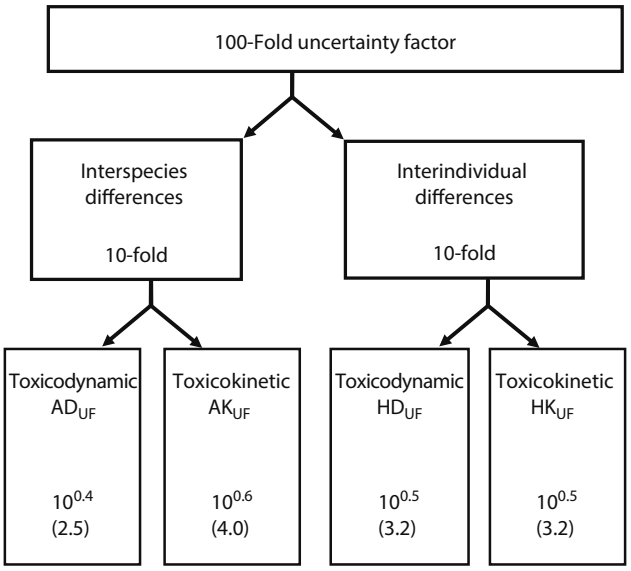
The relationship between kinetics, dose, and toxicity is probably the single most important contribution that kinetics can make to the field of toxicity testing. Although a few therapeutic drugs show nonlinear kinetics at the doses normally given to humans, the plasma levels of foreign chemicals in humans are usually well below those necessary to saturate

any protein-mediated reactions. In contrast, the maximum dose in toxicity tests is designed to show some degree of toxicity, and nonlinear kinetics are a distinct possibility. At doses above saturation, the body load of free compound increases steeply with increase in dose, and plasma and tissue concentrations of the chemical will also be considerably higher than predicted by extrapolation from lower doses. Nonlinear kinetics may result in an extremely steep dose–response curve for the toxic effect and must be taken into account when extrapolating effects detected in animal studies to humans.

A possible consequence of saturation of elimination is that normally minor pathways of metabolism may become of major significance. Thus, if a chemical undergoes metabolism by two routes, one with a low K_m (high affinity) and the other with a high K_m (low affinity), then at low doses, most chemical in the cell is eliminated by the high-affinity route, but more of the chemical will be eliminated by the low-affinity enzyme at plasma levels that saturate the high-affinity enzyme.

Different risk assessment approaches have been used traditionally for cancer (and other nonthreshold effects), compared with adverse effects believed to show a biological threshold.[2–4]

- Toxicokinetic data are sometimes incorporated into the risk assessment process for genotoxic carcinogens by the use of a PBPK model that corrects the external administered doses in the animal study to internal doses and also allows for differences between animals and humans in the relationship between external dose and target organ dose.
- For threshold toxicity, risk assessment involves estimating a level of human intake that would be without appreciable health effects, that is, a health-based guidance value such as a reference dose (RfD), an acceptable daily intake (ADI), or a tolerable daily intake (TDI). The guidance value is usually derived by dividing the intake of animals treated at the no-observed-adverse effect level (NOAEL) (expressed in mg kg^{-1} body weight) by an appropriate uncertainty factor. A default uncertainty factor of 100 has been applied for over 50 years, with a 10-fold factor to allow for extrapolation from animals to humans and a 10-fold factor to allow for interindividual differences in the exposed human population.[3,4] The use of chemical-specific adjustment factors (CSAFs)[90–92] allows appropriate toxicokinetic or mechanistic data to replace part of the relevant 10-fold default factor (Figure 5.13). This subdivision has the potential to replace uncertainty with scientific data and has been used in recent evaluations of the sweetener cyclamate (see cyclohexylamine mentioned later) and of dioxins, but replacement of one of the factors requires extensive data. The use of such a scheme would produce a more secure and scientific basis for the establishment of an ADI/TDI/RfD and will encourage the investment of time and money necessary to generate of such data.



Chemical-specific data can be used to replace a default uncertainty factor (UF)

A—animal to human; H—human variability; D—toxicodynamics; K—toxicokinetics

FIGURE 5.13 Subdivision of the 10-fold uncertainty factors to allow for species differences and human variability in toxicokinetics or toxicodynamics.[92] The total composite factor would be the product of any chemical-specific adjustment values and the remaining default uncertainty factors that had not been replaced, for example, if the interspecies toxicokinetic UF were replaced by chemical specific value of 20, then the total factor would be $2.5 \times 20 \times 3.2 \times 3.2 = 500$.

Extrapolation of animal toxicokinetic data to humans may be by either a PBPK model or compartmental modeling methods.[93,94] The physiological approach relies on the scale-up between animals and humans of parameters such as tissue volume and blood flow and their relationship to body weight.[95] PBPK models may be scaled up from animals to humans based on the known physiological differences. Alternatively, the plasma kinetics in various species may be fitted by compartmental modeling and then scaled up empirically according to the body mass of the species studied and extrapolated to humans.[96,97]

In general, species differences in basic physiological processes such as cardiac output and relative tissue weights usually result in lower clearances and longer half-lives in humans than in animals. Thus, comparisons of animals and humans on the basis of plasma levels or AUC values, rather than intake or exposure data (expressed per kg body weight), remove important variables from interspecies comparisons and provide a more secure basis for the safety assessment.[2,9,98]

The increased use of kinetic data, especially when combined with the knowledge of the mechanism of toxicity, allows the safety of potentially toxic chemicals to be based on scientific principles and understanding.[2,5–7,11] The following examples illustrate the contribution of toxicokinetic studies

to understanding the biological basis of toxicity and to the interpretation of toxicity data in terms of risk assessment.

Cyclohexylamine

Cyclohexylamine is a metabolite of the intense sweetener cyclamate, which is formed by the intestinal bacteria in the lower gut. Cyclohexylamine produces testicular toxicity when given chronically to rats, but not to mice.[99] Toxicokinetic studies indicated that the plasma clearance was higher in mice than in rats, and that rats but not mice showed evidence of nonlinear kinetics at high doses.[100] The steady-state concentrations in the plasma and testes during chronic administration confirmed dose-dependent kinetics in the rat, which coincided with the dose–response for testicular atrophy in this species.[100] These toxicokinetic data thus provide a possible explanation for the steepness of the dose–response curve in the rat and the apparent species difference in sensitivity.

Cyclohexylamine is an indirectly acting sympathomimetic amine in rats,[101] but it does not increase blood pressure in humans following its formation from cyclamate metabolism.[102] The apparent difference in response arises from the different concentration–time profiles when cyclohexylamine is absorbed rapidly following oral administration of a bolus dose or when it is formed slowly by the intestinal microflora from cyclamate,[102] illustrating further the importance of kinetics in the interpretation of dose–effect relationships.

The ADI for cyclamate established by the Joint Expert Committee on Food Additives (JECFA)[103] and the Scientific Committee on Foods (SCF) in Europe[104] is based on the NOAEL for testicular toxicity of cyclohexylamine in a 90-day study in rats. There are extremely wide person-to-person differences in the conversion of cyclamate into cyclohexylamine, and this has to be taken into account in calculating an ADI for cyclamate. The majority of the population cannot form cyclohexylamine, but 3%–4% of the population metabolizes 20% or more of the daily intake. Early evaluations[103,104] used a value of 18.9% metabolism with a 100-fold uncertainty factor. A recent study[105] showed that up to 85% of the daily intake could be converted to cyclohexylamine on individual days during chronic intake, but that the highest detected 7-day average was 58%. The most recent SCF evaluation[106] used a value of 85% metabolism and reduced the uncertainty factor to 32 because human variability in kinetics had been taken into account by the use of a worst-case % metabolism.

Dioxins (TCDD: 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin)

A major problem with deriving a health-based guidance value for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from animal toxicity data is that there is considerable accumulation due to the very long half-life. The usual 10-fold default uncertainty used for extrapolating from rats to humans would be inadequate for TCDD, because there is an approximately 90-fold difference in half-life between rats (about 30 days) and humans (about 7.5 years). Evaluations of dioxins by the JECFA[107] and the SCF[108,109] illustrate how toxicokinetic data can be used (and to some extent misused) in risk assessment.

These risk assessments have been based on the body burden, rather than the daily intake because this would take into account species differences in half-life and accumulation. Based on the relationship

$$\text{Extent of accumulation} = \frac{1.44 \times t_{1/2}}{T}$$

a 90-fold difference in half-life would give a 90-fold difference in body burden at steady-state.

The steady-state body burden of TCDD was estimated in rats and humans using the following relationship:

$$\text{Steady-state body burden (ng kg}^{-1}) = \frac{\left\{\left[\text{Dose}\left(\text{ng kg}^{-1}\right) \times t_{1/2}\left(\text{days}\right)\right] \times \text{F}\right\}}{\text{Ln } 2}$$

where

Dose is the daily administered dose or intake
F is the fraction absorbed (highly lipid-soluble compounds such as TCDD are incompletely absorbed from the gut and a bioavailability of 50% was assumed)
$t_{1/2}$ is the species-specific half-life of TCDD

This relationship is based on Equation 5.33, using 1 day as the dose interval, with the body burden as ($C_{mean} \times V$) and 1/k converted to $t_{1/2}$/ln 2 ($t_{1/2}$/0.693).

Because interspecies differences are taken into account by the use of body burden as a dose metric, the usual default uncertainty factor of 4.0 (Figure 5.13) was replaced by a CSAF of 1.0.[107–109] In addition, it was recognized that the most sensitive human would not be more sensitive than the rat strains used to define the NOAEL, so that the interspecies and human variability toxicodynamic CSAFs were set at 1.0. In consequence, only the factor of 3.2 for human variability in toxicokinetics was applied to the rodent NOAEL, instead of the usual default factor of 100-fold.

These recent risk assessments of dioxins illustrate how suitable chemical-specific data can be used quantitatively in derivation of a health-based guidance value. Despite this welcome scientific development, a number of difficulties remained and were only partially resolved:

1. The majority of human exposure to dioxin-like compounds (polyhalogenated dibenzodioxins, polyhalogenated dibenzofurans, and coplanar polychlorinated biphenyls) is to congeners other than TCDD. This is taken into account by the use of toxic equivalency factors (TEFs),[110] which relate the in vivo potency of the individual congener in animals to that of TCDD. The application of the relationship

given earlier for TCDD to non-TCDD congeners assumes that the 90-fold species difference in the half-life of TCDD applies equally to the TEFs of other congeners.

2. The JECFA and SCF assessments were based on postnatal effects in male rats following in utero exposure to a single oral dose given on day 15 of gestation (GD15).[111–115] Initially, these studies could not be interpreted because of potential and undefined differences in the distribution of the body burden following a bolus dose and following slow accumulation from the diet. TCDD and related compounds are highly lipid soluble and show very high fat to blood concentration ratios, such that the majority of the body burden at steady-state is in adipose tissue and not in potential sites of toxicity, such as the fetus. The toxicokinetic studies on TCDD performed in pregnant rats specifically to address this issue[116,117] allowed the fetal concentrations on GD16 to be estimated following different methods of exposure. Risk assessments were based on conversion of the maternal body burden in rats following a bolus oral dose on GF15,[111–113,115] or following a loading dose and weekly doses,[114] into a chronic maternal daily intake that would give the same fetal concentrations at steady-state. Extrapolation to humans was made using the relationship given earlier and the known species differences in half-life.
3. The risk of cancer was determined from occupational cohort studies (see Ref. 107 for details) in which the body burden was estimated some time after exposure had ceased, and the relationship between exposure and cancer risk was determined by back-extrapolation of the body burden to the period of occupational exposure. The average half-life in humans was used for back-extrapolation, but TCDD is a known enzyme inducer,[52] and if enzyme induction had occurred at occupational exposures, then a shorter half-life should have been used to back-extrapolate. A shorter half-life would result in a higher estimate of the body burden during the period of occupational exposure so that any cancer risk could have been overestimated by the use of an inappropriately long half-life (because the association would be with lower predicted body burdens).
4. The best dose metric for a chemical with a very long half-life would be the steady-state concentration in plasma, which is given by

$$C_{ss\ mean} = \frac{\text{Dose} \times F}{T \times CL}$$

Because *CL* is not known for humans, the JECFA and SCF used the half-life, which had the effect of changing $C_{ss\ mean}$ into $Ab_{ss\ mean}$. However, half-life is affected by both *CL* and *V*: rearrangement of Equation 5.16 gives the relationship between half-life, *CL* and *V*:

$$t_{1/2} = \frac{0.693 \times V}{CL}$$

The risk assessments of TCDD based on estimated body burdens in animals and humans assume that the pattern of tissue distribution is similar, so that the concentration at the site of action represents a similar proportion of the body burden in animals and humans. However, rats and humans have different body compositions, with fat representing about 10% of the body mass of rats, but about 20%–25% of humans. In consequence, the value of *V* would be over twofold higher per kg body weight in humans, so that the concentrations in all tissues, including sites of toxicity would be twofold lower in humans for any given body burden. Body composition was not taken into account in the JECFA[107] and SCF[108,109] evaluations, which ignored the contribution of differences in *V* to differences in half-life.

SELF-ASSESSMENT EXERCISES

5.1 A new chemical has been administered to rats and humans by both oral and intravenous routes. Basic toxicokinetic measurements (extrapolated to infinity) are given as follows:

	Rat	Human
Intravenous		
Dose (mg kg^{-1})	10	1
AUC (μg mL^{-1} min)	2000	500
Terminal slope (min^{-1})	0.0025	0.001
% Dose excreted unchanged in urine	1	15
Oral		
Dose (mg kg^{-1})	100	1
AUC (μg mL^{-1} min)	8000	490
Terminal slope (min^{-1})	0.0025	0.001

Calculate appropriate toxicokinetic parameters and suggest biochemical and physiological mechanisms that could explain the species difference.

5.2 The pharmaceutical company for which you work has synthesized a new antianxiety drug, which is a basic compound, structurally related to the old drug debrisoquine. The parent drug, which is the active form, causes enzyme (cytochrome P450) induction and liver enlargement; the hydroxylated metabolite, which is formed on incubation of the drug with liver microsomes, is inactive. After an oral dose, 40% is excreted in the urine within 24 h as the parent compound, 40% is in urine as a hydroxylated metabolite, and 20% is in feces as the parent drug. After an intravenous dose, 80% is in urine as the parent drug,

and 20% is in urine as the metabolite. What advice would you give the company about the following issues:

a. Is the drug likely to be toxic after oral dosage?
b. Would the oral and intravenous doses associated with toxicity be the same?
c. What are the likely sources of variability in kinetics in young physically healthy adults (20–30 years old)?
d. Would the kinetics be different in the elderly (70–80 years old)?
e. How much would a 50% decrease in liver or kidney function affect the kinetics, and would the toxicity be increased or decreased?
f. How much would a 50% increase in liver or kidney function affect the kinetics, and would the toxicity be increased or decreased?
g. Should the pharmaceutical group develop a slow-release formulation, and would this be likely to affect the toxicity?

5.3 The company you work for has developed a novel opioid for the treatment of intractable pain. The drug is 20 times more potent than morphine in relation to both analgesia and respiratory depression when given to rats by intravenous injection, and binding studies show that it has a high and similar affinity for μ-receptors of rats and humans. Initial kinetic studies in humans after a single intravenous bolus dose of 10 mg gave the following data:

Time after Dose (h)	Plasma Concentration (ng mL^{-1})
0.5	367
1.0	336
2	283
4	200
6	141
10	71
24	6.3

The area under the plasma concentration–time curve (AUC) extrapolated to infinity was 2310 ng mL^{-1} h. Urine was collected over a period of 2–4 h after dosing and contained a total of 1.85 mg of the parent drug and 0.1 mg of a hydroxy metabolite: the plasma concentration of parent drug at 3 h was 238 ng mL^{-1}.

After a single oral dose of 10 mg, the maximum plasma concentration occurred at 8 h and was only 48 ng mL^{-1}; the blood concentration reached 6 ng mL^{-1} by 36 h. The AUC to infinity was 1155 ng mL^{-1} h.

Plot the intravenous data on graph paper. Calculate appropriate pharmacokinetic parameters to describe the elimination rate, clearance, distribution, and absorption of the drug. Describe the probable overall fate of the drug in the body (e.g., routes of elimination).

Your research director needs the following advice:

a. What extra studies/data could support your description of the fate of the drug?
b. What route(s) of administration should the company use for its first trials of clinical effect for pain relief?
c. How should the drug be administered to provide relief of chronic pain?

Hints and Clues

Question 5.1

a. Calculate clearance (per kg body weight)—why is it different? (See b.)
b. Use urinary excretion data to think about pathways of elimination.
c. Use clearance and *terminal slope* (k or β—we don't know) to calculate the apparent volume of distribution.
d. Use AUC data to calculate bioavailability.
e. Are terminal rates different after oral dosage? What would it mean if they were?
f. What are the likely causes of differences between species? Could clearance and bioavailability be interrelated (if so how)?
g. Would scaling to body surface area affect the calculations and conclusions? If so, how?

Question 5.2

a. Use urinary excretion data to interpret the potential for exposure (or not) of the liver to the parent compound (obviously, the dose will affect the response—but is toxicity possible?).
b. Use urinary excretion data to calculate bioavailability. What processes are giving rise to the low bioavailability?
c. Variability in adults—what are the routes of elimination? What is the relevance of debrisoquine?
d. Consider 50% changes in liver in relation to bioavailability and clearance. Then, consider changes in renal function similarly. Will kidney function affect bioavailability?
e. A slow-release formulation is necessary when a drug has a very short half-life (e.g., 3–4 h or less). There is information on the rate of elimination in the question—what can you conclude about half-life? (*Clue*—could it be 24 h?)

Question 5.3

a. You can calculate clearance, apparent volume of distribution, and half-life from the intravenous data. But what route is important for elimination? (*Clue*—use urine data to calculate renal clearance and compare with plasma clearance.)
b. The extra studies should relate to kinetics. (*Clue*—what studies would we normally have before giving the first dose to humans?)
c. What is happening with the oral data? What is the extent of absorption? Why are blood levels at 36 h higher after oral dosage? (*Clue*—you can calculate the concentration at 36 h after iv dosage using the exponential terms derived from the iv data.)

d. Phase 1 studies (initial human studies) are usually by the oral route. Is this likely to produce analgesia or side effects with this compound?
e. Chronic pain relief requires the maintenance of constant concentrations of the analgesic. Which route would be likely to give this profile? If oral dosage could not give effective plasma levels without unacceptable side effects (such as constipation), how could you give the drug parenterally to provide similar constant concentrations?

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6 Physiologically Based Pharmacokinetic and Toxicokinetic Models

Harvey J. Clewell III, Rebecca A. Clewell, and Melvin E. Andersen

CONTENTS

INTRODUCTION

Physiologically based pharmacokinetic (PBPK) models provide sets of equations that simulate the time courses of chemicals and their metabolites in various tissues throughout the body.* The interest in PBPK modeling in toxicology and pharmacology arose from the need to relate internal concentrations of active compounds at their target sites with the doses of chemical given to an animal or human subject [1]. The reason, of course, is a fundamental tenet in pharmacology or toxicology that both beneficial and adverse responses to compounds are related to the concentrations of active chemicals reaching target tissues rather than the amounts of chemical at the site of absorption regimen [1–3]. The relationships between tissue dose and administered dose can be complex, especially in high-dose toxicity testing studies, with multiple, repeated daily dosing, or when metabolism or toxicity at routes of entry alters uptake processes for various routes of exposure. PBPK models of all kinds are primarily a tool to assess chemical dosimetry at target tissues for a wide range of exposure situations.

In PBPK modeling, compartments correspond to discrete tissues or to groupings of tissues with appropriate volumes, blood flows, and pathways for the metabolism of test chemicals [4]. These PBPK models include pertinent biochemical and physicochemical constants for metabolism and solubility in each compartment. Routes of dosing (routes of administration) are included in their proper relationship to the overall physiology. For instance, dermally absorbed compounds penetrate the skin, enter the mixed venous blood, and then travel through the heart and lungs to the arterial blood for distribution. Orally absorbed compounds move through intestinal tissues and portal blood to the liver before moving to the mixed venous blood for distribution to the remainder of the body. The equations that form the basis of the PBPK model also account for the time sequence of dose input into test subjects and permit input by multiple routes if necessary for specific exposure situations. Each compartment in the model is described with a mass balance differential equation whose terms mathematically represent biological processes. The set of equations is solved by numerical integration to simulate tissue time-course concentrations of chemicals and their metabolites.

Among the opportunities offered by PBPK approaches are (1) creating models from physiological, biochemical, and anatomical information, entirely separate from collection of detailed concentration time-course curves; (2) evaluating mechanisms by which biological processes govern disposition of a wide range of compounds by comparison of PK results with model predictions; (3) using chemicals as probes of the biological processes to gain more general information on the way chemical characteristics govern the importance of various transport pathways in the body; (4) applying the models in risk assessments for setting exposure standards; and (5) using annotation of a modeling database as a repository of information on the toxicity and kinetics of specific compounds.

Some PBPK models account for interactions of circulating compounds with specific receptors or the covalent interactions of chemicals with tissue constituents. Modeling these reversible and irreversible molecular interactions with cell constituents is the initial step in developing physiologically based pharmacodynamic (PBPD) models for effects of chemicals on biological processes [5]. Several excellent reviews are available that focus on the early stages of the development of PBPK modeling approaches [6–10], including a volume on PBPK modeling in chemical risk assessment [11], and a comprehensive review of the application of PBPK modeling in toxicology has been performed [12]. A number of recent publications provide additional insights into the application of PBPK modeling in risk assessment [13–18], drug development [19–24], and food/nutrition research [25]. Some of the aims of this chapter are to provide an overview of the fundamental concepts of PBPK modeling, the range of applications of PBPK modeling, and the insights that can be derived from the application of PBPK models to the distribution of chemicals in intact animals.

The advent of biologically structured PBPK models had a dramatic influence on the nature of the experiments conducted to determine PK behavior and to estimate tissue dosimetry. In PBPK descriptions, time-course behavior is not an intrinsic property of the organism accessible only by direct experimentation. Instead, it is a composite behavior, governed by more fundamental physiological and biochemical processes. More importantly, these fundamental processes

* Toxicokinetics (TK) is the quantitative study of factors that control the time course for absorption, distribution, metabolism, and excretion (ADME) of toxic compounds within the body. The time course of drugs, on the other hand, has traditionally been referred to as pharmacokinetics (PK). Similarly, the effect of a drug or toxic compound in the target tissue has been referred to as pharmacodynamics (PD) and toxicodynamics (TD), respectively. This practice, of course, ignores the wisdom of Paracelsus: "…only the dose differentiates a poison and a remedy." To avoid this false distinction, the terms *biokinetic* and *biodynamic* have sometimes been used.

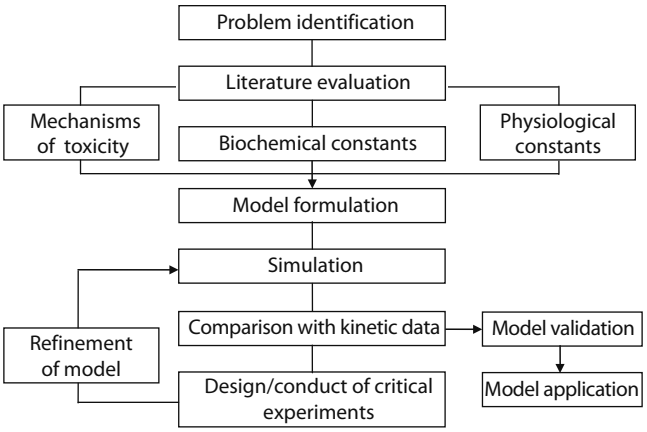


FIGURE 6.1 Schematic of the steps involved in the development of PBPK models.

can be studied in simpler systems to obtain the necessary PBPK model parameters in experiments separate from the collection of time-course concentration curves. Based on these parameters and an appropriate model structure, tissue time-course behaviors can be predicted by computer simulation with PBPK models and compared to data as a test of model performance.

The basic approach to PBPK model development is illustrated in Figure 6.1. The process of model development begins with the identification of the chemical exposure and toxic effect of concern, as well as the species and target tissue in which it is observed. Literature evaluation involves the integration of available information about the mechanism of toxicity; the pathways of chemical metabolism; the nature of the toxic chemical species (i.e., whether the parent chemical, a stable metabolite, or a reactive intermediate produced during metabolism is responsible for the toxicity); the processes involved in absorption, transport and excretion; the tissue partitioning and binding characteristics of the chemical and its metabolites; and the physiological parameters (i.e., tissue weights and blood flow rates) for the species of concern (i.e., the experimental species and the human). Using this information, the investigator develops a PBPK model that expresses mathematically a conception of the animal–chemical system [26]. To the extent that the structure of the model reflects the important determinants of the kinetics of the chemical, the result of this approach is a model that can predict the qualitative behavior of an experimental time course without having been based directly on it [27]. Refinement of the model to incorporate additional insights gained from comparison with experimental data yields a model that can be used for quantitative extrapolation well beyond the range of experimental conditions on which it was based. The model itself can then be used to help design critical experiments to collect data needed for its own refinement [28]. In particular, a properly validated PBPK model can be used to perform the high-to-low dose, dose route, and interspecies extrapolations necessary for estimating human risk on the basis of toxicology studies [29].

History of PBPK Modeling

Inhalation anesthesiologists have maintained a long tradition on understanding the role of ventilation rates, blood flow rates, and tissue solubility on the uptake and distribution of volatile anesthetics to the central nervous system. In the 1920s, Haggard [30,31] quantitatively described the importance of physiological factors for the uptake of ethyl ether into the body during the first few breaths. Accomplishing this analysis required writing an equation for the relationship between inhaled ether and the concentration of ether in blood. Tools for solving this equation over time were not available, so the mathematical analysis was limited to the first few breaths when venous concentrations remained small.

In the 1930s, Teorell [32,33] provided a set of equations for uptake, distribution, and elimination of drugs from the body. These papers are rightly regarded as providing the first physiological model for drug distribution. However, computational methods were not available to solve the sets of equations at this time. Exact mathematical solutions for distribution of compounds in the body could be obtained only for simplified models in which the body was reduced to a small number of compartments that did not correspond directly with specific physiological compartments. Over the next 30 years, PK modeling focused on these simpler descriptions with exact solutions rather than on developing models more concordant with the structure and content of the biological system itself. These approaches are sometimes referred to as *data-based* compartmental modeling since the work generally took the form of a detailed collection of time-course blood/excreta concentrations at various doses (Figure 6.1). Time-course curves were analyzed by assuming particular model structures and estimating a small number of model parameters by curve fitting. In the earliest of these models, all processes for metabolism, distribution, and elimination were treated as first order (i.e., they increased in direct proportion to the concentration of the chemical species). Two areas of concern that particularly affected data-based compartmental PK modeling arose in the 1960s and early 1970s: (1) the saturation of elimination pathways and (2) the possibility that blood flow rather than metabolic capacity of an organ might limit clearance. Saturation led to models that were not first order, making it difficult to derive exact solutions to the sets of equations. Blood flow–limited metabolism in an organ meant that the removal rate constant from a central compartment could not increase indefinitely as the metabolic capacity increased.

More complete PBPK models for inhalation were later provided by Kety [34], Mapleson [35], and Riggs [36]. In these models, body tissues were lumped together based on blood perfusion rates, giving sets of tissues referred to as richly perfused or poorly perfused. Mapleson [35] solved the set of equations using an analog computer to give solutions to the complete time course within the various tissue groups. These analog computer PBPK models for inhaled gases and vapors were extended by Fiserova-Bergerova and colleagues [37–39] to focus on compounds in the occupational environment and to describe the metabolism of these compounds in

the liver. The extension to include metabolism was particularly important for subsequent work in toxicology because most compounds of interest in occupational toxicology are metabolized, and metabolites are often involved in toxic responses.

Data-based compartmental models were brought to toxicology and risk assessment in a series of innovative studies by the late Dr. Perry Gehring (1938–2003) and his colleagues at the Dow Chemical Company in Midland, Michigan, in order to examine PK behavior where specific elimination pathways, both metabolic and excretory, become saturated at high doses [40–42]. In these studies, nonlinear data-based compartmental models were ingeniously applied to a series of compounds of toxicological and commercial importance including herbicides [43,44], solvents [45], plastic monomers [46,47], and hydrocarbons [48,49]. The final piece of technology needed to bring a full PBPK approach to studying factors that determine chemical disposition came with the rapid development of digital computation by the engineering community and the availability of these tools within the research laboratory.

Scientists trained in chemical engineering and computational methods developed PBPK models for chemotherapeutic compounds, that is, chemicals used in cancer therapy [4]. Many of these compounds are highly toxic and have therapeutic efficacy by being slightly more toxic to rapidly growing cells (the cancer cells) than to normal tissues. Initial successes with methotrexate [50] led to PBPK models for other compounds, including 5-fluorouracil [51] and cisplatin [52]. These seminal contributions showed the ease with which realistic descriptions of physiology and relevant pathways of metabolism could be incorporated into PBPK models for chemical disposition and paved the way for more extensive use of PBPK modeling in toxicology and chemical risk assessment. These models took advantage of the increasing availability of digital computation on main frame computers for solving sets of simultaneous differential equations.

Ramsey and Andersen [53] applied a PBPK modeling approach to describe the disposition of styrene in rats and humans for a range of concentrations and for several routes of administration. One of these two scientists (J. C. Ramsey) was a member of the PK group developing nonlinear PK models for chemicals at Dow Chemical Company and solving these models with a modern software package for solving sets of MB-DEs by numerical integration. The other (M. E. Andersen) had worked in inhalation toxicology laboratories at the Wright-Patterson Air Force Base, Ohio, and developed a steady-state analysis of PBPK models for inhalation of metabolized vapors [2]. This interinstitutional collaboration with styrene [53,54] relied on advances from inhalation anesthesia, data-based compartmental modeling, pharmaceutics, chemical engineering, and digital computation, to create PBPK models that would support extrapolation across species, between exposure routes, and from high to low doses. Using scale-up methods common for engineering models [55], the interspecies PBPK model for styrene (Figure 6.2) was able to predict blood and exhaled air time-course curves for oral and intravenous dosing in the rat and for inhalation exposures in human volunteers. This ability to support extrapolation to untested (and sometimes untestable) conditions is an essential part of risk assessment and has made these PBPK models attractive tools in human health risk assessments of various kinds [11,29]. In the styrene PBPK model, the liver was split off as a separate compartment (i.e., rather than embedded in a central compartment), metabolism in the liver was saturable (i.e., followed Michaelis–Menten kinetics), and styrene clearance from tissues was directly based on blood flow and metabolic characteristics of tissues. From that point in time, the application of PBPK modeling to environmental risk assessment has gained widespread acceptance in both the scientific and regulatory communities.

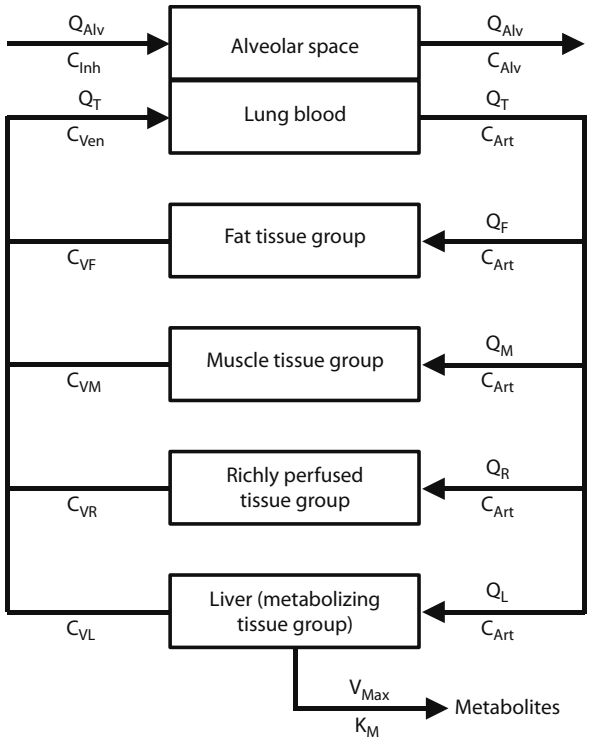


FIGURE 6.2 Diagram of a PBPK model for styrene. In this description, groups of tissues are defined with respect to their volumes, blood flows (Q), and partition coefficients for the chemical. The uptake of vapor is determined by the alveolar ventilation (QALV), the cardiac output (QT), the blood/air partition coefficient (PB), and the concentration gradient between arterial and venous pulmonary blood (C_{Art} and C_{Ven}). The dashed line (no dashed lines, author) reflects the fact that the lung compartment is described by a steady-state equation assuming that diffusion between the alveolar air and lung blood is fast compared to ventilation and perfusion. Metabolism is described in the liver with a saturable pathway defined by a maximum velocity (V_{max}) and affinity (K_M). The mathematical description assumes equilibration between arterial blood and alveolar air as well as between each of the tissues and the venous blood exiting from that tissue. (Adapted from Ramsey, J.C. and Andersen, M.E., *Toxicol. Appl. Pharmacol.*, 73, 159, 1984.)

PBPK Model Example: Styrene

The experience with styrene serves as a useful example of the advantages of the PBPK modeling approach. In this case, blood and tissue time-course curves of styrene had been obtained for rats exposed to four different concentrations of 80, 200, 600, and 1200 ppm [56]. Data were obtained during a 6-h exposure period and for 18 h after cessation of the exposure. The initial analysis of these data had been based on a simple compartmental model, which had a zero-order input related to the amount of styrene inhaled, a two-compartment description of the rat, and linear metabolism in the central compartment. The compartmental model was successful with lower concentrations but was unable to account for the more complex behavior at higher concentrations (note the different behavior of the data at the two concentrations shown in Figure 6.3).

In an attempt to provide a more successful description, a PBPK model was developed with a realistic equilibration process for pulmonary uptake and Michaelis–Menten saturable metabolism in the liver. A diagram of the PBPK model that was used by Ramsey and Andersen [53] to describe styrene inhalation in both rats and humans is shown in Figure 6.2. In this diagram, the boxes represent tissue compartments and the lines connecting them represent blood flows. The model contained several *lumped* tissue compartments: fat tissues, poorly perfused tissues (muscle, skin, etc.), richly perfused tissues (viscera), and metabolizing tissues (liver). The fat tissues were described separately from the other poorly perfused tissues due to their much higher partition coefficient for styrene, which leads to different kinetic properties, while the liver was described separately from the other richly perfused tissues due to its key role in the metabolism of styrene.

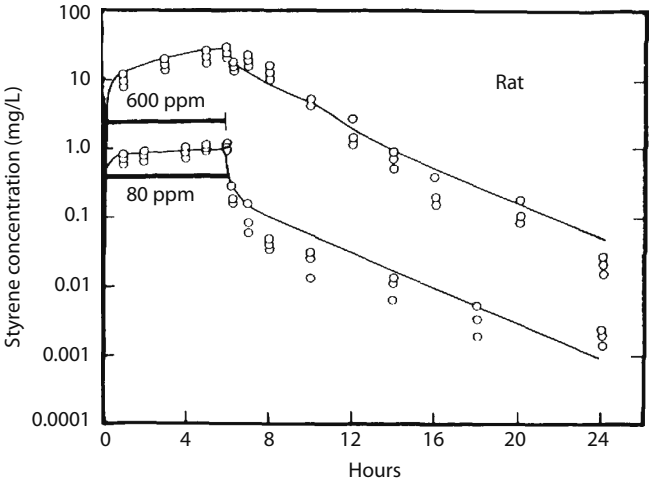


FIGURE 6.3 Model predictions (solid lines) and experimental blood styrene concentrations in rats during and after 6 h exposures to 80 and 600 ppm styrene. The thick bars represent the chamber air concentrations of styrene and are shown to highlight the nonlinearity of the relationship between administered and internal concentrations. The model contains sufficient biological realism to predict the very different behaviors observed at the two concentrations.

Each of these tissue groups was defined with respect to their blood flow, tissue volume, and their ability to store (partition) the chemical of interest.

The model equations represented by the diagram are described in the original publication [53]. For each compartment, the various time-dependent chemical transport and metabolic processes are described as a system of simultaneous differential equations. As an example, the differential equation defining the liver compartment in Figure 6.2 is shown as follows:

$$dA_L/dt = Q_L \times \left(C_{Art} - C_L/P_L\right) - V_{max} \times C_L/P_L/\left(K_M + C_L/P_L\right)$$

where

- A_L is the amount of chemical in the liver (mg)
- C_{Art} is the concentration of chemical in the arterial blood (mg/L)
- C_L is the concentration of chemical in the liver (mg/L)
- Q_L is the total (arterial plus portal) blood flow to the liver (L/h)
- P_L is the liver/blood partition coefficient
- V_{max} is the maximum rate of metabolism (mg/h)
- K_M is the concentration at half-maximum rate of metabolism (mg/L)

Although the model diagram in Figure 6.3 shows a lung compartment, a steady-state approximation for the equilibration of lung blood with alveolar air was used in the mathematical formulation of the model to eliminate the need for an actual lung tissue compartment. This simple model structure, with realistic constants for the physiological, partitioning, and metabolic parameters, very accurately predicted the behavior of styrene in both fat and blood of the rat at all concentrations. Figure 6.3 compares the model-predicted time course in the blood with the experimental data for the highest and lowest exposure concentrations in the rat studies.

The structure of the PBPK model for styrene reflects the generic mammalian architecture. Organs are arranged in a parallel system of blood flows with total blood flow through the lungs. This model can easily be scaled up to examine styrene kinetics for other mammalian species. In the case of styrene, exposure experiments had also been conducted with human volunteers [49]. In order to model these data, the PBPK model parameters were changed to human physiological values, the human blood/air partitioning was determined from human blood samples, and the metabolism was scaled allometrically so that capacity (V_{max}) was related to basal metabolic rate (BW raised to the 0.7 power) and affinity (K_m) was the same in the human as in the rat, 0.36 mg/L. Ramsey and coauthors [49] measured both venous blood and exhaled air concentrations in these human volunteers. Although the rat PBPK model was developed for blood and fat, not for exhaled air, the physiologically based description automatically provides information on expected exhaled air concentrations. It was straightforward then to predict expected exhaled air concentrations in humans and compare

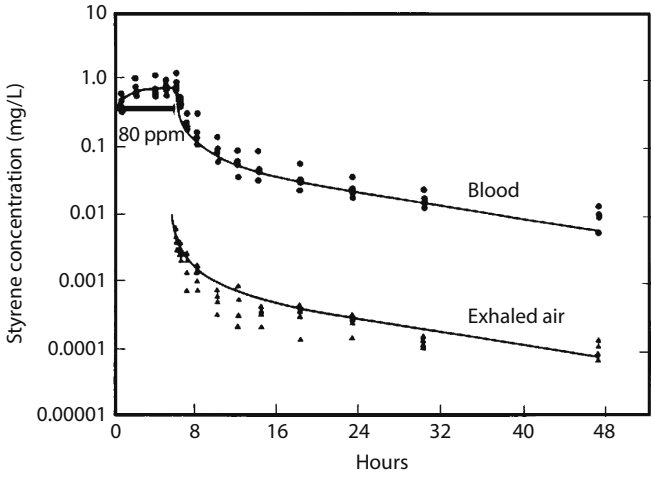


FIGURE 6.4 Model predictions and experimental blood and exhaled air concentrations in human volunteers during and after 6 h exposures to 80 ppm styrene. The model is identical to that used for rats. The model parameters have been changed to values appropriate for humans on the basis of physiological and biochemical information and have not been adjusted to improve the fit to the experimental data.

the predictions with the concentrations measured during the experiments (Figure 6.4).

A similar comparison of the model's predictions with another human data set from Stewart et al. [57] also demonstrated the ability of the PBPK structure to support extrapolation of styrene kinetics from the rat to the human (Figure 6.5).

The specific structure of a particular model is driven by the need to estimate the appropriate measure of tissue dose under the various exposure conditions of concern in both the experimental animal and the human. Before the model can be used in risk assessment, it has to be validated against kinetic, metabolic, and toxicity data and, in many cases, refined based on comparison with the experimental results. Importantly, the model itself can frequently be used to help design critical experiments to collect data needed for its own validation. Perhaps the most desirable feature of a PBPK model is that it provides a conceptual framework for employing the scientific method: Hypotheses can be described in terms of biological processes, quantitative predictions can be made on the basis of the mathematical description, and the model (hypothesis) can be revised on the basis of comparison with targeted experimental data. Refinement of the model to incorporate additional insights gained from comparison with experimental data yields a model that can be used for quantitative extrapolation well beyond the range of experimental conditions on which it was based.

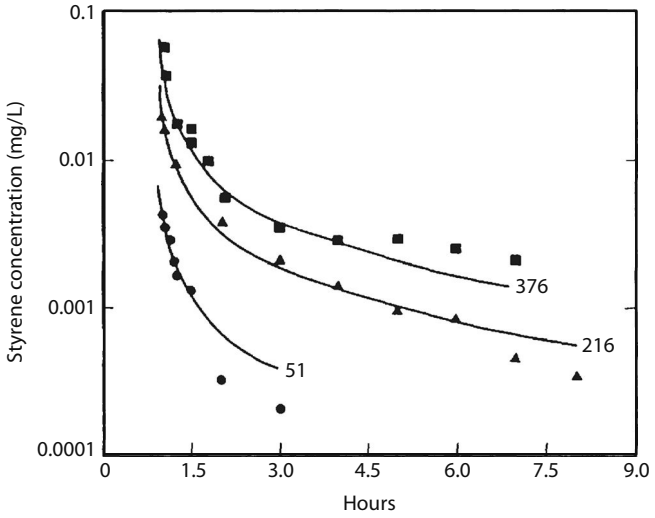


FIGURE 6.5 Model predictions and experimental exhaled air concentrations in human volunteers following 1 h exposures to 51, 216, and 376 ppm styrene.

Modeling Philosophy

This basic PBPK model for styrene has several tissue groups that were lumped according to their perfusion and partitioning characteristics. In the mathematical formulation, each of these several compartments is described by a single mass balance differential equation. It would be possible to describe individual tissues in each of the lumped compartments, if necessary. This detail is usually unnecessary unless some particular tissue in a lumped compartment is the target tissue. One might, for example, want to separate brain from other richly perfused tissues if the model were for a chemical that had a toxic effect on the central nervous system [58–60]. Other examples of additional compartments include the addition of placental and mammary compartments to model pregnancy and lactation [61–63]. The interactions of chemical mixtures can even be described by including compartments for more than one chemical in the model [64–66]. Increasing the number of compartments does increase the number of differential equations required to define the model. However, the number of equations does not pose any problem due to the power of modern desktop computers.

On the other hand, as the number of compartments in the PBPK model increases, the number of input parameters increases correspondingly. Each of these parameters must be estimated from experimental data of some kind. Fortunately, the values of many of these can be set within narrow limits from nonkinetic experiments. The PBPK model can also help to define those experiments that are needed to improve parameter estimates by identifying conditions where the sensitivity of the model to the parameter is the greatest [67]. The demand that the PBPK fit a variety of data also restricts the parameter values that will give a satisfactory fit to experimental data. For example, the styrene model (described earlier) was required to reproduce both the high and low concentration behaviors, which appeared qualitatively different, using the same parameter values. If one were independently fitting single curves with a model, the different parameter values obtained under different conditions would be relatively uninformative for extrapolation.

As the renowned statistician George Box has said, "All models are wrong but some are useful [68]." Even a relatively complex description such as a PBPK model will sometimes

fail to fit reliable experimental data. When this occurs, the investigator needs to think how the model might be changed, that is, what extra biological aspects must be added to the physiological description to bring the predictions in line with experimental observation. In the case of the work with styrene cited earlier, continuous 24-h styrene exposures could not be modeled with a time-independent maximum rate of metabolism, and induction of enzyme activity had to be included to yield a satisfactory representation of the observed kinetic behavior [54].

When a PBPK model is unable to adequately describe kinetic data, the nature of the discrepancy can provide the investigator with additional insight into time dependencies in the system. This insight can then be utilized to reformulate the biological basis of the model and improve its fidelity to the data. The resulting model may be more complicated, but it will still be useful if the pertinent kinetic constants can be estimated for human tissues. Indeed, as long as the model maintains its biological basis, the additional parameters can often be determined directly from separate experiment, rather than estimated by fitting the model to kinetic data. As the models become more complex, they necessarily contain larger numbers of physiological, biochemical, and biological constants. The crucial task during model development is to keep the description as simple as possible and to ensure the identifiability of new parameters that are added to the model; every attempt should be made to obtain or verify model parameters from experimental studies separate from the modeling exercises themselves [69].

MODEL DEVELOPMENT

The following section explores some of the key issues associated with the development of PBPK models. It is meant to provide a general understanding of the basic design concepts and mathematical forms underlying the PBPK modeling process and is not meant to be a complete exposition of the PBPK modeling approach for all possible cases. It must be understood that the specifics of the approach can vary greatly for different types of compounds and for different applications.

Model building is an art and is best understood as an iterative process in the spirit of the scientific method [26,70,71]. The literature includes many examples of successful PBPK models for a wide variety of compounds that provide a wealth of insight into various aspects of the PBPK modeling process [12]. These should be consulted for further detail on the approach for applying the PBPK methodology in specific cases.

Tissue Grouping

The first aspect of PBPK model development that will be discussed is determining the extent to which the various tissues in the body may be grouped together. Although tissue grouping is really just one aspect of model design, which is discussed in the next section, it provides a simple context for introducing the two alternative approaches to PBPK model development: *lumping* and *splitting* (Figure 6.6). In the context of tissue grouping, the guiding philosophy in the lumping approach can be stated as "Tissues that are pharmacokinetically and toxicologically indistinguishable may be grouped together." In this approach, model development begins with information at the greatest level of detail that is practical, and decisions are made to combine physiological elements (tissues and blood flows) to the extent justified by their similarity [72]. The common grouping of tissues into richly (or rapidly) perfused and poorly (or slowly) perfused on the basis of their perfusion ratio (ratio of blood flow to tissue volume) is an example of the lumping approach. The contrasting philosophy of splitting is "Tissues that are pharmacokinetically or toxicologically distinct must be separated." This approach starts with the simplest reasonable model structure and increases the model's complexity only to the extent required to reproduce data on the compound of concern for the application of interest. Lumping (starting with a large number of compartments and then testing whether they can be combined) requires the greater initial investment in data collection and, if taken to the extreme, could paralyze model development. On the other hand, splitting (starting with a small number of compartments and increasing complexity only if the simple model fails) is more efficient but runs a greater risk of overlooking compound-specific determinants of disposition.

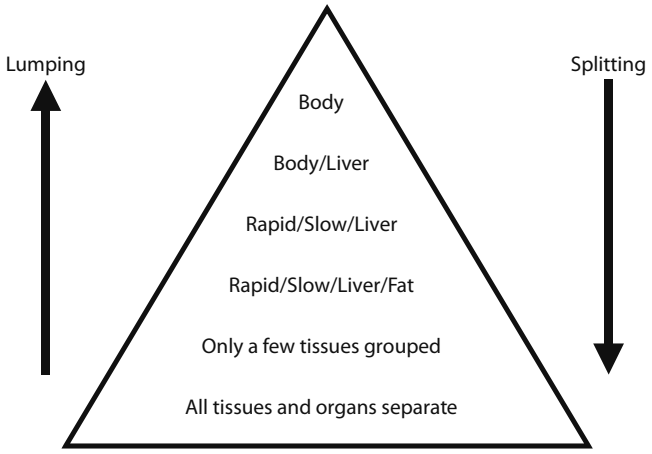


FIGURE 6.6 The role of lumping and splitting processes in PBPK model development.

There are two alternatives for determining whether tissues are kinetically distinct or can be lumped together. In the first approach, the tissue rate constants are compared. The rate constant (k_t) for a tissue is similar to the perfusion ratio except that the partitioning characteristics of the tissue are also considered:

$$k_T = \frac{Q_T}{\left(P_T * V_T\right)}$$

where

Q_T is the blood flow to the tissue (L/h)

P_T is the tissue/blood partition coefficient for the compound

V_T is the volume of the tissue (L)

Thus, the units of the tissue rate constant are the same as for the perfusion ratio, h^{-1}, but the rate constant more accurately reflects the kinetic characteristics of a tissue for a particular chemical. It was the much smaller rate constant for fat in the case of a lipophilic chemical such as styrene that required the separation of the fat compartment from the other poorly perfused tissues (muscle, skin, etc.) in its PBPK model [53]. The tissue time constant, 1/k, provides a measure of the time required for loading or unloading of compound under idealized conditions.

The second, less rigorous, approach for determining whether tissues should be lumped together is simply to compare the performance of the model with the tissues combined and separated. The reliability of this approach depends on the availability of data under conditions where the tissues being evaluated would be expected to have an observable impact on the kinetics of the compound. Sensitivity analysis can sometimes be used to determine the appropriate conditions for such a comparison [67].

Model Design Principles

There is no easy rule for determining the structure and level of complexity needed in a particular modeling application. The wide variability of PBPK model design for different chemicals can be seen by comparing the diagram of the PBPK model for methotrexate [50], shown in Figure 6.7, with the diagram for the styrene PBPK model shown in Figure 6.3. Model elements that are important for a volatile lipophilic chemical such as styrene (lung, fat) do not need to be considered in the case of a nonvolatile water-soluble compound such as methotrexate. Similarly, while kidney excretion and enterohepatic recirculation are important determinants of the kinetics of methotrexate, only metabolism and exhalation are significant for styrene. The decision of which elements to include in the model structure for a specific chemical and application draws on all of the modeler's experience and knowledge of the animal–chemical system.

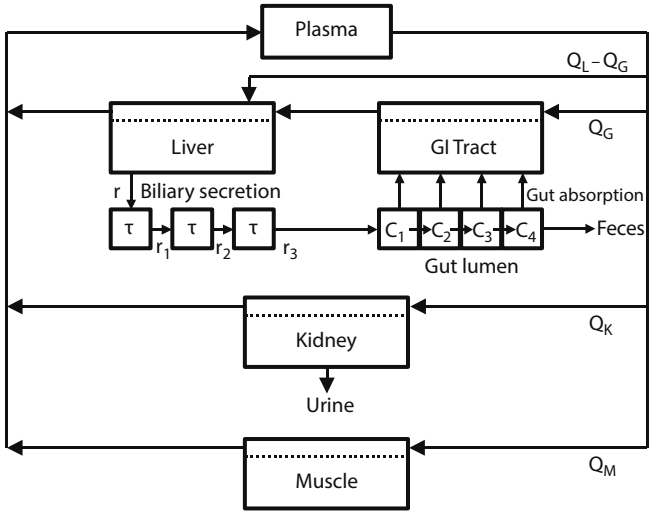


FIGURE 6.7 PBPK model for methotrexate. (Adapted from Bischoff, K.B. et al., *J. Pharm. Sci.*, 60, 1128, 1971.)

The alternative approaches to tissue grouping discussed earlier are actually reflections of two competing criteria that must be balanced during model design: parsimony and plausibility. The principle of parsimony simply states that a model should be as simple as possible for the intended application (but no simpler). This *splitting* philosophy is related to that of Occam's razor: "Entities should not be multiplied unnecessarily." That is, structures and parameters should not be included in the model unless they are needed to support the application for which the model is being designed.

There is no easy rule for determining the structure and the level of complexity needed in a particular modeling application. The decision regarding which elements to include in the model structure for a specific compound and application draws on all of the modeler's experience and knowledge of the animal–chemical system. For example, model elements such as inhalation and fat storage, which are important for a volatile lipophilic chemical such as styrene [53], do not need to be considered in the case of a nonvolatile water-soluble compound such as methotrexate [50,73]. Similarly, while kidney excretion and enterohepatic recirculation are important determinants of the kinetics of methotrexate (Figure 6.7), they are not needed in the model of styrene. As another example, a simple description of inhalation uptake as a one-compartment gas exchange (Figure 6.3) may be adequate for some model applications, as in the case of modeling of the systemic uptake of a lipophilic vapor like styrene. However, a more complicated description is required in the case of water-soluble vapors, to account for a *wash-in, wash-out* effect in the upper respiratory tract [74,75].

The desire for parsimony in model development is driven not only by the desire to minimize the number of parameters whose values must be identified, but also by the recognition that as the number of parameters increases, the potential for unintended interactions between parameters also increases. A generally accepted rule of software engineering warns that it is relatively easy to design a computer program that is too complicated to be completely comprehended by the developer. As a model becomes more complex, it becomes increasingly difficult to validate, even as the level of concern for the trustworthiness of the model should increase.

Countering the desire for model parsimony is the need for plausibility of the model structure. The credibility of a PBPK model's predictions of kinetic behavior under conditions different from those under which the model was validated rests to a large extent on the correspondence of the model design to known physiological and biochemical structures, and an accurate description of the mode of action for the effects of the chemical [1,76,77]. In general, the ability of a model to adequately simulate the behavior of a physical system depends on the extent to which the model structure is homomorphic (having a one-to-one correspondence) with the essential features determining the behavior of that system [26].

Model Identification

The process of determining the most appropriate structure for the model, which is also known as model identification, begins with the selection of those model elements that the modeler considers to be minimum essential determinants of the behavior of the particular animal–chemical system under study, from the viewpoint of the intended application of the model. Comparison with appropriate data, relevant to the intended purpose of the model, then can provide insights into defects in the model that must be corrected either by reparameterization or by changes to the model structure. Unfortunately, it is not always possible to separate these two elements. In models of biological systems, estimates of the values of model parameters will always be uncertain, due to both biological variation and experimental error. At the same time, the need for biological realism unavoidably results in models that are overparameterized, that is, they contain more parameters than can be identified from the kinetic data the model is used to describe.

As an example of the interaction between model structure and parameter identification, the two metabolic parameters, V_{max} and K_m, in the model for styrene discussed earlier could both be identified relatively unambiguously in the case of the rat. Indeed, as pointed out previously, the inclusion of capacity-limited metabolism in the model was necessary in order to reproduce the available data at both low and high exposure concentrations. In the case of the human, however, data were not available at sufficiently high concentrations to saturate metabolism. Therefore, only the ratio, V_{max}/K_m, would actually be identifiable. The use of the same model structure, including a two-parameter description of metabolism, in the human as in the rat was justified by the knowledge that similar enzymatic systems are responsible for the metabolism of chemicals such as styrene in both species. However, if the model were to be used to extrapolate to higher concentrations in the human, the potential impact of the uncertainty in the values of the individual metabolic parameters would have to be carefully considered.

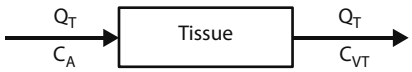
Model identification is the selection of a specific model structure from several alternatives, based on the conformity of the model's predictions to experimental observations. The practical reality of model identification in the case of biological systems is that regardless of the complexity of the model, there will always be some level of *model error* (lack of homomorphism), which will result in systematic discrepancies between the model and the experimental data. This model structural deficiency interacts with deficiencies in the identifiability of the model parameters, potentially leading to misidentification of the parameters or misspecification of structures. This most dangerous aspect of model identification is exacerbated by the fact that, in general, adding equations and parameters to a model increases the model's degrees of freedom, improving its ability to reproduce data, regardless of the validity of the underlying structure. Therefore, when a particular model structure improves the agreement of the model with kinetic data, it can be said that the model structure is only *consistent* with the kinetic data; it cannot be said that the model structure has been *proved* by its consistency with the data. In such circumstances, it is imperative that the physiological or biochemical hypothesis underlying the model structure is tested using nonkinetic data.

Elements of Model Structure

The process of selecting a model structure can be broken down into a number of elements associated with the different aspects of uptake, distribution, metabolism, and elimination. In addition, there are several general model structure issues that must be addressed, including mass balance and allometric scaling. The following section treats each of these elements in turn.

Storage Compartments

Naturally, any tissues that are expected to accumulate significant quantities of the compound or its metabolites need to be included in the model structure. As discussed earlier, these storage tissues can be grouped together to the extent that they have similar time constants. The muscle tissue in the methotrexate model (Figure 6.7) is an example of a storage compartment. The generic mass balance equation for storage compartments such as these is



$$\frac{dA_T}{dt} = Q_T * C_A - Q_T * C_{VT}$$

where

A_T is the mass of compound in the tissue (mg)
C_A is the concentration of compound in the arterial blood reaching the tissue (mg/L)
C_{VT} is the concentration of the compound in the venous blood leaving the tissue (mg/L)

Thus, this mass balance equation simply states that the rate of change in the amount of compound in the tissue with respect to time (dA_T/dt) is equal to the difference between the rate at which the compound enters the tissue and the rate at which the compound leaves the tissue. We can then calculate the concentration of the compound in the storage tissue (C_T) from the amount in the tissue and the tissue volume (V_T):

$$C_T = \frac{A_T}{V_T}$$

In PBPK models, it is common to assume *venous equilibration*, that is, in the time that it takes for the blood to perfuse the tissue, the compound is able to achieve its equilibrium distribution between the tissue and blood. Therefore, the concentration of the compound in the venous blood can

be related to the concentration in the tissue by the equilibrium tissue/blood partition coefficient, P_T:

$$C_{VT} = \frac{C_T}{P_T}$$

Therefore, we obtain a differential equation in A_T:

$$dA_T/dt = Q_T{*}C_A - Q_T{*}A_T/(P_T{*}V_T)$$

If desired, we can reformulate this mass balance equation in terms of concentration:

$$dA_T/dt = d(C_T{*}V_T)/dt = C_T{*}dV_T/dt + V_T{*}dC_T/dt$$

If (and only if) V_T is a constant (i.e., the tissue does not grow during the simulation), $dV_T/dt = 0$, and

$$dA_T/dt = V_T{*}dC_T/dt$$

so we have the alternative differential equation:

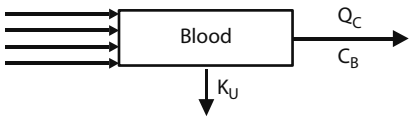
$$dC_T/dt = Q_T{*}(C_A - C_T/P_T)/V_T$$

This alternative mass balance formulation, in terms of concentration rather than amount, is popular in the pharmacokinetic literature. However, in the case of models with compartments that change volume over time (e.g., in a model incorporating growth of a single or multiple tissues), it is preferable to use the formulation in terms of amounts in order to avoid the need for the additional term reflecting the change in volume ($C_T{*}dV_T/dt$).

Depending on the compound, many different tissues can potentially serve as important storage compartments. The use of a fat storage compartment is typically required for lipophilic compounds. The gut lumen can also serve as a storage site for compounds subject to enterohepatic recirculation, as in the case of methotrexate. Important storage sites for metals, on the other hand, can include the kidney, red blood cells, intestinal epithelial cells, skin, bone, and hair [78]. Transport to and from a storage compartment does not always occur via the blood, as was described earlier, for example, in some cases, the storage is an intermediate step in an excretion process (e.g., hair, intestinal epithelial cells). As with methotrexate, it may also be necessary to use multiple compartments in series, or other mathematical devices, to model plug flow (i.e., a time delay between entry and exit from storage).

Blood Compartment

The description of the blood compartment can vary considerably from one PBPK model to another depending on the role the blood plays in the kinetics of the compound being modeled. In some cases, the blood may be treated as a simple storage compartment, with a mass balance equation describing the summation (Σ) of the venous blood flows from the various tissues and the return of the total arterial blood flow (Q_C) to the tissues, as well as any urinary clearance (if, as in the case of glomerular filtration, clearance is described as occurring from the blood compartment):



$$\frac{dA_B}{dt} = \Sigma\left(Q_T * C_{VT}\right) - Q_C * C_B - K_U * C_B$$

where

A_B is the amount of compound in the blood (mg)
Q_C is the total cardiac output (L/h)
C_B is the concentration of compound in the blood (mg/L)
K_U is the urinary clearance (L/h)

The value for K_U can often be estimated from the unbound fraction in plasma (f_{ub}) and the glomerular filtration rate (GFR), unless active transport processes contribute to renal elimination. An alternative method for estimating human renal clearance based on rat renal clearance and incorporating species-specific physiological differences in GFR has been proposed [79,80].

Usually, concentrations are measured in plasma or serum and not in whole blood; hence, plasma or serum is the reference fluid for the derived pharmacokinetic parameters such as clearances and volumes of distribution. However, whole blood, not plasma or serum, is flowing through the vessels of the human body. Therefore, provided that there is evidence in support of fast equilibration of the compound between red blood cells and plasma [81], whole blood rather than plasma is the more appropriate reference fluid for calculating and interpreting clearances and volumes of distribution. For this reason, parameterization of the PBPK model is often performed in terms of blood flows. To compare calculated plasma concentrations to experimental data, the calculated blood concentration must then be divided by the blood-to-plasma ratio (BPR), which is often measured experimentally.

In cases where a compound is not taken up by the red blood cells, plasma flow can be used in place of blood flow in the model [50]. For some compounds, where exchange between plasma and red blood cells is slow compared to tissue perfusion, it may be necessary to model the red blood cells as a storage compartment in communication with the plasma via diffusion-limited transport. Typically, however, exchange between red blood cells and plasma is fast compared to tissue distribution, and the blood can be treated as a single compartment.

For some chemicals, such as methotrexate, all of the chemical is present in the plasma rather than the red blood cells, so plasma flows and volumes are used instead of blood. For other chemicals, it may be necessary to model the red blood cells as a storage compartment in communication with the plasma via diffusion-limited transport. If the blood is

an important storage compartment for a compound, it may be necessary to carefully evaluate data on tissue concentrations, particularly the richly perfused tissues, to determine whether compound in the blood perfusing the tissue could be contributing to the measured tissue concentration. For other compounds, such as styrene, the amount of compound actually in the blood may be relatively small, in which case only the concentration may be of interest. In this case, instead of having a true blood compartment, a steady-state approximation can be used to estimate the concentration in the blood at any time. Assuming the blood is at steady state with respect to the tissues,

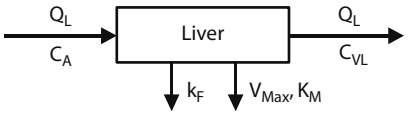
$$\frac{dA_B}{dt} = 0$$

Therefore, solving the blood equation for the concentration,

$$C_B = \frac{\Sigma\left(Q_T * C_{VT}\right)}{Q_C}$$

Metabolism/Elimination

The liver is frequently the primary site of metabolism, though other tissues such as the kidney, placenta, lung, skin, and blood may be important metabolism sites depending on the chemical. The following equation is an example of the mass balance equation for the liver in the case of a compound that is metabolized through both saturable and nonsaturable components:



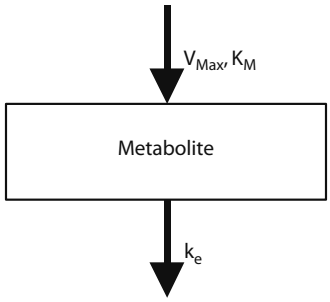
$$dA_L/dt = Q_L*(C_A - C_{VL}) - k_F* C_{Lfree}* V_L - V_{max}*C_{Lfree}/(K_m + C_{Lfree})$$

where

Q_L is the total blood flow (arterial and portal) to the liver
C_{Lfree} is the free (unbound) concentration in the liver

In the equation given earlier, the first term on the right-hand side of the equation represents the mass flux associated with transport in the blood and is essentially identical to the case of the storage compartment described previously. In the case of the liver, however, it should be noted that there are two sources of blood flow: the arterial flow directly to the liver and the portal flow that first perfuses the intestines. When appropriate, these two blood flows can be differentiated in the model (Figure 6.7).

The second term in the equation given earlier describes metabolism by a linear (first-order) pathway with rate constant k_F (h^{-1}), and the third term represents metabolism by a saturable (Michaelis–Menten) pathway with capacity V_{max} (mg/h) and affinity K_m (mg/L). If it were desired to model a water-soluble metabolite produced by this saturable pathway, an equation for its formation and elimination could also be added to the model:



$$dA_M/dt = R_{stoch} * V_{max} * C_{Lfree}/(K_m + C_{LFree}) - k_e * A_M$$

$$C_M = \frac{A_M}{V_D}$$

where

A_M is the amount of metabolite in the body (mg)
R_{stoch} is the stoichiometric fractional yield of the metabolite (unitless)
(times the ratio of its molecular weight to that of the parent compound if the modeling is in terms of weight rather than moles)
k_e is the rate constant for the clearance of the metabolite from the body (h^{-1})
C_M is the concentration of the metabolite in the plasma (mg/L)
V_D is the apparent volume of distribution for the metabolite (L)

The definition of free concentration in the liver (C_{Lfree}) in these equations is not as straightforward as it may at first appear. In cases where hepatic clearance is relatively low, the free concentration in the liver is often assumed to be equal to the free (unbound) concentration in the blood, that is, $C_{Lfree} = f_u * C_B$. However, when hepatic clearance is high, the free concentration in the liver can be drawn well below the free concentration in the blood, and such an assumption may be inappropriate. An alternative assumption that is often made is that the free concentration in the liver can be estimated by dividing the total liver concentration by the liver/blood partition coefficient, that is, $C_{Lfree} = C_L/P_L$ [50]. This approximation is particularly useful for compounds whose metabolism is limited by hepatic blood flow at low concentrations. If the intent is to use in vitro estimates of the metabolic parameters (V_{max}, K_m, k_f) in the model, then the definition of the in vivo free concentration should be consistent with the conditions under which the in vitro estimates of intrinsic clearance were obtained. In principle, this could require adjusting for differences in binding between the in vitro medium and the tissue in vivo, although such adjustments are seldom performed in practice.

Clearance may occur through urinary or fecal excretion, exhaled air, or even through loss via hair. This loss may often be successfully described using first-order clearance terms. However, more elaborate descriptions are sometimes

required for chemicals that are substrates for transporters that transfer the chemical against a concentration gradient. Some transporters in the kidney and bile can increase the clearance of xenobiotics, while others, such as those responsible for reabsorption, may decrease clearance [82].

Metabolite Compartments

In principle, the same considerations that drive decisions regarding the level of complexity of the PBPK model for the parent chemical must also be applied for each of its metabolites, and their metabolites, and so on. As in the case of the parent chemical, the first and most important consideration is the purpose of the model. If the concern is direct parent chemical toxicity and the chemical is detoxified by metabolism, then there is no need for a description of metabolism beyond its role in the clearance of the parent chemical. The models for styrene and methotrexate discussed earlier are examples of parent chemical models. Similarly, if reactive intermediates produced during the metabolism of a chemical are responsible for its toxicity, as in the case of methylene chloride, a very simple description of the metabolic pathways might be adequate [64]. The cancer risk assessment model for methylene chloride described the rate of metabolism for two pathways: the glutathione conjugation pathway, which was considered responsible for the carcinogenic effects, and the competing P450 oxidation pathway, which was considered protective.

If one or more of the metabolites are considered to contribute to the toxicity of a compound, it may also be necessary to provide a more complete description of the kinetics of the metabolites themselves [83–85]. Fortunately, the metabolism of xenobiotic compounds often produces metabolites that are relatively water soluble, simplifying the description needed. In many cases, a classical one-compartment description may be adequate for describing the metabolite kinetics [86,87]. In other cases, however, the description of the metabolite (or metabolites) may have to be as complex as that of the parent compound. For example, in the case of teratogenicity from all-trans-retinoic acid, both the parent compound and several of its metabolites are considered to be toxicologically active; therefore, in developing the PBPK model for this compound, it was necessary to include a fairly complete description of the metabolic pathways [85]. On the other hand, if reactive intermediates produced during the metabolism of a compound are responsible for toxicity, a very simple description of the metabolic pathways might be adequate [64].

Target Tissues

Typically, a PBPK model used in risk assessment applications will include compartments for any target tissues for the toxicity of the compound. The target tissue description may in some cases need to be fairly complicated, including such features as in situ metabolism, binding, and pharmacodynamic processes in order to provide a realistic measure of biologically effective tissue exposure. For example, whereas the lung compartment in the styrene model was represented only by a steady-state description of alveolar vapor exchange, the PBPK model for methylene chloride that was applied to perform a cancer risk assessment [64] included a two-part lung description in which alveolar vapor exchange was followed by a lung tissue compartment with in situ metabolism. This more complex lung compartment was required to describe the dose–response for methylene chloride–induced lung cancer, which was assumed to result from the metabolism of methylene chloride in lung clara cells.

In other cases, describing a separate compartment for the target tissue may be unnecessary. For example, the styrene model described earlier could be used to relate acute exposures associated with neurological effects without the necessity of separating out a brain compartment. Instead, the concentration or area under the concentration curve (AUC) of styrene in the blood could be used as a metric, on the assumption that the relationship between brain concentration and blood concentration would be the same under all exposure conditions, routes, and species, namely, that the concentrations would be related by the brain/blood partition coefficient. In fact, this is probably a reasonable assumption across different exposure conditions in a given species. However, while tissue/air partition coefficients for volatile lipophilic chemicals appear to be similar in dog, monkey, and man [88], human blood/air partition coefficients appear to be roughly half of those in rodents [89]. Therefore, the human brain/blood partition would probably be about twice that in the rodent. Nevertheless, if the model were to be used for extrapolation from rodents to humans, this difference could easily be factored into the analysis as an adjustment to the blood metric, without the need to actually add a brain compartment to the model.

A fundamental issue in determining the nature of the target tissue description required is the need to identify the toxicologically active form of the chemical. In some cases, a chemical may produce a toxic effect directly, either through its reaction with tissue constituents (e.g., ethylene oxide) or through its binding to cellular control elements (e.g., dioxin). Often, however, it is the metabolism of the chemical that leads to its toxicity. In this case, toxicity may result primarily from reactive intermediates produced during the process of metabolism (e.g., chlorovinyl epoxide produced from the metabolism of vinyl chloride) or from the toxic effects of stable metabolites (e.g., trichloroacetic acid produced from the metabolism of trichloroethylene).

The specific nature of the relationship between tissue exposure and response depends on the mechanism, or mode of action, involved. Rapidly reversible effects may result primarily from the current concentration of the compound in the tissue, while longer-term effects may depend on both the concentration and the duration of the exposure. In general, the appropriate measure of tissue exposure for one toxic effect of a compound may be different from the appropriate measure for another of its effects. For example, the mitogenic effect of a compound may depend on the prolonged maintenance of a relatively high concentration sufficient to occupy a receptor in the target tissue, while cytotoxicity may result from transient high rates of metabolism occurring shortly after dosing. In such a case, PBPK modeling of the concentration

time course in the target tissue for different dosing routes or regimens might be necessary. For developmental toxicity, the concentration time course might also have to be convoluted with the window of susceptibility for a particular gestational event. The evaluation of the various modes of action for the beneficial and toxic effects of a compound is the most important step in a pharmacokinetic analysis and a principal determinant of the structure and level of detail that will be required in the PBPK model.

Uptake Routes

Each of the relevant uptake routes for the compound must be described in the model. Often, there are a number of possible ways to describe a particular uptake process, ranging from simple to complex. As with all other aspects of model design, the competing goals of parsimony and realism must be balanced in the selection of the level of complexity to be used. The following examples are meant to provide an idea of the variety of model code that can be required to describe the various possible uptake processes.

Intravenous Administration (Bolus Dosing)

$$A_{B0} = \text{Dose} * BW$$

where

A_{B0} is the amount of compound in the blood at the time of dosing (t = 0)
Dose is administered dose (mg/kg)
BW is body weight (kg)

Intravenous Administration (Infusion)

For example, in the case where a steady-state approximation has been used to eliminate the blood compartment,

$$C_B = \left(Q_L * C_{VL} + \cdots + Q_F * C_{VF} + k_{IV}\right)$$

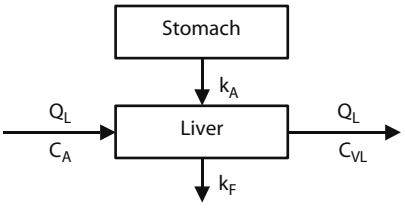
where

$k_{IV} = \text{Dose} * BW/t_{IV}$ $(t < t_{IV})$
$= 0$ $(t > t_{IV})$
t_{IV} is the duration of time over which the infusion takes place (h)

In this case, the model code must be written with a *switch* to change the value of k_{IV} to zero at $t = t_{IV}$.

Oral Gavage

For a compound that is completely absorbed in the stomach,



$$A_{ST0} = \text{Dose} * BW$$

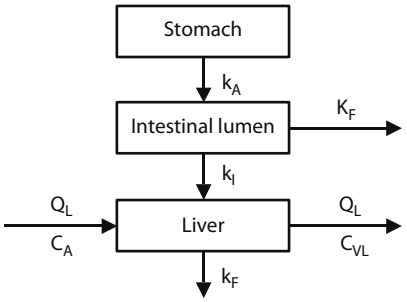
$$dA_{ST}/dt = -k_A * A_{ST}$$

$$dA_L/dt = Q_L*(C_A - C_L/P_L) - k_F * C_L * V_L/P_L + k_A * A_{ST}$$

where

A_{ST0} is the amount of compound in the stomach at the beginning of the simulation
A_{ST} is the amount of compound in the stomach at any given time
k_A is a first-order rate constant (h^{-1}) describing uptake from the stomach

For a compound that is incompletely absorbed,



$$A_{ST0} = \text{Dose} * BW$$

$$dA_{ST}/dt = -k_S * A_{ST}$$

$$dA_I/dt = k_S * A_{st} - k_I * A_I - K_F * A_I/V_I$$

$$dA_L/dt = Q_L * (C_A - C_{VL}) - k_F * C_{Lfree} * V_L + k_I * A_I$$

where

A_I is the amount of compound in the intestinal lumen (mg)
k_I is the rate constant for intestinal absorption (h^{-1})
K_F is the fecal clearance (L/h)
V_I is the volume of the intestinal lumen (L)

The rate of fecal excretion of the compound is then

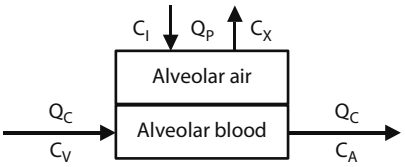
$$dA_F/dt = K_F * A_I/V_{Is}$$

The examples described earlier are highly simplified descriptions. For example, they describe uptake from the stomach or intestinal lumen directly to the liver, when in fact the uptake is into the tissues of the gastrointestinal (GI) tract with subsequent transport to the liver in the portal blood. While this simplification is adequate in some cases, a more accurate description may be necessary in others, such as when metabolism in the gut tissues is important [87] or portal blood flow could limit uptake. The equations shown earlier also do not describe biliary excretion or other processes that might be important determinants of the intestinal concentration and fecal excretion of a compound over time. Again, these processes would need to be included in the full model description for compounds where these processes are important [85]. Note, also, that this simple formulation does not consider the

plug flow of the intestinal contents and will not reproduce the delay that actually occurs in the appearance of a compound in the feces. Such a delay could be added using a delay function available in common simulation software, or multiple compartments could be used to simulate plug flow, as shown in the diagram of the methotrexate model (Figure 6.7).

Inhalation

For compounds that are volatile, it is necessary to describe the exchange of vapor between the lung air and blood in the alveolar region. This is true even if the compound is not administered by inhalation, because exhalation can be an important route of clearance for volatile compounds regardless of the dose route:



$$\frac{dA_{AB}}{dt} = Q_C * (C_V - C_A) + Q_P * (C_I - C_X)$$

where

- A_{AB} is the amount of compound in the alveolar blood (mg)
- C_V is the concentration of compound in the pooled venous blood (mg/L)
- C_A is the concentration of compound in the alveolar (arterial) blood (mg/L)
- Q_P is the alveolar (not total pulmonary) ventilation rate (L/h)
- C_I is the concentration of compound in the inhaled air (mg/L)
- C_X is the concentration of compound in the alveolar air (mg/L)

Assuming the alveolar blood is at steady state with respect to the other compartments,

$$\frac{dA_{AB}}{dt} = 0$$

Also, assuming lung equilibration (i.e., the blood in the alveolar region has reached equilibrium with the alveolar air prior to exhalation),

$$C_X = \frac{C_A}{P_B}$$

Substituting into the equation for the alveolar blood and solving for C_A,

$$C_A = \frac{(Q_C * C_V + Q_P * C_I)}{(Q_C + Q_P/P_B)}$$

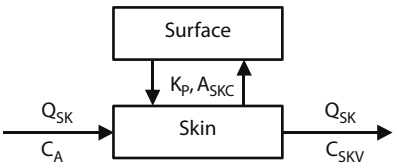
Note that the rate of elimination of the compound by exhalation is just $Q_P * C_X$. The alveolar ventilation rate, Q_P, does not include the *dead-space* volume (the portion of the inhaled air that does not reach the alveolar region) and is therefore roughly 70% of the total respiratory rate. The concentration C_X represents the *end-alveolar* air concentration; in order to estimate the average exhaled concentration (C_{EX}), the dead-space contribution must be included:

$$C_{EX} = 0.3 * C_I + 0.7 * C_X$$

PBPK models including more detailed physiological descriptions of inhalation exposures developed to understand toxicological effects of reactive vapors in the nasal cavity have been reviewed [90].

Dermal

A simple model can be used to describe dermal absorption from a constant-concentration vehicle on the skin surface:



$$dA_{SK}/dt = K_P * A_{SFC} * (C_{SFC} - C_{SK}/P_{SKV})/1000 + Q_{SK} * (C_A - C_{SK}/P_{SKB})$$

where

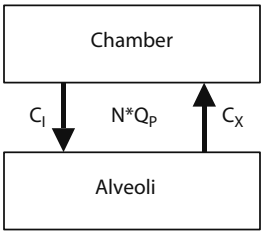
- A_{SK} is the amount of compound in the skin (mg)
- K_P is the skin permeability coefficient (cm/h)
- A_{SFC} is the skin surface area (cm^2)
- C_{SFC} is the concentration of compound on the surface of the skin (mg/L)
- C_{SK} is the concentration of the compound in the skin (mg/L)
- P_{SKV} is the skin/vehicle partition coefficient (i.e., the vehicle containing the compound on the surface of the skin)
- Q_{SK} is the blood flow to the skin region (L/h)
- C_A is the arterial concentration of the compound (mg/L)
- P_{SKB} is the skin/blood partition coefficient

Due to addition of this compartment, the equation for the blood in the model must also be modified to add a term for the venous blood returning from the skin ($+ Q_{SK} * C_{SK}/P_{SKB}$), and the blood flow and volume parameters for the slowly perfused tissue compartment must be reduced by the amount of blood flow and volume for the skin. Approaches used to include the dermal exposure route in PBPK models have been reviewed [12]. Several reviews discuss the wide variety of available compartment models and provide guidance for choosing among them [91,92].

Experimental Apparatus

In some cases, in addition to compartments describing the animal–chemical system, it may also be necessary to include

model compartments that describe the experimental apparatus in which measurements were obtained. An example of such a case is modeling a closed-chamber gas uptake experiment [93–96]. In a gas uptake experiment, several animals are maintained in a small enclosed chamber while the air in the chamber is recirculated, with replenishment of oxygen and scrubbing of carbon dioxide. A small amount of a volatile chemical is then allowed to vaporize into the chamber, and the concentration of the chemical in the chamber air is monitored over time. In this design, any loss of the chemical from the chamber air reflects uptake into the animals [93]. In order to simulate the change in the concentration in the chamber air as the chemical is taken up into the animals, an equation is required for the chamber itself:



$$\frac{dACH}{dt} = N * QP * (CX - CI)$$

$$CI = \frac{ACH}{VCH}$$

where

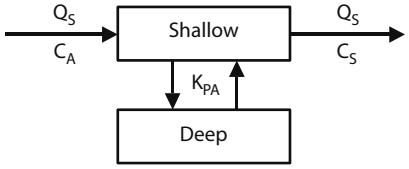
ACH is the amount of chemical in the chamber (mg)
N is the number of animals in the chamber
CX is the concentration of chemical in the air exhaled by the animals (mg/L)
QP is the alveolar ventilation rate for a single animal (L/h)
CI = the chamber air concentration (mg/L)
VCH is the volume of air in the chamber (L)

Distribution/Transport

Chemical transfer between the blood and tissue compartments may be governed by passive diffusion (flow or diffusion limited) or active transport. Many published PBPK models are flow limited, that is, they assume that the rate of tissue uptake of the chemical is limited only by the flow of the chemical to the tissue in the blood. While this assumption is generally reasonable, for some chemicals and tissues, the uptake may instead be limited by other factors such as diffusion. Examples of tissues for which diffusion-limited transport has often been described include the skin, placenta, mammary glands, brain, and fat [61,62,97,98]. Some chemicals may be transported against the concentration gradient through energy-dependent processes. These processes are sometimes limited by the availability of transporter proteins, and such saturable processes are often well described using Michaelis–Menten-type kinetics [82,99–101].

Diffusion-Limited Transport

Most of the PBPK models in the literature are flow-limited models, that is, they assume that the rate of tissue uptake of the chemical is limited only by the flow of the chemical to the tissue in the blood. While this assumption appears to be reasonable in general, for some chemicals and tissues, uptake may instead be diffusion limited. Examples of tissues for which diffusion-limited transport has often been described include the skin, placenta, brain, and fat. The model compartments described thus far have all assumed flow-limited transport. If there is evidence that the movement of a compound between the blood and a tissue is limited by diffusion, a two-compartment description of the tissue can be used with a *shallow* exchange compartment in communication with the blood and a diffusion-limited *deep* compartment that represents the actual tissue:



$$dA_S/dt = Q_S * (C_A - C_S) - K_{PA} * (C_S - C_D/P_D)$$

$$dA_D/dt = K_{PA} * (C_S - C_D/P_D)$$

where

A_S is the amount of the compound in the shallow compartment (mg)
Q_S is the blood flow to the shallow compartment (L/h)
C_S is the concentration of the compound in the shallow compartment (mg/L)
K_{PA} is the permeability–area product for diffusion-limited transport (L/h)
C_D is the concentration of the compound in the deep compartment (mg/L)
P_D is the tissue/blood partition coefficient
A_D is the amount of compound in the deep compartment (mg)

Saturable Tissue Binding

When there is evidence that saturable binding is an important determinant of the distribution of a compound into a tissue (such as evidence of dose-dependent tissue partitioning), a simple description of tissue binding can be added to the model. In this description, only free (unbound) compound is considered to be available for transport or clearance at any given moment in time. For example, in the case of saturable binding in the liver,

$$dA_L/dt = V_L dC_L/dt = Q_L * (C_A - C_{Lfree}/P_{Lfree}) - k_F * C_{Lfree}/P_{Lfree}$$

where

A_L is the total (free plus bound) concentration of the compound in the liver (mg)
C_{Lfree} is the concentration of free (unbound) compound in the liver (mg/L)
P_{Lfree} is the liver/blood partition coefficient for free (unbound) compound
k_F is the rate constant for metabolism (h^{-1})

The apparent complication in adding this equation to the model is that the change in the total amount of compound in the tissue (dA_L/dt) is needed for the mass balance, but the determinants of the kinetics are described in terms of the free concentration in the tissue (C_{Lfree}). To solve for free in terms of total, we note that

$$C_L = C_{Lfree} + C_{Lbound}$$

where C_{Lbound} is the concentration of bound compound in the tissue (mg/L).

We can describe the saturable binding with an equation similar to that for saturable metabolism,

$$C_{Lbound} = B_m * C_{Lfree}/(K_d + C_{Lfree})$$

where

B_m is the binding capacity (mg/L)
K_d is the binding affinity (mg/L)

Substituting this equation in the previous one,

$$C_L = C_{Lfree} + B_m * C_{Lfree}/(K_d + C_{Lfree})$$

Rewriting this equation to solve for the free concentration in terms of only the total concentration would result in a quadratic equation, the solution of which could be obtained with the quadratic formula. However, taking advantage of the iterative algorithm by which these PBPK models are exercised (as will be discussed later), it is not necessary to go to this effort. Instead, a much simpler implicit equation can be written for the free concentration (i.e., an equation in which the free concentration appears on both sides):

$$C_{Lfree} = \frac{C_L}{\left(1 + B_m/\left(K_d + C_{Lfree}\right)\right)}$$

In an iterative algorithm, this equation can be solved at each time step using the previous value of C_{Lfree} to obtain the new value! A new value of C_L is then obtained from the mass balance equation for the liver using the new value of C_{Lfree}, and the process is repeated. An underlying assumption for this simple description of tissue binding is that the binding is rapidly reversible compared to the movement or clearance of free compound, such that equilibrium between free and bound chemicals can be maintained.

In fact, at least two different computational approaches have been used to describe saturable binding in PBPK models. The concentration of free compound can be estimated either, as described earlier, from solving a conservation equation for total mass that apportions the total amount of compound between free and bound forms using the equilibrium dissociation constants, K_ds, and binding maxima, B_ms [102], or by explicitly including both *on* (association) and *off* (dissociation) rate constants, k_a and k_d [101,103]. In the latter case, the dissociation binding constant (with units of concentration) is the ratio of the two rate constants, k_d/k_a. The advantage of the rate constant approach is that it does not require the assumption that binding is fast compared to transport and clearance.

While these two computational approaches have usually been applied within tissues, they can also be readily applied to binding in blood if the concentrations and affinities of binding proteins are known. However, in most cases, protein binding in blood is linear (not saturable at relevant concentrations) and can be characterized by a single parameter, fraction unbound, rather than estimates of concentrations of binding sites and their affinities for binding. Nevertheless, there are cases where protein binding is more appropriately described as a nonlinear process [104]. Alternative descriptions of blood binding will be discussed in more detail in the next section.

Binding in Blood

Protein binding in blood can be a key determinant of disposition, affecting compound availability for uptake into target tissues as well as for clearance. A high fraction bound in the blood also gives rise to concerns regarding potential competitive binding by other compounds that could produce a transient increase in concentrations to potentially toxic levels. Methodologies to estimate binding and approaches for the quantitative description of this binding in pharmacokinetic models have been areas of intense interest over the past four to five decades. Consideration of blood binding faces two parallel challenges: first, when compounds are bound in capillary blood, what fraction should be regarded as available for transport into tissue, and, second, how does the binding in blood influence blood/tissue partitioning. Historically, these questions have been addressed both in empirical descriptions and in PBPK models, and some care is required to reconcile the different approaches and arrive at a consistent quantitative treatment of blood binding and transport of compound throughout the body in both types of modeling approaches.

In the standard description of clearance of a compound from blood by tissue metabolism, binding in the blood is assumed to be linear and the fraction unbound, f_u, is simply multiplied by the intrinsic clearance, leading to a straightforward relationship:

$$CL = Q_T * f_u * CL_{int}/(Q_T + f_u * CL_{int})$$

In this relationship, the maximal tissue clearance, even with a low fraction unbound, is total tissue blood flow. That is, all of the compound in the blood, whether bound or unbound, becomes available for clearance as long as the intrinsic clearance is sufficiently large. Note that in this description, the fraction unbound is not a function of the clearance. In fact, the derivation of this equation rests on the assumption that dissociation of bound compound in the blood is fast

compared to the rate of tissue clearance. If the uptake of a compound into the tissue is limited by the rate of dissociation of the compound from binding proteins in the blood, the earlier simple formula will overestimate its clearance.

In PBPK models, blood concentrations of bound and free compounds can be described separately, as discussed in the previous section, and only free compound is generally considered to be available to participate in processes such as diffusion, metabolism, tissue reaction, and intercompartmental transfer. As in the case of tissue binding, the relationship of free to bound concentrations in the blood is generally calculated by knowledge of dissociation binding constants (K_ds) and maximum concentrations of binding proteins (B_ms), as described previously. An example of this approach has been described in the case of a PBPK model for estradiol [104]. This approach differs from the simple clearance formula given earlier in that the fraction unbound can be a nonlinear function of the concentration of the compound in the blood. It shares, however, the assumption that the *on* and *off* rates for binding are fast compared to rates of clearance. The alternative description of binding described in the previous section, which explicitly includes the binding rate constants, would be preferred in cases where this assumption may be violated.

Pharmacokinetic models need to carefully consider the manner in which binding can be introduced into the basic equations and track free concentrations at sites of action or at least within the plasma. Introduction of blood binding in PBPK models raises some conceptual challenges, especially in comparing conventional empirical approaches with PBPK approaches intended to track thermodynamically free concentrations throughout the body. Although simple descriptions are often adequate, careful consideration of the underlying kinetic processes and their relative rates for the compound and tissues of interest is required to assure that a particular modeling approach is appropriate [105].

Tissue/Blood Partitioning

Regardless of the manner in which blood binding is implemented in PBPK models, another challenge arises in describing equilibration between blood and tissues. In general, both the blood and the tissues will contain free and bound forms of the compound. For equilibration, only the free compound in the plasma diffuses across the tissue capillary interface into the tissue, and at equilibrium between blood and tissue, the free concentration in the plasma and the tissue is expected to be equal (except in the case of active transport). However, the equilibrium relationship of the concentrations in tissues compared to the blood or plasma is typically described with empirical partition coefficients based on measurements of total concentrations of the compound. Differential binding in plasma and tissue will therefore influence apparent tissue partitioning.

The relationship between apparent tissue/blood partitioning and binding in blood vs. tissue can be straightforwardly described, as long as no other factors affect distribution. Assuming that (a) there is no need to adjust for the effect of clearance and (b) there is no evidence of active transport of the compound between blood and tissue, the free fraction in the tissue, f_{ut}, can then be estimated:

$$P_{tb} = P_{tp}/BPR = (f_{up}/f_{ut})/BPR$$

where

P_{tb} is the tissue/blood partition coefficient
P_{tp} is the tissue/plasma partition coefficient
BPR is the blood-to-plasma ratio
f_{up} is the fraction unbound in the plasma
f_{ut} is the fraction unbound in the tissue
then, $f_{ut} = f_{up}/(P_{tb} * BPR)$

In fact, there are quite a number of different determinants of the apparent partitioning between blood and tissues:

- Partitioning due to lipophilicity
- Plasma binding
- Tissue binding
- Active transport
- Clearance processes
- BPR (for converting tissue/plasma partitions to tissue/blood)

A measured or estimated partition coefficient may reflect any combination of these factors, and the modeler must be aware of this potential complexity in attempting to use a particular set of data on a compound. For example, if partition coefficients have been estimated from quantitative structure–activity relationship (QSAR), they are likely to primarily reflect lipophilic partitioning and may need to be adjusted for differences in binding in plasma and tissues. Alternatively, the model can be designed to separately describe lipophilic partitioning, using the estimated partition coefficients, and binding, based on other data. In vitro–derived partition coefficients, on the other hand, may reflect both thermodynamic partitioning and binding, although disruption of the tissue architecture may alter the binding characteristics of the tissue. It is also necessary to ensure that no metabolic clearance of the compound occurs in either blood or tissue during the measurements. In vivo–derived partition coefficients may reflect lipophilicity, binding, and active transport, as well as the effect of any clearance processes [106]. Due to this complexity, it may in some cases be preferable to estimate model parameters for tissue partitioning by fitting the in vivo blood and tissue concentration data rather than trying to use it directly to calculate *partition coefficients* for the model. This latter method can be misleading for compounds with dose-dependent, tissue-specific binding, such as dioxin [107].

Model Parameterization

Once the model structure has been determined, it still remains to identify the values of the input parameters in the model.

Physiological Parameters

Estimates of the various physiological parameters needed in PBPK models are available from a number of sources in the literature, particularly for the human, monkey, dog, rat, and mouse [4,108–112]. Estimates for the same parameter often vary widely, however, due both to experimental differences and to differences in the animals examined (age, strain, and activity). Ventilation rates and blood flow rates are particularly sensitive to the level of activity [108,109]. Data on some important tissues are relatively poor, particularly in the case of fat tissue. Table 6.1 shows typical values of a number of physiological parameters in several species.

Biochemical Parameters

For volatile chemicals, partition coefficients may be measured using a relatively simple in vitro technique known as vial equilibration [88,89,113,114]. Partition coefficients for nonvolatile compounds can be determined by either in vivo or in vitro measurements [106,115,116]. Alternatively, partition coefficients can be estimated from QSAR modeling [117–129].

Metabolism parameters can be obtained from parent chemical disappearance (or metabolite formation) curves in intact cells, tissue homogenate, or microsomal fractions [130–140]. Rapid in vivo approaches may also be used to estimate metabolic constants based on steady-state extraction [54] or gas uptake experiments [93,95,96,141,142], as well as information on the total amount of chemical metabolized in a particular exposure situation [143]. Determination of stable end-product metabolites after exposure can also be useful in some cases [94,144].

Allometry

The different types of physiological and biochemical parameters in a PBPK model are known to vary with BW in different ways [145]. Typically, the parameterization of PBPK models is simplified by assuming standard allometric scaling [109,146], as shown in Table 6.2, where the scaling factors, b, can be used in the following equation:

$$Y = aX^b$$

where

Y is the value of the parameter at a given BW, X (kg)
a is the scaled parameter value for a 1 kg animal

While standard allometric scaling provides a useful starting point, or hypothesis, for cross-species scaling, it is not sufficiently accurate for some applications, such as risk assessment. In the case of the physiological parameters, the

TABLE 6.1
Typical Physiological Parameters for PBPK Models

Species		Mouse	Rat	Monkey	Human
Ventilation					
Alveolar	(L/h-1 kg)[a]	29.0[b]	15.0[b]	15.0[b]	15.0[b]
Blood flows					
Total	(L/h-1 kg)[a]	16.5[c]	15.0[c]	15.0[c]	15.0[c]
Muscle	(fraction)	0.18	0.18	0.18	0.18
Skin	(fraction)	0.07	0.08	0.06	0.06
Fat	(fraction)	0.03	0.06	0.05	0.05
Liver (arterial)	(fraction)	0.035	0.03	0.065	0.07
Gut (portal)	(fraction)	0.165	0.18	0.185	0.19
Other organs	(fraction)	0.52	0.47	0.46	0.45
Tissue volumes					
Body weight	(kg)	0.02	0.3	4.0	80.0
Body water	(fraction)	0.65	0.65	0.65	0.65
Plasma	(fraction)	0.04	0.04	0.04	0.04
RBCs	(fraction)	0.03	0.03	0.03	0.03
Muscle	(fraction)	0.34	0.36	0.48	0.33
Skin	(fraction)	0.17	0.195	0.11	0.11
Fat	(fraction)	0.10[d]	0.07[d]	0.05[d]	0.21
Liver	(fraction)	0.046	0.037	0.027	0.023
Gut tissue	(fraction)	0.031	0.033	0.045	0.045
Other organs	(fraction)	0.049	0.031	0.039	0.039
Intestinal lumen	(fraction)	0.054	0.058	0.053	0.053

[a] Scaled allometrically: $QC = QCC * BW^{0.75}$.
[b] Varies significantly with activity level (range, 15.0–40.0).
[c] Varies with activity level (range, 15.0–20.0).
[d] Varies substantially (lower in young animals, higher in older animals).

TABLE 6.2
Standard Allometric Scaling for PBPK Model Parameters

Parameter Type (Units)	Scaling (Power of Body Weight)
Volumes	1.0
Flows (volume per time)	0.75
Ventilation (volume per time)	0.75
Clearances (volume per time)	0.75
Metabolic capacities (mass per time)	0.75
Metabolic affinities (mass per volume)	0
Partition coefficients (unitless)	0
First-order rate constants (inverse time)	−0.25

species-specific parameter values are generally available in the literature [4,108–112] and can be used directly in place of the allometric estimates. For compound-specific parameters, in vitro data for metabolism, distribution, or absorption relevant to the species under consideration are used in most of the cases in preference to allometric estimates. However, allometric scaling might provide first parameter estimates when data are lacking.

Parameter Optimization

In many cases, important parameter values needed for a PBPK model may not be available in the literature. In such cases, it is necessary to measure them in new experiments, to estimate them by QSAR techniques, or to identify them by optimizing the fit of the model to an informative data set. Even in the case where an initial estimate of a particular parameter value can be obtained from other sources, it may be desirable to refine the estimate by optimization. For example, given the difficulty of obtaining accurate estimates of the fat volume in rodents, a more reliable estimate may be obtained by examining the impact of fat volume on the kinetic behavior of a lipophilic compound [98]. Of course, being able to uniquely identify a parameter from a kinetic data set rests on two key assumptions: (1) that the kinetic behavior of the compound under the conditions in which the data were collected is sensitive to the parameter being estimated and (2) that other parameters in the model that could influence the observed kinetics have been determined by other means and are held fixed during the estimation process. When it is necessary to estimate multiple parameters from in vivo pharmacokinetic data, using sensitivity analysis to verify that sufficient data are available and optimizing on individual parameters before performing a global optimization have been recommended [147].

The actual approach for conducting a parameter optimization can range from simple visual fitting, where the model is run with different values of the parameter until the best correspondence appears to be achieved or by a quantitative mathematical algorithm. The most common algorithm used in optimization is the least-squares fit. To perform a least-squares optimization, the model is run to obtain a set of predictions at each of the times a data point was collected. The square of the difference between the model prediction and data point at each time is calculated, and the results for all of the data points are summed. The parameter being estimated is then modified, and the sum of squares is recalculated. This process is repeated until the smallest possible sum of squares is obtained, representing the best possible fit of the model to the data.

In a variation on this approach, the square of the difference at each point is divided by the square of the prediction. This variation, known as relative least squares, is preferable in the case of data with an error structure that can be described by a constant coefficient of variation (i.e., a constant ratio of the standard deviation to the mean). The former method, known as absolute least squares, is preferable in the case of data with a constant variance. From a practical viewpoint, the absolute least-squares method tends to give greater weight to the data at higher concentrations and results in fits that look best when plotted on a linear scale, while the relative least-squares method gives greater weight to the data at lower concentrations and results in fits that look best when plotted on a logarithmic scale.

A generalization of this weighting concept is provided by the extended least-squares method. In the extended least-squares algorithm, the heteroscedasticity parameter can be varied from 0 (for absolute weighting) to 2 (for relative weighting) or can be estimated from the data. In general, setting the heteroscedasticity parameter from the knowledge of the error structure of the data is preferable to estimating it from a data set.

A common example of identifying PBPK model parameters by fitting kinetic data is the estimation of tissue partition coefficients from experiments in which the concentration of compound in the blood and tissues is reported at various time points. Using an optimization approach, the predictions of the model for the time course in the blood and tissues could be optimized with respect to the data by varying the model's partition coefficients. There is little difference in the strength of the justification for estimating the partition coefficients in this way as opposed to estimating them directly from the data (by dividing the tissue concentrations by the simultaneous blood concentration). In fact, the direct estimates would probably be used as initial estimates in the model when the optimization was started.

A major difficulty in performing parameter optimization results from correlations between the parameters. When it is necessary to estimate parameters that are highly correlated, it is best to generate a contour plot of the objective function (sum of squares) or confidence region over a reasonable range of values of the two parameters [148]. An example of a contour plot for two of the metabolic parameters in a PBPK model for methylene chloride is shown in Figure 6.8. The contours in the figure outline the joint confidence region for the joint values of the two parameters, and the fact that the confidence region is aligned diagonally reflects correlation between the two parameters.

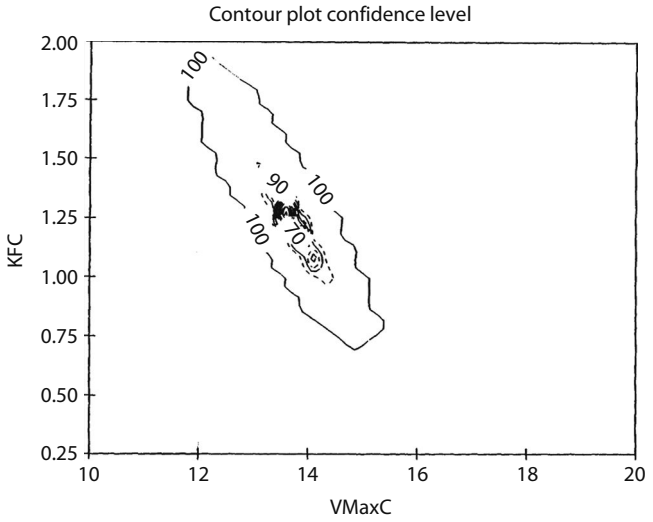


FIGURE 6.8 Contour plot for correlated metabolic parameters in the PBPK model for methylene chloride. KFC is the first-order rate constant for metabolism by glutathione conjugation, and VMaxC is the maximum rate of metabolism by oxidation.

Mass Balance Requirements

One of the most important mathematical considerations during model design is the maintenance of mass balance. Simply put, the model should neither create nor destroy mass. This seemingly obvious principle is often violated unintentionally during the process of model development and parameterization. A common violation of mass balance, which typically leads to catastrophic results, involves failure to exactly match the arterial and venous blood flows in the model. As described earlier, the movement of compound in the blood (in units of mass per time) is described as the product of the concentration of compound in the blood (in units of mass per volume) times the flow rate of the blood (in units of volume per time). Therefore, to maintain mass balance, the sum of the blood flows leaving any particular tissue compartment must equal the sum of the blood flows entering the compartment. In particular, to maintain mass balance in the blood compartment (regardless of whether it is actually a compartment or just a steady-state equation), the sum of the venous flows from the individual tissue compartments must equal the total arterial blood flow leaving the heart:

$$\Sigma Q_T = Q_C$$

Another obvious but occasionally overlooked aspect of maintaining mass balance during model development is that if a model is modified by splitting a tissue out of a lumped compartment, the blood flow to the separated tissue (and its volume) must be subtracted from that for the lumped compartment. Moreover, even though a model may initially be designed with parameters that meet the earlier requirements, mass balance may unintentionally be violated later if the parameters are altered during model execution. For example, if the parameter for the blood flow to one compartment is increased, the parameter for the overall blood flow must be increased accordingly or an equivalent reduction must be made in the parameter for the blood flow to another compartment. Particular care must be taken in this regard when the model is subjected to sensitivity or uncertainty analysis; inadvertent violation of mass balance during Monte Carlo sampling can lead to erroneous sensitivity results [67].

A similar mass balance requirement must be met for transport other than blood flow. For example, if the compound is cleared by biliary excretion, the elimination of compound from the liver in the bile must exactly match the appearance of compound in the gut lumen in the bile. Put mathematically, the same term for the transport must appear in the equations for the two compartments, but with opposite signs (positive vs. negative). For example, if the following equation were used to describe a liver compartment with first-order metabolism and biliary clearance,

$$dA_L/dt = Q_L*(C_A - C_L/P_L) - k_F * C_L * V_L/P_L - K_B * C_L$$

where K_B is the biliary clearance rate (L/h).

The equation for the intestinal lumen would then need to include the term $+K_B * C_L$.

As a model grows in complexity, it becomes increasingly difficult to assure its mass balance by inspection. Therefore, it is a worthwhile practice to check for mass balance by including equations in the model that add up the total amount of compound in each of the model compartments, including metabolized and excreted compound, for comparison with the administered or inhaled dose.

Model Diagram

As described in the previous sections, the process of developing a PBPK model begins by determining the essential structure of the model based on the information available on the compound's toxicity, mechanism of action, and pharmacokinetic properties. The results of this step can usually be summarized by an initial model diagram, such as those depicted in Figures 5.3 and 5.7. Generally, a well-constructed model diagram, together with a table of the input parameter values and their definitions, is all that an accomplished modeler should need in order to create the mathematical equations defining a PBPK model. In general, there should be a one-to-one correspondence of the boxes in the diagram to the mass balance equations (or steady-state approximations) in the model. Similarly, the arrows in the diagram correspond to the transport or metabolism processes in the model. Each of the arrows connecting the boxes in the diagram should correspond to one of the terms in the mass balance equations for both of the compartments it connects, with the direction of the arrow pointing from the compartment in which the term is negative to the compartment in which it is positive. Arrows connected only to a single compartment, which represent uptake and excretion processes, are interpreted similarly.

The model diagram should be labeled with the names of the key variables associated with the compartment or process

represented by each box and arrow. Interpretation of the model diagram is also aided by the definition of the model input parameters in the corresponding table. The definition and units of the parameters can indicate the nature of the process being modeled (e.g., diffusion-limited vs. flow-limited transport, binding vs. partitioning, and saturable vs. first-order metabolism).

MODEL IMPLEMENTATION

The previous sections have focused on the process of designing the PBPK model structure needed for a particular application. At this point, the model consists of a number of mathematical equations: differential equations describing the mass balance for each of the compartments and algebraic equations describing other relationships between model variables. The next step in model development is the coding of the mathematical form of the model into a form that can be executed on a computer. There are many options available for performing this process, ranging from programming languages such as FORTRAN, C, and MATLAB® to more sophisticated simulation software packages such as acslX and Berkeley Madonna.

Mathematical Formulation

Mathematically, a PBPK model is represented by a system of simultaneous ordinary differential equations. Each of the differential equations describes the mass balance for one of the *state variables* (compartments) in the model. There may also be additional differential equations to calculate other necessary model outputs, such as the AUC in a particular compartment, which is simply the integral of the concentration over time. The resulting system of equations is referred to as simultaneous because the time courses of the compound in the various compartments are so interdependent that solving the equations for any one of the compartments requires information on the current status of all the other compartments, that is, the equations for all of the compartments must be solved at the same time. This kind of mathematical problem, in which a system is defined by the conditions at time zero together with differential equations describing how it evolves over time, is known as an initial value problem, and matrix decomposition methods can be used to obtain the simultaneous solution.

A number of numerical algorithms are available for solving such problems. They all have in common that they are step-wise approximations, that is, they begin with the conditions at time zero and use the differential equations to predict how the system will change over a small time step, resulting in an estimate of the conditions at a slightly later time, which serves as the starting point for the next time step. This iterative process is repeated as long as necessary to simulate the experimental scenario.

The more sophisticated methods, such as the Gear algorithm (named after the mathematician, David Gear, who developed it), use a predictor–corrector approach, in which the corrector step essentially amounts to *predicting backward* after each step forward, in order to check how closely the algorithm is able to reproduce the conditions at the previous time step. This approach allows the time step to be increased automatically when the algorithm is performing well and to be shortened when it is having difficulty, such as when conditions are changing rapidly. However, due to the wide variation of the time constants (response times) for the various physiological compartments (e.g., fat vs. richly perfused), PBPK models often represent *stiff* systems. Stiff systems are characterized by state variables (compartments) with widely different time constants, which cause difficulty for predictor–corrector algorithms. The Gear algorithm was specifically designed to overcome this difficulty. It is therefore generally recommended that the Gear algorithm (or an alternative stiff system solver such as the Rosenbrock method) be used for executing PBPK models. An implementation of the Gear algorithm is available in a popular Fortran subroutine, LSODE, developed by Alan Hindmarsh at Lawrence Livermore National Laboratory and available in the public domain [149].

Regardless of the specific algorithm selected, the essential nature of the solution will be a step-wise approximation. However, all of the algorithms made available in computer software are convergent, that is, they can stay arbitrarily close to the true solution, given a small enough time step. On modern personal computers, even large PBPK models can be run to more than adequate accuracy in a reasonable timeframe.

Model Coding

Once a PBPK model structure has been defined with a model diagram or expressed as a set of mass balance equations, it can be coded into a computer language, such as FORTRAN, C, or MATLAB, or into a simulation language, such as acslX or Berkeley Madonna. The advantage of a simulation language is that it frees the modeler from the task of implementing the code necessary to solve the set of mass balance differential equations over time. In either case, the model code should be written and annotated in such a way to facilitate understanding by individuals other than the original program developer. The model can then be exercised for particular conditions necessary to reproduce available data sets collected by the investigator or available in the literature. The model can also be used to make specific risk calculations for particular exposure situations. As part of the model documentation, it is imperative to provide other potential users with the detailed steps that are required to reproduce these figures and calculations. This step usually entails provision of *scripts* that allow reproduction of the figures and calculations by entering specified programming commands or by invoking specified sets of instructions (procedures).

There are a number of available simulation languages and other multipurpose models that can be used to construct PBPK models for chemicals and their metabolites, including acslX, Berkley Madonna, and MATLAB (Table 6.3). Some of these are code based (i.e., require text that defines the equations of

TABLE 6.3
PBPK Modeling Software

Package	Source	Website
General-purpose high-level scientific computing software. These high-level programming language packages are very general modeling tools. They are not specifically designed for PBPK modeling but do offer more complexity to the user.		
acslX	AEgis Technologies Group, Inc.	http://www.aegistg.com
Berkeley Madonna	University of California at Berkeley	http://www.berkeleymadonna.com
MATLAB/Simulink	MathWorks, Inc.	http://www.mathworks.com
GNU Octave	University of Wisconsin	http://www.octave.org
MLAB	Civilized Software, Inc.	http://www.civilized.com
Biomathematical modeling software. These packages have been specifically designed for modeling biological systems, and some are user-friendly. Their usefulness in PBPK modeling is determined by their graphical interfaces, computational speed, and language flexibility. Some of these packages provide mixed-effects (population) capabilities allowing for the analysis of sparse data sets.		
ADAPT II	Biomedical Simulations Resource, USC	http://bmsr.usc.edu
ModelMaker	ModelKinetix	http://www.modelkinetix.com
NONMEM	University of California at San Francisco and Globomax Service Group	http://www.globomaxservice.com
SAAM II	SAAM Institute, Inc.	http://www.saam.com
SCoP	Simulation Resources, Inc.	http://www.simresinc.com
Stella	High Performance Systems, Inc.	http://www.hps-inc.com
WinNonlin	Pharsight Corp.	http://www.pharsight.com
WinNonMix	Pharsight Corp.	http://www.pharsight.com
Toxicokinetic software. These packages were designed specifically for PBPK and PBTK modeling and are extremely flexible. They are based on modeling languages developed in the aerospace industry for modeling complex systems.		
SimuSolv	Dow Chemical	Not maintained or subject to further development
Physiologically based custom-designed software. These are custom-designed proprietary software programs specifically for biomedical systems or applications. They provide a high level of biological detail; however, they are not easily customized.		
GastroPlus	Simulations Plus, Inc.	http://www.simulations-plus.com
Pathway Prism	Physiome Sciences, Inc.	http://www.physiome.com
Physiolab	Entelos, Inc.	http://www.entelos.com

Source: Rowland, M. et al., *AAPS J.*, 6, 56, 2004.

the model) and others utilize a graphical interface for model construction in which boxes and arrows serve as input rather than equations. Code-based representations ease the task of model verification. Model verification is the process of determining whether the model is accurately calculating the desired outputs. The verification step entails ensuring that the equations are appropriate to describe the model and checking the mass balance for all compounds tracked by the model. The validation step involves establishing that the model reproduces within some error available in PK data on the substance relevant to the purposes for which the model was developed.

Currently, there exists a very diverse group of modeling software packages that vary in both complexity and range of application. Because of these diversities, there is a software package designed for every user from the expert to the first-time modeler. However, not all modeling packages are created equal, and some of the more user-friendly software can lack the capabilities of the more complex programs. Consequently, no single software package available can meet all needs of all users, and the diverse complexity of the programs can often make converting a model from one program to be used in another rather difficult. Table 6.3 provides a list of some of the available software packages that may be used for PBPK modeling [19].

A list of additional pharmacokinetic software may be located at http://boomer.org/pkin/soft.html. However, not all of this software listed on this website is PBPK software.

The first three programs listed in Table 6.3 are popular for modeling of environmental compounds. AcslX is an updated version of Advanced Continuous Simulation Language (ACSL), one of the first widely used PBPK modeling

platforms [150]. It has a graphical as well as a text interface with automatic linkage to the integration algorithm. It allows for the use of discrete blocks (for scheduled events) and script files (m-files) and automatically sorts equations in the derivative block. Sensitivity analysis and Monte Carlo analysis are supported. A fully functional free demo is available.

Berkeley Madonna has many of the same features as acslX; however, it does not allow for the use of discrete blocks or script files. It does currently have both an optimization and a sensitivity analysis feature, but does not have a Monte Carlo feature.

MATLAB is a powerful engineering programming language that has a text interface, but not a graphical interface. It does allow for the use of script files but not discrete blocks. It does not sort the code in the model so the user must be very careful not to use a parameter or variable before it has been defined. This can often be a problem for systems of simultaneous differential equations, when one variable is used in another equation to define a variable that is used in the equation for the first variable. An optimization package is available through an add-on toolbox, but sensitivity analysis and Monte Carlo analysis must be performed through the use of script files. A graphic interface for MATLAB is available through the Simulink add-on. The use of discrete blocks is also added with the use of Simulink. Since Simulink uses only a graphical interface, there is no code to be viewed for model verification.

Coding Example: ACSL

The following section contains typical elements of the ACSL code for a PBPK model, interspersed with comments, which will be written in *italics* to differentiate them from the actual model code. The code shown is for an early version of ACSL; however, with only minor modifications, it could be run in either acslX or Berkeley Madonna. During a typical modeling session, the input parameters (constants) in the model are changed, the model is run, and the model outputs are displayed or plotted. The model used in this example is a simple multiroute model for volatile chemicals, similar to the styrene model discussed earlier, except that it also has the ability to simulate closed-chamber gas uptake experiments.

Typical Elements in an ACSL Source (.CSL) File

An ACSL source file follows the structure defined in the Standard for Continuous Simulation Languages (just like there's a standard for FORTRAN). Thus, for example, there will generally be an INITIAL block defining the initial conditions followed by a DYNAMIC block which contains DISCRETE and/or DERIVATIVE subblocks that define the model. In addition, conventions which have been generally adopted by the PBPK modeling community (most of which started with John Ramsey at Dow Chemical during the development of the styrene model) help to improve the readability of the code. The following file shows typical elements of "Ramseyan code."

The first line in the code must start with the word "PROGRAM." The rest of the line is ignored by the ACSL translator and can be used to identify the model:

```
PROGRAM:  PHYSIM93—  Physiologically  Based
  Pharmacokinetic Model
```

Lines starting with an exclamation point (and portions of lines to the right of one) are ignored by the ACSL translator and can be used for comments:

```
! Generic model for halogenated methanes
```

The first section of an ACSL source file is the INITIAL block, which is used to define parameters and perform calculations that do not need to be repeated during the course of the simulation:

```
INITIAL ! Beginning of pre-execution section
```

Only parameters defined in a CONSTANT statement can be changed during a session using the SET command:

```
LOGICAL CC ! Flag set to.TRUE. for closed chamber
runs

! Physiological parameters (rat)

CONSTANT QPC=14. ! Alveolar ventilation rate (L/h)
CONSTANT QCC=14. ! Cardiac output (L/h)
CONSTANT QLC=0.25 ! Fractional blood flow to liver
CONSTANT QFC=0.09 ! Fractional blood flow to fat
CONSTANT BW=0.22 ! Body weight (kg)
CONSTANT VLC=0.04 ! Fraction liver tissue
CONSTANT VFC=0.07 ! Fraction fat tissue

!— — — -Chemical specific parameters (styrene)

CONSTANT PL=3.46 ! Liver/blood partition coefficient
CONSTANT PF=86.5 ! Fat/blood partition coefficient
CONSTANT PS=1.16 ! Slowly perfused tissue/blood
  partition
CONSTANT PR=3.46 ! Richly perfused tissue/blood
  partition
CONSTANT PB=40.2 ! Blood/air partition coefficient
CONSTANT MW=104. ! Molecular weight (g/mol)
CONSTANT VMAXC=8.4 ! Maximum velocity of
  metabolism (mg/h-1 kg)
CONSTANT KM=0.36 ! Michaelis–Menten constant
  (mg/L)
CONSTANT KFC=0. ! First order metabolism (/h-1 kg)
CONSTANT KA=0. ! Oral uptake rate (/h)

!— — — -Experimental parameters

CONSTANT PDOSE=0. ! Oral dose (mg/kg)
CONSTANT IVDOSE=0. ! IV dose (mg/kg)
CONSTANT CONC=1000. ! Inhaled concentration (ppm)
CONSTANT CC =.FALSE.! Default to open chamber
CONSTANT NRATS=3. ! Number of rats (for closed
  chamber)
```

```
CONSTANT KLC=0. ! First order loss from closed chamber (/h)
CONSTANT VCHC=9.1 ! Volume of closed chamber (L)
CONSTANT TINF =.01 ! Length of IV infusion (h)
```

It is an understandable requirement in ACSL to define when to stop and how often to report. The parameter for the reporting frequency ("communication interval") is assumed by the ACSL translator to be called CINT unless you tell it otherwise using the CINTERVAL statement. The parameter for when to stop can be called anything you want, as long as you use the same name in the TERMT statement (see the following text), but the Ramseyan convention is TSTOP:

```
CONSTANT TSTOP=24. ! Length of experiment (h)
CONSTANT POINTS=96 ! Number of points in plot
   CINT=TSTOP/POINTS ! Communication interval
```

The calculation of CINT shown earlier is motivated by wanting a certain plot resolution regardless of the duration of the run, but wanting to save disk space for long runs. If accuracy of calculation is important, using a fixed interval may be preferable:

```
CINTERVAL CINT=0.1
```

The following parameter name is generally used to define the length of inhalation exposures (the name LENGTH is also used by some):

```
CONSTANT TCHNG=6. ! Length of inhalation exposure (h)
```

The INITIAL block is a useful place to perform logical switching for different model applications, in this case between the simulation of closed-chamber gas uptake experiments and normal inhalation studies. It is also sometimes necessary to calculate initial conditions for one of the integrals ("state variables") in the model (the initial amount in the closed chamber in this case):

```
IF (CC) RATS=NRATS ! Closed chamber simulation
IF (CC) KL=KLC
IF (.NOT.CC) RATS=0. ! Open chamber simulation
IF (.NOT.CC) KL=0.
! (Turn off chamber losses so concentration in chamber remains constant)
IF (PDOSE.EQ.0.0) KA=0. ! If not oral dosing, turn off oral uptake
VCH=VCHC-RATS*BW    ! Net chamber air volume (L)
AI0=CONC*VCH*MW/24450. ! Initial amount in chamber (mg)
```

After all the constants have been defined, calculations using them can be performed. In contrast to the DERIVATIVE block (of which more later), the calculations in the INITIAL block are performed in the order written, just like in FORTRAN or MATLAB, so a variable must be defined before it can be used.

Note how allometric scaling is used for flows (QC, QP) and metabolism (VMAX, KFC). Also note how the mass balance for the blood flows and tissue volumes is maintained by the model code. Run-time changes in the parameters for fat and liver are automatically balanced by changes in the slowly and richly perfused compartments, respectively. The fractional blood flows add to one, but the fractional tissue volumes add up to only 0.91, allowing 9% of the BW to reflect nonperfused tissues:

```
!— — — Scaled parameters

QC=QCC*BW**0.74 ! Cardiac output
QP=QPC*BW**0.74 ! Alveolar ventilation
QL=QLC*QC ! Liver blood flow
QF=QFC*QC ! Fat blood flow
QS=0.24*QC-QF ! Slowly-perfused tissue blood flow
QR=0.76*QC-QL ! Richly-perfused tissue blood flow
VL=VLC*BW ! Liver volume
VF=VFC*BW ! Fat tissue volume
VS=0.82*BW-VF ! Slowly-perfused tissue volume
VR=0.09*BW-VL ! Richly-perfused tissue volume
VMAX=VMAXC*BW**0.7 ! Maximum rate of metabolism
KF=KFC/BW**0.3 ! First-order metabolic rate constant
DOSE=PDOSE*BW ! Oral dose
IVR=IVDOSE*BW/TINF ! Intravenous infusion rate
```

An END statement is required to delineate the end of the initial block:

```
END ! End of initial section
```

The next (and often last) section of an ACSL source file is the DYNAMIC block, which contains all of the code defining what is to happen during the course of the simulation:

```
DYNAMIC ! Beginning of execution section
```

ACSL possesses a number of different algorithms for performing the simulation, which mathematically speaking consists of solving an initial value problem for a system of simultaneous linear differential equations. (although it is easier to just refer to it as integrating). Available methods include the Euler, Runge–Kutta, and Adams–Moulton, but the tried and true choice of most PBPK modelers is the Gear predictor–corrector, variable step-size algorithm for stiff systems, which PBPK models often are (stiff, that is):

```
ALGORITHM IALG=2 ! Use Gear integration algorithm
```

One of the structures which can be used in the DYNAMIC block is called a DISCRETE block. The purpose of a DISCRETE block is to define an event which is desired to occur at a specific time or under specific conditions. The integration algorithm then keeps a lookout for the conditions and executes the code in the DISCRETE block at the proper moment during the execution of the model. An example of a pair of discrete blocks that are used to control repeated dosing in another PBPK model is shown:

```
DISCRETE DOSE1    ! Schedule events to turn exposure on and off daily
INTERVAL DOSINT=24.    ! Dosing interval
          !(Set interval larger than TSTOP to prevent multiple exposure)
```

```
  IF (T.GT.TMAX) GOTO NODOSE
  IF (DAY.GT.DAYS) GOTO NODOSE
     CONC = CI    ! Start inhalation exposure
   TOTAL = TOTAL + DOSE    ! Administer oral dose
   TDOSE = T !    Record time of dosing
  SCHEDULE DOSE2.AT. T + TCHNG    ! Schedule end
    of exposure
  NODOSE..CONTINUE
  DAY = DAY + 1.
   IF (DAY.GT.7.) DAY = 0.5
END ! of DOSE1

DISCRETE DOSE2
        CONC = 0.    ! End inhalation exposure
END ! of DOSE2
```

Within the DYNAMIC block, a group of statements defining a system of simultaneous differential equations is put in a DERIVATIVE block. If there's only one it doesn't have to be given a name:

```
DERIVATIVE    ! Beginning of derivative definition block
```

The main function of the derivative block is to define the "state variables" that are to be integrated. They are identified by the INTEG function. For example, in the code below, AI is defined to be a state variable which is calculated by integrating the equation defining the variable RAI, using an initial value of AI0. For most of the compartments, the initial value is zero:

```
!— — — — -CI = Concentration in inhaled air (mg/L)
        RAI = RATS*QP*(CA/PB-CI) - (KL*AI) ! Rate
equation
      AI = INTEG(RAI,AI0)    ! Integral of RAI
      CI = AI/VCH*CIZONE    ! Concentration in air
 CIZONE = RSW((T.LT.TCHNG).OR.CC,1.,0.)
        CP = CI*24450./MW    ! Chamber air concentra-
tion in ppm
```

Any experienced programmer would shudder at the previously showncode, because several variables appear to be used before they have been calculated (e.g., CIZONE is used to calculate CI and CI is used to calculate RAI). However, within the derivative block, writing code is almost too easy because the translator will automatically sort the statements into the proper order for execution. That is, there's no need to be sure a variable is calculated before it is used.

The downside of the sorting is you can't be sure that two statements will be calculated in the order you want just because you place them one after the other. Also, because of the sorting (as well as the way the predictor–corrector integration algorithm hops forward and backward in time), IF statements won't work right. The RSW function earlier works like an IF statement, setting CIZONE to 1 whenever T (the default name for the time variable in ACSL) is less than TCHNG, and setting CIZONE to 0 (and thus turning off the exposure) whenever T is greater than or equal to TCHNG.

Another way to be sure statements are executed in order is to put them in a PROCEDURAL, which is left alone by the sorter:

```
PROCEDURAL
  if (recovery) then
    goto out
  elseif (t.gt.tendexp) then
    recovery =.true.
  endif
  out..continue
END !of procedural
```

The double dot (..) after the word "out" identifies it as a label, that is, a point that the program can be told to jump to, as in the if statement earlier.

The following blocks of statements each define one of the compartments in the model. These statements can be compared with the mathematical equations described in the previous sections of the manual. One of the advantages of models written in ACSL following the Ramseyan convention is that they are easier to comprehend and reasonably self-documenting:

```
!— — MR = Amount remaining in stomach (mg)
  RMR = -KA*MR
  MR = DOSE*EXP(-KA*T)
```

Note that the stomach could have been defined as one of the state variables:

```
MR = INTEG(RMR,DOSE)
```

But instead the exact solution for the simple integral has been used directly.

Similarly, instead of defining the blood as a state variable, the steady-state approximation is used:

```
!— — CA = Concentration in arterial blood (mg/L)
CA = (QC*CV+QP*CI)/(QC+[QP/PB])
AUCB = INTEG(CA,0.)

!— — AX = Amount exhaled (mg)
CX = CA/PB ! End-alveolar air concentration (mg/L)
CXPPM = (0.7*CX+0.3*CI)*24450./MW    !    Average
   exhaled air concentration (ppm)
RAX = QP*CX
AX = INTEG(RAX,0.)

!— — AS = Amount in slowly perfused tissues (mg)
RAS = QS*(CA-CVS)
AS = INTEG(RAS,0.)
CVS = AS/(VS*PS)
CS = AS/VS

!— — AR = Amount in rapidly perfused tissues (mg)
RAR = QR*(CA-CVR)
AR = INTEG(RAR,0.)
CVR = AR/(VR*PR)
CR = AR/VR
```

```
!— — AF = Amount in fat tissue (mg)
RAF = QF*(CA-CVF)
AF = INTEG(RAF,0.)
CVF = AF/(VF*PF)
CF = AF/VF

!— — AL = Amount in liver tissue (mg)
RAL = QL*(CA-CVL) - RAM + RAO
AL = INTEG(RAL,0.)
CVL = AL/(VL*PL)
CL = AL/VL
AUCL = INTEG(CL,0.)

!— — AM = Amount metabolized (mg)
RAM = (VMAX*CVL)/(KM+CVL) + KF*CVL*VL
AM = INTEG(RAM,0.)

!— — AO = Total mass input from stomach (mg)
RAO = KA*MR
AO = DOSE-MR

!— — IV = Intravenous infusion rate (mg/h)
IVZONE = RSW(T.GE.TINF,0.,1.)
IV = IVR*IVZONE

!— — CV = Mixed venous blood concentration (mg/L)
CV = (QF*CVF + QL*CVL + QS*CVS + QR*CVR + IV)/QC

!— — TMASS = mass balance (mg)
TMASS = AF+AL+AS+AR+AM+AX+MR

!— — DOSEX = Net amount absorbed (mg)
DOSEX = AI+AO+IVR*TINF-AX
```

Last, but definitely not least, you have to tell ACSL when to stop:

```
TERMT(T.GE.TSTOP)  !Condition for terminating
   simulation

END   ! End of derivative block
END   ! End of dynamic section
```

Another kind of code section, the TERMINAL block, can also be used here to execute statements that should only be calculated at the end of the run (e.g., increase the concentration, go back to a labelled statement in the INITIAL block, and start again).

```
END   ! End of program
```

MODEL EVALUATION

The following section discusses various issues associated with the evaluation of a PBPK model. Once an initial model has been developed, it must be evaluated on the basis of its conformance with experimental data. In some cases, the model may be exercised to predict conditions under which experimental data should be collected in order to verify or improve model performance. Comparison of the resulting data with the model predictions may suggest that revision of the model will be required. Similarly, a PBPK model designed for one compound or application may be adapted to another compound or application, requiring modification of the model structure and parameters. It is imperative that revision or modification of a model is conducted with the same level of rigor applied during initial model development, and that structures are not added to the model with no justification other than that they improve the agreement of the model with a particular data set.

In addition to comparing model predictions to experimental data, model evaluation includes assessing the biological plausibility of the model structures and input parameters, and the resulting confidence that can be placed in extrapolations performed by the model [151]. Both elements of testing the model, kinetic validation and mechanistic validation, are necessary to provide confidence in the model. Unfortunately, there is a temptation to accept kinetic validation alone, particularly when data for mechanistic validation are unavailable. It should be remembered, however, that the simple act of adding equations and parameters to a model will, in itself, increase the flexibility of the model to fit data. Therefore, every attempt should be made to obtain additional experimental data to provide support for the mechanistic hypothesis underlying the model structure.

Model Documentation

In cases where a model previously developed by one investigator is being evaluated for use in a different application by another investigator, adequate model documentation is critical for the evaluation of the model. The documentation for a PBPK model should include sufficient information about the model so that an experienced modeler could accurately reproduce its structure and parameterization. Usually the suitable documentation of a model will require a combination of one or more *box-and-arrow* model diagrams together with any equations that cannot be unequivocally derived from the diagrams. Model diagrams should clearly differentiate blood flow from other transport (e.g., biliary excretion) or metabolism, and arrows should be used where the direction of transport could be ambiguous. All tissue compartments, metabolism pathways, routes of exposure, and routes of elimination should be clearly and accurately presented. All equations should be dimensionally consistent and in standard mathematical notation. Generic equations (e.g., for tissue i) can help to keep the description brief but complete. The values used for all model parameters should be provided, with units. If any of the listed parameter values are based on allometric scaling, a footnote should provide the BW used to obtain the allometric constant as well as the power of BW used in the scaling.

Model Validation

Internal validation (verification) consists of the evaluation of the mathematical correctness of the model [71]. It is best accomplished on the actual model code but, if necessary, can be performed on appropriate documentation of the model structure and parameters, as described earlier (assuming, of course, that the actual model code accurately reflects the

model documentation). A more important issue regards the provision of evidence for external validation (sometimes referred to as evaluation). The process of evaluating the sufficiency of the model for its intended purpose requires a demonstration of the ability of the model to predict the behavior of experimental data different from that on which it was based.

Whereas a simulation is intended simply to reproduce the behavior of a system, a model is intended to confirm a hypothesis concerning the nature of the system. Therefore, model validation should demonstrate the ability of the model to predict the behavior of the system under conditions that test the principal aspects of the underlying hypothetical structure. While quantitative tests of goodness of fit may often be a useful aspect of the verification process, the more important consideration may be the ability of the model to provide an accurate prediction of the general behavior of the data in the intended application.

Where only some aspects of the model can be verified, it is particularly important to assess the uncertainty associated with the aspects that are untested. For example, a model of a compound and its metabolites that is intended for use in cross-species extrapolation to humans would preferably be verified using data in different species, including humans, for both the parent compound and the metabolites. If only parent compound data are available in the human, the correspondence of metabolite predictions with data in several animal species could be used as a surrogate, but this deficiency should be carefully considered when applying the model to predict human metabolism.

In some cases, it is necessary to use all of the available data to support model development and parameterization. Unfortunately, this type of modeling can easily become a form of self-fulfilling prophecy: models are logically strongest when they fail, but psychologically most appealing when they succeed [70]. Under these conditions, model verification can be particularly difficult, putting an additional burden on the investigators to substantiate the trustworthiness of the model for its intended purpose. Nevertheless, a combined model development and verification can often be successfully performed, particularly for models intended for interpolation, integration, and comparison of data rather than for true extrapolation.

Parameter Verification

In addition to evaluating the performance of the model against experimental data, the model should be evaluated in terms of the plausibility of its parameters. This evaluation is particularly important in the case of PBPK models, where the parameters generally possess biological significance and can therefore be evaluated for plausibility independent of the context of the model. The source of each model input parameter value should be identified, whether it was obtained from prior literature, determined directly by experiment, or estimated by fitting a model output to experimental data. Parameter estimates derived independently of tissue time course or dose–response data are preferred. To the extent feasible, the degree of uncertainty regarding the parameter values should also be evaluated. The empirically derived *law of reciprocal certainty* states that the more important the model parameter, the less certain will be its value. In accordance with this principle, the most difficult, and typically most important, parameter determination for PBPK models is the characterization of the metabolism parameters.

When parameter estimation has been performed by optimizing model output to experimental data, the investigator must assure that the parameter is adequately identifiable from the data [71]. Due to the confounding effects of model error, overparameterization, and parameter correlation, it is quite possible for an optimization algorithm to obtain a better fit to a particular data set by modifying a parameter that in fact should not be identified on the basis of that data set. Also, when an automatic optimization routine is employed, it should be restarted with a variety of initial parameter values to assure that the routine has not stopped at a local optimum. These precautions are particularly important when more than one parameter is being estimated simultaneously, since the parameters in biologically based models are often highly correlated, making independent estimation difficult. Estimates of parameter variance obtained from automatic optimization routines should be viewed as lower-bound estimates of true parameter uncertainty since only a local, linearized variance is typically calculated. In characterizing parameter uncertainty, it is probably more instructive to determine what ranges of parameter values are clearly inconsistent with the data than to accept a local, linearized variance estimate provided by the optimization algorithm.

It is usually necessary for the investigator to repeatedly vary the model parameters manually to obtain a sense of their identifiability and correlation under various experimental conditions, although some simulation languages include routines for calculating parameter sensitivity and covariance or for plotting confidence region contours. Sensitivity analysis and Monte Carlo uncertainty analysis techniques can serve as useful methods to estimate the impact of input parameter uncertainty on the uncertainty of model outputs. However, as stated earlier, care should be taken to avoid violation of mass balance when parameters are varied by sensitivity or Monte Carlo algorithms, particularly where blood flows are affected.

Sensitivity Analysis

To the extent that a particular PBPK model correctly reflects the physiological and biochemical processes underlying the pharmacokinetics of a compound, exercising the model can provide a means for identifying the most important physiological and biochemical parameters determining the pharmacokinetic behavior of the compound under different conditions [67]. The technique for obtaining this information is known as sensitivity analysis and can be performed by two different methods. Analytical sensitivity coefficients are defined as the ratio of the change in a model output to the change in a model parameter that produced it. To obtain

a sensitivity coefficient by this method, the model is run for the exposure scenario of interest using the preferred values of the input parameters, and the resulting output (e.g., brain concentration) is recorded. The model is then run again with the value of one of the input parameters varied slightly. Typically, a 1% change is appropriate. The ratio of the resulting incremental change in the output to the change in the input represents the sensitivity coefficient. It is usually more convenient to use log-normalized sensitivity coefficients, which represent the ratio of the fractional change in output to the fractional change in input. For example, if a 1% increase in an input parameter resulted in a 0.5% decrease in the output, the log-normalized sensitivity coefficient would be −0.5. Log-normalized sensitivity coefficients >1.0 in absolute value represent amplification of input error and indicate that any uncertainty in the value of the parameter will result in even greater uncertainty in the output. An alternative approach is to conduct a Monte Carlo analysis, as described later, and then to perform a simple correlation analysis of the model outputs and input parameters. This type of approach is often referred to as global sensitivity analysis [152]. Both methods have specific advantages. The analytical sensitivity coefficient most accurately represents the functional relationship of the output to the specific input under the conditions being modeled. The advantage of the global sensitivity analysis is that it also reflects the impact of interactions between the parameters during the Monte Carlo analysis.

Uncertainty and Variability Analysis

Evaluations of the uncertainty and/or the variability associated with the predictions of a PBPK model are often performed using the Monte Carlo simulation approach [153–155]. In a Monte Carlo simulation, a probability distribution for each of the PBPK model parameters is randomly sampled, and the model is run using the chosen set of parameter values. This process is repeated a large number of times until the probability distribution for the desired model output has been created. Generally speaking, 1000 iterations or more may be required to ensure the reproducibility of the mean and standard deviation of the output distributions as well as the 1st through 99th percentiles. To the extent that the input parameter distributions adequately characterize the uncertainty in the inputs, and assuming that the parameters are reasonably independent, the resulting output distribution will provide a useful estimate of the uncertainty associated with the model outputs. If simulations are performed so that the probability distribution for PBPK model parameters represents the variability expected in the human population, then the Monte Carlo analysis will result in the simulation of the pharmacokinetics expected for a population.

Due to its physiological structure, many of the parameters in a PBPK model are interdependent. For example, the blood flows must add up to the total cardiac output, and the tissue volumes (including those not included in the model) must add up to the BW. In addition, some physiological parameters are naturally correlated, such as cardiac output and respiratory ventilation rate, and these correlations should be taken into account during the Monte Carlo analysis.

In performing a Monte Carlo analysis, it is important to distinguish uncertainty from variability. As it relates to the impact of pharmacokinetics in risk assessment, uncertainty can be defined as the possible error in estimating the *true* value of a parameter for a representative (*average*) person. Variability, on the other hand, should only be considered to represent true interindividual differences. Understood in these terms, uncertainty is a defect (lack of certainty) that can typically be reduced by experimentation, and variability is a fact of life that must be considered regardless of the risk assessment methodology used. An elegant approach for separately documenting the impact of uncertainty and variability is 2D Monte Carlo, in which distributions for both uncertainty and variability are developed and multiple Monte Carlo runs are used to convolute the two aspects of overall uncertainty. Unfortunately, in practice, it is often difficult to differentiate the contribution of variability and uncertainty to the observed variation in the reported measurements of a particular parameter [156]. A hierarchical Bayesian approach, known as Markov chain Monte Carlo (MCMC) simulation, has the potential to refine prior estimates of parameter uncertainty and variability on the basis of the agreement of model predictions to experimental data. MCMC has been used to characterize the uncertainty and variability in PBPK model predictions [157].

Although sophisticated methods of uncertainty analysis can provide valuable information, very simple techniques can also be used as a way to effectively communicate uncertainty. By simply determining the minimum and maximum value of an uncertain but important model parameter, and performing a simulation for a range of values, the impact of that parameter on pharmacokinetic predictions can be illustrated. When predicting human pharmacokinetics based on animal data, there is always uncertainty. Quantifying key uncertainties and providing a range of possible outcomes that could all be reasonable based on the current knowledge of the pharmacokinetic properties of the compound will allow for more informed decision-making.

Collection of Critical Data

As with model development, the best approach to model evaluation is within the context of the scientific method. The most effective way to evaluate a PBPK model is to exercise the model to generate a quantitative hypothesis, that is, to predict the behavior of the system of interest under conditions *outside the envelope* of the data used to develop the model (at shorter/longer durations, higher/lower concentrations, different routes, different species, etc.). In particular, if there is an element of the model that remains in question, the model can be exercised to determine the experimental design under which the specific model element can best be tested. For example, if there is uncertainty regarding whether uptake into a particular tissue is flow or diffusion limited, alternative forms of the model can be used to compare predicted

tissue concentration time courses under each of the limiting assumptions under various experimental conditions. The experimental design and sampling time that maximize the difference between the predicted tissue concentrations under the two assumptions can then serve as the basis for the actual experimental data collection. Once the critical data have been collected, the same model can also be used to support a more quantitative experimental inference. In the case of the tissue uptake question just described, not only can the a priori model predictions be compared with the observed data to test the alternative hypotheses, but the model can also be used a posteriori to estimate the quantitative extent of any observed diffusion limitation (i.e., to estimate the relevant model parameter by fitting the data). If, on the other hand, the model is unable to reproduce the experimental data under either assumption, it may be necessary to reevaluate other aspects of the model structure. The key difference between research and analysis is the iterative nature of the former. It has wisely been said, "If we knew when we started what we had to do to finish, they'd call it search, not research."

Model Revision

An attempt to model the metabolism of allyl chloride [67] serves as an excellent example of the process of model refinement and validation. As mentioned earlier, in a gas uptake experiment, several animals are maintained in a small enclosed chamber while the air in the chamber is recirculated, with replenishment of oxygen and scrubbing of carbon dioxide. A small amount of a volatile chemical is then allowed to vaporize into the chamber, and the concentration of the chemical in the chamber air is monitored over time. In this design, any loss of the chemical from the chamber air reflects uptake into the animals. After a short period of time during which the chemical achieves equilibration with the animals' tissues, any further uptake represents the replacement of chemical removed from the animals by metabolism. Analysis of gas uptake data with a PBPK model has been used successfully to determine the metabolic parameters for a number of chemicals [93].

In an example of a successful gas uptake analysis, Gargas et al. [94] described the closed-chamber kinetics of methylene chloride using a PBPK model, that included two metabolic pathways: one saturable, representing oxidation by cytochrome P450 enzymes, and one linear, representing conjugation with glutathione (Figure 6.9). As can be seen in this figure, there is a marked concentration dependence of the observed rate of loss of this chemical from the chamber. The initial decrease in chamber concentration in all of the experiments results from the uptake of chemical into the animal tissues. Subsequent uptake is a function of the metabolic clearance in the animals, and the complex behavior reflects the transition from partially saturated metabolism at higher concentrations to linearity in the low-concentration regime. The PBPK model reproduced this complex behavior with a single set of parameters because the model structure appropriately captures the concentration dependence of the rate of metabolism.

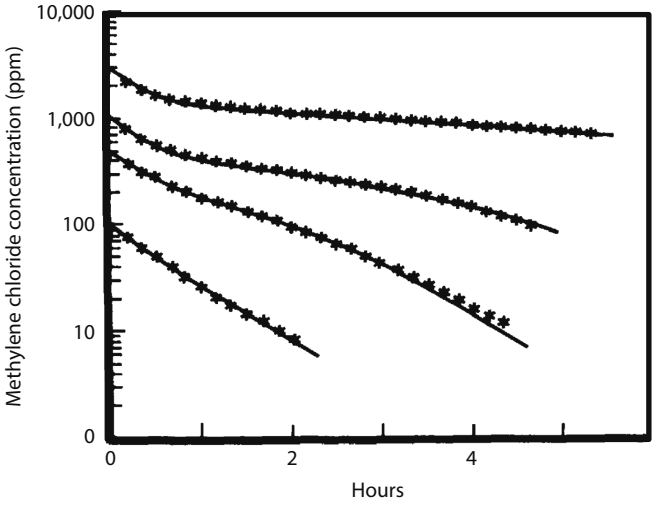


FIGURE 6.9 Gas uptake experiment. Concentration (ppm) of methylene chloride in a closed, recirculated chamber containing three Fischer 344 rats. Initial chamber concentrations were (top to bottom) 3000, 1000, 500, and 100 ppm. Solid lines show the predictions of the model for a V_{max} of 4.0 mg/h/kg, a K_m of 0.3 mg/L, and a first-order rate constant of 2.0/h/kg, while symbols represent the measured chamber atmosphere concentrations.

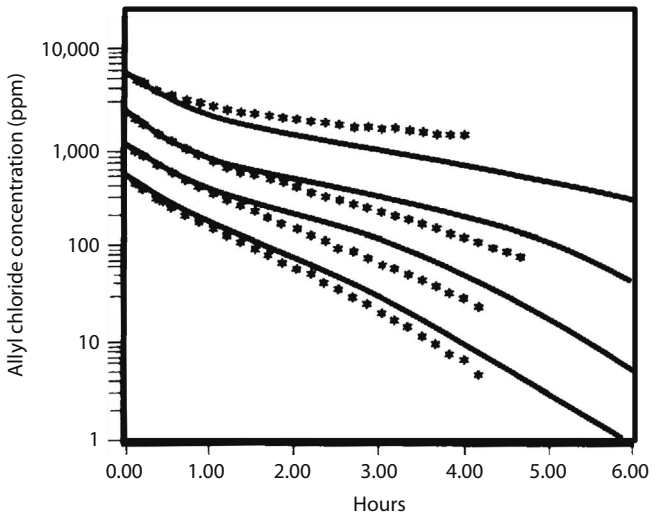


FIGURE 6.10 Model failure. Concentration (ppm) of allyl chloride in a closed, recirculated chamber containing three Fischer 344 rats. Initial chamber concentrations were (top to bottom) 5000, 2000, 1000, and 500 ppm. Symbols represent the measured chamber atmosphere concentrations. The curves represent the best result that could be obtained from an attempt to fit all of the data with a single set of metabolic constants using the same closed-chamber model structure as in Figure 6.9.

A similar analysis of gas uptake experiments with allyl chloride using the same model structure was less successful. The smooth curves shown in Figure 6.10 are the best fit that could be obtained to the observed allyl chloride chamber concentration data assuming a saturable pathway and a first-order pathway with parameters that were independent of concentration. Using this model structure, there were large systematic

errors associated with the predicted curves. The model predictions for the highest initial concentration were uniformly lower than the data, while the predictions for the intermediate initial concentrations were uniformly higher than the data. A much better fit could be obtained by setting the first-order rate constant to a lower value at the higher concentration; this approach would provide a better correspondence between the data and the model predictions, but would not provide a basis for extrapolating to different exposure conditions.

The nature of the discrepancy between the PBPK model and the data for allyl chloride suggested the presence of a dose-dependent limitation on metabolism not included in the model structure. This indication was consistent with other experimental evidence indicating that the conjugative metabolism of allyl chloride depletes glutathione, a necessary cofactor for the linear conjugation pathway. The conjugation pathway for reaction of methylene chloride and glutathione regenerates glutathione, but in the case of allyl chloride, glutathione is consumed by the conjugation reaction. To adequately reflect the biological basis of the kinetic behavior, it was necessary to model the time dependence of hepatic glutathione. To accomplish this, the mathematical model of the closed-chamber experiment was expanded to include a more complete description of the glutathione-dependent pathway. The expanded model structure used for this description [158] included a zero-order production of glutathione and a first-order consumption rate that was increased by reaction of the glutathione with allyl chloride; glutathione resynthesis was inversely related to the instantaneous glutathione concentration. This description provided a much improved correspondence between the data and predicted behavior (Figure 6.11). Of course, the improvement in fit was obtained at the expense of adding several new glutathione-related parameters to the model. To ensure that the improved fit is not just a consequence of the additional parameters providing more freedom to the model for fitting the uptake data, a separate test of the hypothesis underlying the added model structure (depletion of glutathione) was necessary. Therefore, the expanded model was also used to predict both allyl chloride and hepatic glutathione concentrations following constant-concentration inhalation exposures. Model predictions for end-exposure hepatic glutathione concentrations compared very favorably with actual data obtained in separate experiments (Table 6.4).

Note: Glutathione depletion data were graciously supplied by John Waechter, Dow Chemical Co., Midland, Michigan. For the purpose of this comparison, the basal glutathione consumption rate in the model was adjusted to obtain rough agreement with the controls in each experiment. This basal consumption rate was then used to simulate the associated exposure.

Reiterating the key points of this example as follows:

1. A PBPK model that had successfully described experimental results for a number of chemicals was unable to reproduce similar kinetic data on another chemical.

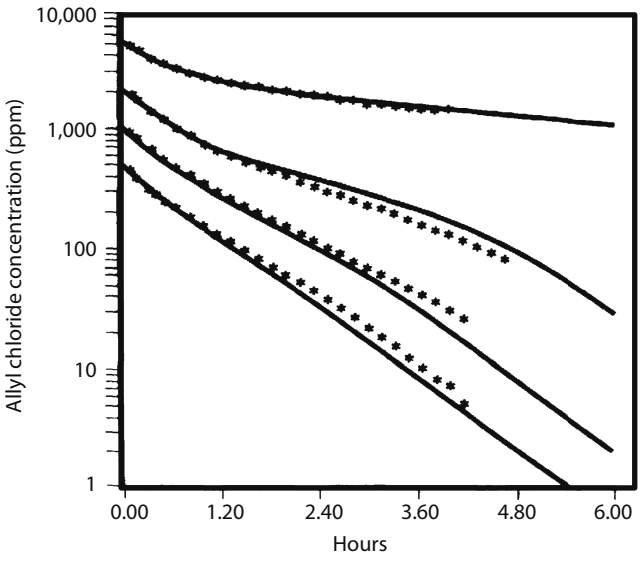


FIGURE 6.11 Cofactor depletion. Symbols represent the same experimental data as in Figure 6.10. The curves show the predictions of the expanded model, which not only included depletion of glutathione by reaction with allyl chloride, but also provided for regulation of glutathione biosynthesis on the basis of the instantaneous glutathione concentration, as described in the text.

2. A hypothesis was developed that depletion of a necessary cofactor was affecting metabolism. This hypothesis was based on
   a. The nature of the discrepancy between the model predictions and the kinetic data
   b. Other available information about the nature of the chemical's biochemical interactions
3. The code for the PBPK model was altered to include additional mass balance equations describing the depletion of this cofactor, and its resynthesis, as well as the resulting impact on metabolism.

TABLE 6.4
Predicted Glutathione Depletion Caused by Inhalation Exposure to Allyl Chloride

Concentration (ppm)	Depletion (μM)	
	Observed	Predicted
0	7080 ± 120	7088
10	7290 ± 130	6998
0	7230 ± 80	7238
100	5660 ± 90	5939
0	7340 ± 180	7341
1000	970 ± 10	839
0	6890 ± 710	6890
2000	464 ± 60	399

4. The modification to the model was then tested in two ways:
   a. By testing the ability of the new model structure to simulate the kinetic data that the original model was unable to reproduce
   b. By testing the underlying hypothesis regarding cofactor depletion against experimental data on glutathione depletion from a separate experiment

Both elements of testing a model, kinetic validation and mechanistic validation, are necessary to provide confidence in the model. Unfortunately, there is a temptation to accept kinetic validation alone, particularly when data for mechanistic validation are unavailable. It should be remembered, however, that the simple act of adding equations and parameters to a model will, in itself, increase the flexibility of the model to fit data. Therefore, every attempt should be made to obtain additional experimental data to provide support for the mechanistic hypothesis underlying the model structure.

PBPK MODEL APPLICATIONS

The following sections will highlight three areas of application: cancer risk assessment, drug development, and evaluation of developmental toxicity.

Risk Assessment

A properly validated PBPK model can be used to perform the high-to-low dose, dose route, and interspecies extrapolations necessary for estimating human risk on the basis of animal toxicology studies [29,64,84,159–172]. Target tissue dosimetry provided by PBPK modeling is also an essential component in models of pharmacodynamics, such as acetylcholinesterase inhibition [59,173] or mixture interactions [170,174–176]. A number of reviews, some quite extensive, have been published on the application of PBPK modeling in toxicity testing and risk assessment [12–18].

Example of PBPK Modeling in Risk Assessment: Cancer Risk Assessment for Methylene Chloride

In 1985, the National Toxicology Program reported the final results of animal studies indicating that lifetime inhalation of high concentrations (2000 and 4000 ppm) of methylene chloride produced significantly increased incidences of lung and liver tumors in B6C3F1 mice. As a result, the Environmental Protection Agency (EPA) classified methylene chloride as a probable human carcinogen. The risk estimate for methylene chloride was calculated using the approach typically employed by the agency for animal studies: a low-dose linear dose–response model was used to relate tumor outcomes in the bioassay with the dose of methylene chloride in mg/kg/day. Since the studies in this case were inhalation exposures, the doses were calculated as the product of ventilation rate, concentration, and duration of exposure. The animal doses were then decreased by a factor of roughly 13 to obtain the equivalent human doses, calculated as the cube root of the ratio of human BW to bioassay animal BW. This factor, known as the body surface area (BSA) correction, is included in the default EPA approach to account for the possibility that chemical carcinogens could be more potent in humans than in experimental animals.

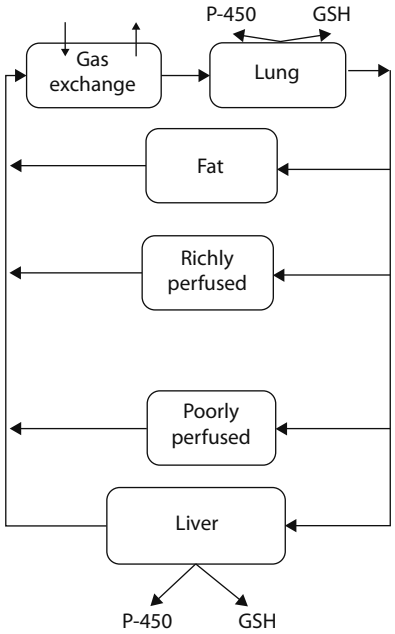


FIGURE 6.12 PBPK model used in the cancer risk assessment for methylene chloride. (Adapted from Andersen, M.E. et al., *Toxicol. Appl. Pharmacol.*, 87, 185, 1987.)

In 1986, Andersen and coworkers at Wright-Patterson Air Force Base and at Dow Chemical Company provided the EPA with their PBPK model for methylene chloride (Figure 6.12) and proposed its use in support of the EPA's carcinogenic risk assessment on that chemical. The suggestion resulted in considerable controversy and eventually led to a workshop sponsored by the National Academy of Science to consider the usefulness of PBPK modeling for chemical risk assessment. The scientific consensus following the workshop was that "relevant PBPK data can be used to reduce uncertainty in extrapolation and risk assessment" [11].

The pharmacokinetic model used in the methylene chloride risk assessment [64] was actually a minor modification of a more extensive model [94,159] that had previously been developed to study the kinetics and metabolism of dihalomethanes. The model was capable of predicting the time course of the parent chemical, as well as the production of metabolites by both a glutathione transferase pathway, which is linear in the physiologically relevant range, and a saturable mixed function oxidase pathway. The model was also capable of describing the production of carbon monoxide during the oxidative metabolism and its subsequent binding to hemoglobin in the blood to produce carboxyhemoglobin (HbCO), which could be measured in experiments to test the model's predictions for saturable metabolism. The model provided a coherent

TABLE 6.5
Comparison of Dose Metrics with Tumor Incidence in Methylene Chloride Bioassays

	Inhalation			Drinking Water	
	Control	2000 ppm	4000 ppm	Control	250 mg/kg/day
Liver					
Tumor incidence[a]	6.7	35	87	19	28
Oxidative pathway[b]	0	3575	3701	0	5197
Conjugation pathway[b]	0	851	1811	0	15
Parent chemical[c]	0	362	771	0	6
Lung					
Tumor incidence	6.7	65	89		
Oxidative pathway	0	1531	1583		
Conjugation pathway	0	123	256		
Parent chemical	0	381	794		

[a] Incidence (percent) for adenomas plus carcinomas.
[b] Average daily amount metabolized in the target tissue divided by the volume of the tissue.
[c] Average daily AUC of methylene chloride in the target tissue.

description of data from both rodents and humans for several routes of exposure and a number of different dihalomethanes.

One of the crucial steps in the pharmacokinetic risk assessment was the determination of the appropriate measure of dose to use in extrapolating from the tumor incidence found in the animal studies to the tumor incidence expected for human exposure. To support the determination of the appropriate dose surrogate, the PBPK model of methylene chloride [159] was expanded to include metabolism in the principal target tissues identified by the National Toxicology Program bioassay: mouse lung and liver. The expanded model could then be used to estimate the amount of oxidation and conjugation expected in these tissues for comparison with the tumor incidence observed in the methylene chloride bioassays (Table 6.5). Both metabolic pathways were thought to produce reactive intermediates that theoretically could be responsible for the carcinogenic activity. However, the calculated dose–response for the tissue dose from the oxidative pathway at the two inhalation bioassay concentrations (2000 and 4000 ppm) was essentially flat, while the number of tumors observed increased (Table 6.5). Moreover, similarly high tissue doses from the oxidative pathway were also predicted at the concentrations used in a negative drinking water study. It was therefore considered very unlikely that the tumor induction was caused by the activity of this metabolic pathway. However, the expected yield of the glutathione-dependent pathway was calculated to increase linearly at the inhalation bioassay concentrations, and very little activity was predicted under the drinking water bioassay conditions. These results were most consistent with the possibility of a reactive glutathione metabolite, presumably chloromethylglutathione, being involved in cancer induction. In this analysis, the possible role of the parent chemical itself in the observed tumorigenicity was discounted due to its chemical inertness and the lack of evidence for any potential mechanism of action.

Once the dose surrogate was selected, the PBPK model predictions for the effective dose to the target tissues at the bioassay concentrations could be used in place of the default *inhaled dose* calculations as inputs for the calculation of carcinogenic potency in the animal bioassay. The same PBPK model, but with human parameter values, was then used to relate an inhaled concentration in the human to the target tissue dose corresponding to a given risk. In these calculations, the risks associated with a given target tissue dose were considered to be equivalent in humans and rodents. The resulting pharmacokinetic risk estimates were roughly a factor of 100 lower than those obtained with the default approach. However, in adopting the pharmacokinetic approach, the EPA included their standard BSA factor for calculating the equivalent human doses, increasing the pharmacokinetic risk estimates by a factor of roughly 13, and resulting in a net reduction from the default risk estimates of about ninefold. Since that time, the use of BSA scaling has been discontinued in cases where cross-species dosimetry is performed with a PBPK model [177].

Drug Development

PBPK models can be developed using pharmacokinetic data typically generated during drug discovery and can be used to address important issues throughout the drug development process. At early stages, a PBPK model can be developed using available in vitro data and verified against in vivo PK data. Because the model links in vitro data to in vivo PK, it can be used to determine which in vitro data are most important for improving the PK of a series of compounds. These early models can be used to understand kinetic properties of a compound. At later stages, PBPK/PD models can be used to predict the therapeutic window and to provide a rational basis to choose the compound most likely to have success

in the clinic. Also, PBPK models can be used to understand the quantitative implications of uncertainties and therefore to prioritize experimentation. During clinical development, PBPK models provide a rational basis for predicting variability of human PK resulting from differences in age, gender, and genetic polymorphisms (e.g., CYP 2D6 weak or strong metabolizers) and from physiological changes resulting from a disease state. Therefore, PBPK models can help to predict the therapeutic window even in subpopulations with PK differing from the norm.

The past 10 years have seen tremendous advances in the capabilities of generic PBPK models that can simulate PK for humans or preclinical species based on a combination of physicochemical properties and in vitro data. Such generic PBPK models can, of course, be constructed using programming packages such as MATLAB, acslX, or Berkeley Madonna. However, powerful commercial PBPK simulation platforms, which incorporate detailed physiologically based absorption models into the traditional whole-body PBPK model, are also now available. Such packages include GastroPlus™ (Simulations Plus Inc., www.simulations-plus.com), SimCyp (Simcyp, www.simcyp.com), and PK-Sim® (Bayer Technology Services, www.pksim.com). These tools allow easy incorporation of preclinical ADME data into a PBPK model for preclinical species and humans. The availability of such tools has greatly simplified the technical use of PBPK models; however, a good understanding of the models and underlying equations is still essential in order to guarantee good interpretation of output.

A number of recent publications provide useful insights into the application of PBPK modeling in drug development [19–24].

Example of PBPK Modeling in Drug Development: Safety Assessment for All-trans Retinoic Acid

A PBPK model for all-trans retinoic acid (ATRA; tretinoin) was developed in order to provide a coherent description of the absorption, distribution, metabolism, and excretion of the compound and its metabolites across species and routes of administration [85]. The goal of developing such a model was to provide a more biologically relevant dose measure than administered dose for assessing the human teratogenic risk from ATRA.

The PBPK model developed for ATRA (Figure 6.13) provided a full physiological description for ATRA, with compartments for plasma, liver, gut, intestinal lumen, fat, skin, richly perfused tissues, slowly perfused tissues, placenta, and embryo. Both oxidation (to the 4-oxo derivative) and glucuronidation of ATRA were described with saturable kinetics. Conversion to the 13-cis isomer (13-cis-RA; isotretinoin) and the subsequent metabolism of that compound were also included (not shown in Figure 6.13). Simpler compartmental descriptions were used for the metabolites of ATRA, since there was no evidence that they preferentially partitioned into any of the body tissues. A third metabolic pathway, side chain oxidation producing CO_2, was also included. Dermal uptake was described by a two-compartment model, with

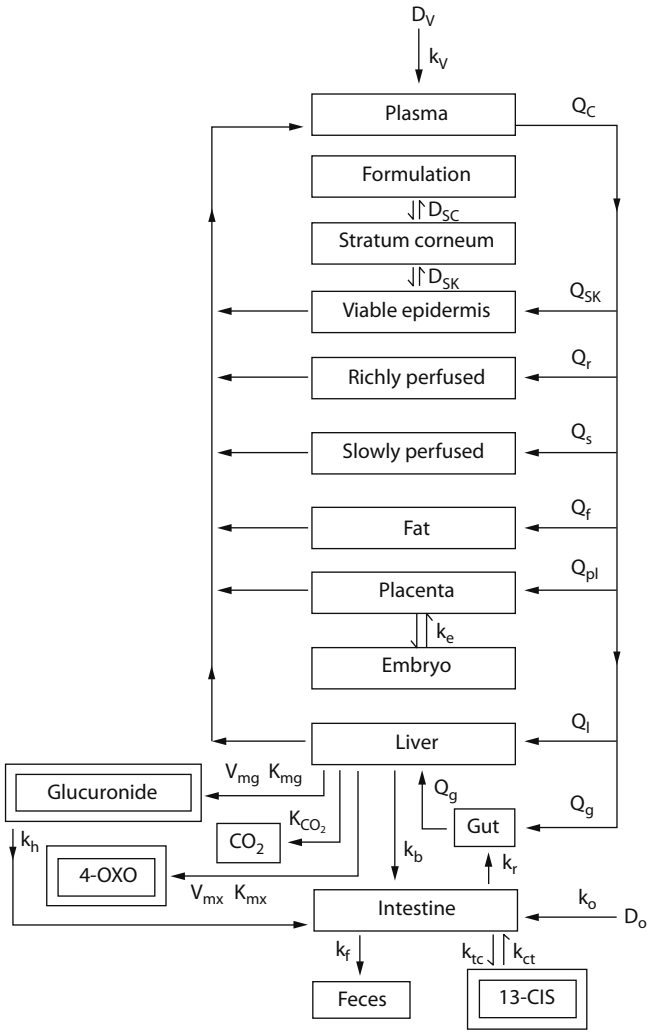


FIGURE 6.13 PBPK model used in safety assessment for all-trans retinoic acid. (Adapted from Clewell, H.J. et al., *J. Am. Acad. Dermatol.*, 36, S77, 1997.)

compartments representing the stratum corneum and viable epidermis. Excretion into urine and that into feces were modeled as first-order processes, with all chemicals being excreted in the feces and only glucuronides being excreted in the urine. Enterohepatic recirculation of ATRA and its metabolites was also described.

A key difference in the metabolism of ATRA across species is the predominance of the oxidative pathway in the rodent, in contrast with the predominance of the glucuronide pathway in the primate; therefore, the kinetic parameters in the human model were based on data from studies with nonhuman primates. The PBPK model for iv dosing of ATRA in the monkey was successfully scaled allometrically to predict the kinetics for oral dosing of human leukemia patients with 1.1 mg/kg ATRA. The only parameters adjusted in this extrapolation were those describing the rate of oral uptake. All other parameters were calculated from those for the monkey, except of course for the use of human physiological parameters. The model was also able to reproduce the observed kinetics for total radioactivity following

topical administration of ATRA. In contrast to the kinetic data, which could provide only an estimate of total exposure to ATRA plus its metabolites, the PBPK model was able to provide separate estimates of internal exposure to ATRA and its active and inactive metabolites. An important result of the modeling was to demonstrate that the low dose rate associated with dermal exposure results in high-efficiency clearance of ATRA to its inactive glucuronide metabolites, as opposed to oral dosing, where the higher dose rate leads to a greater proportion of active oxidative metabolites.

The model was used to simulate oral clinical dosing with ATRA, as well as minimal teratogenic doses of ATRA in the primate and rodent. Based on literature data on the teratogenic potential of the various chemical species, the most appropriate dose surrogate could be either Cmax or AUC for the total concentration of active retinoids. The glucuronides were not included in this dose measure since they do not cross the placenta. On the other hand, based on the activity at retinoic acid receptors, it could be argued that the most appropriate dose surrogate could be the peak concentration or AUC for ATRA alone. In either case, maternal plasma levels were used as a surrogate for fetal levels, based on evidence from animal studies that concentrations achieved in maternal and fetal plasma are similar. The calculated dose surrogates, as well as the estimated peak plasma level for total retinoids (which includes the glucuronides) for several species, are shown in Table 6.6.

One test of a useful dose surrogate is that similar values should be calculated at similar effect levels across species. For a minimal teratogenic effect, all of the dose surrogates in Table 6.6 (including administered dose) are within a factor of two to three across species. The most consistent dose surrogate is the Cmax for total active retinoids, which is essentially constant across species. Based on this dose measure, the internal exposure of patients receiving oral ATRA treatment for cancer is below the threshold for teratogenic effects by about a factor of 7–10. However, this comparison assumes that the maternal plasma concentration profile is representative of fetal exposure. If one takes into consideration the longer period of organogenesis in the human (around 35 days) as compared to the rodent (around 10 days) and assumes, as a worst case, fetal exposure at the maximum maternal concentration throughout the entire period, the margin of safety could be as low as 2–3. It is of interest that the kinetics of 13-*cis*-RA in the human are considerably different from those of ATRA. The compound 13-*cis*-RA has a much longer half-life than ATRA in the human, and oxidation, rather than glucuronidation, is the dominant form of metabolism. The calculated plasma concentrations and AUCs for total active retinoids following oral treatment with 13-*cis*-RA are in the same range as those causing teratogenic effects in animals. This result is consistent with the observation of teratogenic effects associated with the human use of 13-*cis*-RA (isotretinoin; Table 6.6). This PBPK model was used by FDA in their evaluation of the safety of a topical skin treatment containing ATRA, marking the first time a PBPK model had been used in a regulatory decision for a pharmaceutical.

Developmental Toxicity

The physiological structure of PBPK models is particularly useful for examining the effects of changing physiology on target tissue dosimetry, as in the case of exposure during the developmental period [61,62,178–180]. The past decade has seen a significant expansion of PBPK approaches to describe specific life stages in order to examine factors that may affect differential susceptibility of various populations to chemical exposure [87,99,100,162,163,181–191]. Developmental PBPK models show promise for predicting exposure and target tissue dose in the perinatal period, including periods of both gestation and lactation. More than for any other of its applications, the PBPK technologies applied to reproductive and

TABLE 6.6
Comparison of Dose Surrogates for Retinoic Acid Teratogenicity

			All-trans Retinoic Acid		Total Active Retinoids		Total Retinoids
	Route	Dose (mg/kg)	Cmax (ng/mL)	AUC (ng*h/mL)	Cmax (ng/mL)	AUC (ng*h/mL)	Cmax (ng-eq/mL)
Minimal teratogenic doses (all-trans RA)							
Mouse	oral	4.0	1048	1852	2681	12,658	3107
Rat	oral	2.5	943	2029	1918	13,554	2051
Monkey	oral	5.0	1830	3714	2294	5962	4983
Clinical doses (human)							
ATRA	oral	1.1	218	695	264	1088	1237
13-cis	oral	1.1	654[a]	3154[a]	1033	7103	1148

[a] Reflects concentration of 13-cis-RA rather than all-trans RA.

developmental toxicology can (1) reduce the use of animals in toxicity testing and (2) improve estimates of human exposure based on the extrapolation of kinetics across species. The ability to perform these cross-species extrapolation is particularly important, since these populations (i.e., fetus, neonate) are unlikely to be the targets of specific studies in human cohorts. Nevertheless, the application of PBPK techniques to perinatal periods adds to the complexity of model design—the modeler must account for many processes that are often omitted or simplified when describing adult pharmacokinetics.

In general, extension of a PBPK model to gestation or lactation requires revisions to model structures and alternative descriptions of physiological and kinetic parameters. Models describing gestation and lactation will have a larger number of compartments compared to those for the adult. These models may include descriptions of maternal tissues that participate in chemical transfer to the offspring (uterus, placenta, or mammary gland) and some level of detail for the fetus or neonate. New model compartments are necessary. As opposed to the adult, where tissue growth and blood flow often scale directly to BW, many tissues in the pregnant and lactating mother and in the fetus and neonate undergo complex changes in volume and blood flow over time. Biochemical processes that govern chemical kinetics are highly dynamic during the perinatal period. The developing liver and kidney often require time-dependent descriptions of metabolism and clearance. This final section on PBPK modeling applications discusses special considerations for the perinatal period and describes current approaches for describing chemical kinetics during gestation and lactation using PBPK models.

Considerations for Modeling of Gestation

Gestation model structure: At minimum, a gestational model must include both the mother and the fetus and account for transfer of chemicals between them (Figure 6.14). Placental transfer between dam and fetus is bidirectional. The placenta may act as a barrier, a site of metabolism, or a site of accumulation and active transport of chemicals from the mother to the fetus. The complexity of the model description for maternal–fetal kinetics depends on the characteristics of the test chemical. Usually, passive diffusion suffices for describing transfer between the maternal and fetal blood [61,85,178,179]. Other chemicals may require descriptions of the placenta to account for processes, such as active transport and metabolism, which can lead to dose-dependent chemical transfer kinetics [100,185]. Likewise, the description of the fetus can vary from a one-compartment (volume of distribution) model describing fetal blood or plasma [178,181,192–194] to more complex multitissue models that describe target tissue concentrations within the fetus [87,100,163,185,190,191]. As with simpler adult period PBPK models, the structure of life-stage-specific models should provide an appropriate level of detail to accurately describe chemical kinetics in the tissue of interest without adding unnecessary parameters.

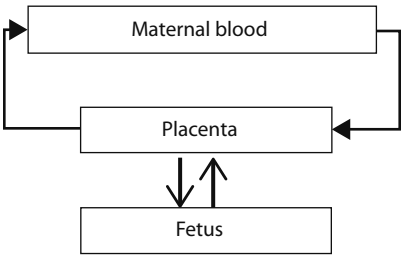


FIGURE 6.14 Simple PBPK model for gestation.

Maternal physiology during gestation: In gestation, several tissues experience rapid growth with changes in tissue volumes over time that cannot be accurately described using allometric scaling. In the rat, the mammary gland and maternal fat volumes increase more or less linearly over the course of gestation. The placenta has a more complex growth curve. The individual rat placenta has three stages of growth: (1) increase in yolk sac to day 10, (2) decrease in yolk sac after day 10, and (3) increase in chorioallantoic placenta from day 10 through term [195,196]. Neither the volumes of these tissues nor their blood flows are proportional to the BW. The fraction of maternal cardiac output to the mammary gland, fat, and yolk sac is likely proportional to tissue volumes; however, the blood flow to the chorioallantoic placenta increases more rapidly than does tissue volume. In rats and mice, these models must account for multiple pups per litter, each with its own placenta. The volume of the individual placenta multiplied by the number of fetuses gives the overall placental weight.

There are two common approaches to implement time-dependent changes in tissue volumes. One model method uses linear interpolation between the measured data to predict tissue volumes over time [61]. Another uses explicit equations to calculate growth curves [63,179]. The output from best-fit equation for tissue volumes over time gives the volumes. Blood flows then follow the volumes.

Maternal BW increases significantly during the course of gestation, primarily from increasing volumes of the fat, placenta, mammary gland, fat, and total fetal/litter volume. Therefore, the total change in maternal BW is the sum of the changes in these four tissue volumes and the initial (prepregnancy) BW (BW_{init}):

$$\text{Total BW} = BW_{init} + \Delta\text{Fat} + \Delta\text{Mammary Gland} + \Delta\text{Placenta} + \Delta\text{Fetal Weight}$$

Total cardiac output also increases over gestation and is modeled in the same way as the BW: cardiac output is described as the sum of the initial cardiac output and the increased blood flow to the fat, mammary gland, and placenta.

Fetal physiology: Fetal growth can be incorporated into the model using linear interpolation to predict the BW between measured data points or by using more explicit equations. The growth of the fetus is complex. In both the rodent and the human, PBPK models generally use a series of equations to describe the increase in fetal weight over time [179,192]. In the method of O'Flaherty et al. [179], a series of three equations describe fetal growth in the rat over the entire period

of gestation. The first equation described fetal growth from conception (day 1) to day 11 with an exponential curve with a starting value of zero and a final value equal to measured fetal weights on day 11. Due to the lack of measured data at earlier time points, the fetal volumes in early gestation were predicted based on the assumption of exponential growth. The following two equations—developed by fitting specific data on tissue volumes over time—described growth from days 11 to 18 and from day 18 to birth (day 21). The series of equations employed by O'Flaherty et al. [179] for describing volume for one rat fetus are shown as follows:

Days 1–11: $BW1_{Fet} = 1 * ((0.1206 * Days)**4.53)$
Days 11–18: $BW1_{Fet} = 1 * ((1.5 * [Days - 10])**2.8)$
Days 18–21: $BW1_{Fet} = (1 * (BW_{FetD18} + (BW_{Pup} - BW_{FetD18})/4) * (Days - 18))$

$BW1_{Fet}$ is the weight of one fetus, days is the number of days postconception, BW_{Pup} is the BW of the pup at birth, and BW_{FetD18} is the value for $BW1_{Fet}$ on day 18 calculated from the equation shown for days 11–18. In species that have multiple fetuses, such as the rat, the model describes the total fetal volume as the sum of the individual fetuses. Any subsequent descriptions of fetal kinetics are then generally calculated as an average for the entire litter, rather than describing each fetus individually. At very early time points (GD 11 in the rat), very little data are available for fetal volume. Some modelers limit simulations to later time points where data are available [61], while others predict early time points assuming exponential growth and passive diffusion of chemicals at early time points where the placenta is not fully developed (<GD 11) [179]. Allometric scaling is generally sufficient for estimating tissue volumes across species and ages in adult rats. However, it is unreliable when applied to the fetus. During fetal development, organs develop at different rates. When tissues are included as separate compartments in a model, these tissue volumes must be explicitly defined as time-varying model parameters.

Fetal exposure: The dose to the fetus depends on maternal kinetics (absorption, clearance, etc.) and placental transfer. Thus, the model must recapitulate maternal blood or plasma kinetics first, before achieving confidence in modeling the maternal–fetal transfer kinetics. Placental transfer is chemical specific. The physicochemical properties of the parent chemical or metabolites generally determine the extent of placental transfer as well as the possible differential retention of chemicals in the fetus. High-molecular-weight chemicals, including highly lipophilic chemicals, may be less efficiently transferred across the placenta. However, once in the fetus, these lipophilic chemicals are likely to have higher concentrations than in the mother (due to decreased placental transfer from the fetus to the mother). Passive diffusion between the maternal and fetal blood often provides a sufficient description of placental transfer for these chemicals.

The functions of the placenta are complex and diverse. The placenta may act as a barrier, a facilitator of transport for essential nutrients, and a site of metabolism. For chemicals that reach higher placental concentrations compared to dam or fetus or ones that are metabolized in the placenta, a placenta compartment provides necessary detail including equations for active transport from the maternal blood or metabolism of the parent chemical, followed by transfer of the chemical from the placenta to the fetus.

Maternal kinetic parameters during gestation: In addition to the many physiological changes occurring during pregnancy, the tissues in the mother undergo changes in the capacity for various metabolic processes. In particular, alterations in serum-binding protein concentrations, GFRs, and hepatic metabolic activity all contribute to different kinetics in the pregnant vs. naive female. Glomerular filtration increases during gestation, leading to increased clearance of chemicals that are eliminated in the urine. Reduced P450 and UGT activity in the liver, however, would result in reduced clearance of chemicals eliminated by phase I and II metabolism.

The use of these altered biochemical parameters in PBPK models is common in the human and rat [87,100,163,192]. Two approaches may be taken to describe these changing biochemical parameters. The parameters may be determined by fitting the model output to data collected in the tissues of pregnant animals. This is feasible in laboratory animals, but not the human. The parameters may also be set for the naive animal, based on ADME data, and then altered using published data on protein activity, protein concentration, or glomerular filtration during gestation. This second method has been used successfully in the rodent and human and is more useful for extrapolating to populations where tissue data are not available.

Kinetic parameters in the fetus: It is customary to describe fetal kinetics using first-order transfer rates between the mother and the fetal blood. Occasionally, however, fetal metabolism may play a role in fetal kinetics. Some phase I and phase II enzymes are present in fetal intestine and liver [197,198] and, especially in the late-term infant, may alter fetal/maternal transfer kinetics. For example, glucuronide conjugation of a chemical substrate in the fetus may reduce placental transfer and fetal clearance. Limited data are available for metabolizing enzyme protein concentrations in the rodent and human fetus. In vitro metabolism data may provide relative activities of fetal metabolizing enzymes for particular substrates compared to the adult. These relative metabolic capacities determined in vitro may be used to predict the fetal parameters for metabolism in vivo, particularly when the parameters are well characterized and validated in the adult.

Example of PBPK Modeling of Gestation: Di-n-Butyl Phthalate

In vivo, di-*n*-butyl phthalate (DBP) is quickly and efficiently hydrolyzed to monobutyl phthalate (MBP) in the gut (prior to absorption). MBP is then metabolized by P450 enzymes or conjugated to glucuronic acid by UDPGT in the liver. In rats, the major metabolites are the MBP and the MBP-glucuronide conjugate (MBP-G). The simple model schematic for the DBP PBPK model (Figure 6.15) shows only the fetus and the

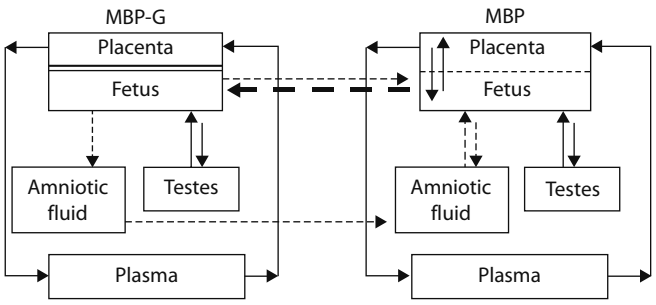


FIGURE 6.15 Model structure for DBP metabolite kinetics in the fetal rat. Dashed arrows indicate first-order processes and clearance rates (deconjugation). Bold dashed arrows represent saturable processes (glucuronide conjugation). Solid arrows represent blood flows to the tissue compartments.

maternal compartments responsible for fetal exposure. Full model structure, including all maternal compartments, is shown elsewhere [87]. The transfer of free MBP used passive diffusion between the placental plasma and the fetal plasma.

In the case of MBP, there was evidence that glucuronide conjugation took place in the fetus itself [87]. Rates for fetal glucuronidation were predicted based on in vitro data and implemented as follows. In vitro, phthalates are similar to steroidal substrates in terms of the isoforms of UDPGT for which they are substrates. Therefore, the fetal UDPGT activity in the model was estimated from the ratio of measured fetal/adult UDPGT activities toward several steroidal substrates from measured in vitro values on GD 17, 18, 19, 20, and 21 [199,200]. These relative activities served as the basis for calculating the value for maximum capacity of fetal UDPGT (VmaxLf) for GD 17, 18, 19, and 20:

$$\text{VmaxLf} = \text{VmaxL} * \text{RAfL} * \text{RMPC} * \text{RLW} * \text{numfet}$$

where VmaxL is the maximum capacity for glucuronide conjugation in the adult male rat (after scaling for BW), RAfL is the in vitro relative activity expressed as the ratio of fetal to maternal activity per mg microsomal protein, RMPC is the ratio of the microsomal protein content of the fetal and maternal liver (mg/g liver), RLW is the ratio of the fetal to maternal liver weight (g), and numfet is the number of fetuses per litter.

Using this description, together with the literature-derived parameters for glucuronide conjugation and hydrolysis, the model successfully described the MBP-G kinetics in the fetal plasma, lending support to the use of in vitro metabolism data in predicting kinetic parameters that change with time.

The final values for VmaxLf were coded into the ACSL model using a TABLE function. That is, a list of values for VmaxLf at specific times was created, and the program used linear interpolation to determine values at intermediate time points.

Considerations for Modeling of Lactation

Model structure: As with gestation, a lactation period mode must include the mother and the neonate and the transfer of chemicals between the two. Lactational exposure is by

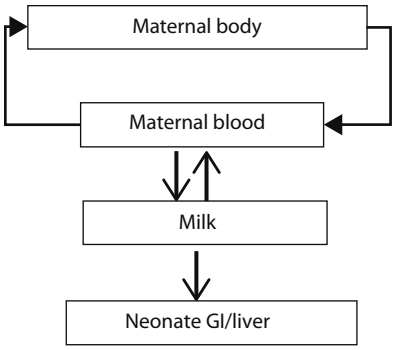


FIGURE 6.16 Simple PBPK model for lactational transfer.

transfer in milk through suckling, a process that is primarily unidirectional—from the mother to the neonate. In rodents, some transfer can occur from the neonate to the dam during grooming. Typically, the maternal model has a compartment for the mammary gland and the milk. Transfer to the neonate is described as a first-order rate, which serves as an oral dose to the neonate. Figure 6.16 shows a simple description of maternal–neonate chemical transfer by suckling.

Maternal physiology during lactation: During lactation, the volumes of some tissues change rapidly. The fat volume decreases from the late gestation weight, while the mammary gland tissue continues increasing after parturition. In these models, the volume of the milk must also be taken into account. Milk production and secretion are highly dynamic. The description of the milk compartment in the mother usually assumes the presence of a relatively static volume (averaging out the daily fluctuations). This residual volume has little effect on maternal BW. Maternal BW during lactation is calculated by adjusting the postbirth BW (end of gestation minus the placenta and fetus) by the change in the fat and mammary tissues and adding the weight of the residual milk [192]. The calculation for the maternal BW during lactation is based on BW_P, the maternal weight at parturition.

$$\text{Maternal BW} = BW_P - \text{placenta} - \text{fetus} + \Delta\,\text{mammary gland} + \Delta\,\text{fat} + \text{residual milk}$$

The maternal cardiac output decreases during lactation compared to gestation, due to the reduced demand in the absence of the uterus/placenta. However, blood flow to the mammary gland increases proportionally to the mammary gland volume.

Neonatal physiology: As opposed to the fetus, a large body of data exists for changes in BW, tissue growth, and even tissue blood flows in neonatal animals. Increases in BW are not linear in early life and must be explicitly described when simulations are performed for more than a single age. Neonatal growth can be incorporated using linear interpolation to predict the BW between measured data points or by using explicit equations [62,99,163,183,192]. Tissue growth during early life depends on the tissue where patterns of growth and development differ substantially. For example, in

the neonatal rat, the fat increases from approximately 2.7% to 11% of the total BW between postnatal days 2 and 16, followed by a gradual decrease to the adult value of 4.61% [111,201]. To the extent that the individual tissues must be explicitly defined in the model, the corresponding model parameters must account for the changing tissue volumes.

Neonatal exposure: The dose to the suckling neonate depends on maternal kinetics (absorption, clearance, etc). Thus, maternal exposure, clearance, and tissue distribution affect the dose of the chemical transferred to the offspring. Furthermore, for long-lived chemicals, fetal exposures significantly affect chemical concentrations in the neonate. In these cases, the prenatal exposure may be accounted for by linking the neonatal model to a gestation model or by accounting for initial tissue concentrations of the chemicals in the state variables (mass balance equations for the tissues) of the neonatal model. Neonatal dose from suckling is a function of the milk chemical concentration and the amount of milk ingested by the neonate. The physicochemical properties of the parent chemical or metabolites determine the extent of uptake into the milk from maternal blood. Highly lipophilic compounds readily concentrate in milk and transfer with breast milk during lactation, providing a potentially important route of postnatal exposure. Modeling uptake of chemicals into the milk usually follows passive diffusion and concentration through blood/milk partition coefficients.

Milk production and secretion are highly dynamic. Temporal variations occur within a single day and across the period of lactation. Daily variation in milk ingestion is typically omitted from models to reduce model complexity. As such, the neonatal dose from suckling is presented as the daily average. The pattern of milk production over lactation follows an inverse U-shape, with an increase after gestation, followed by a decrease near the time of weaning. In the rat and human, data are available for milk production/neonate milk ingestion [202,203]. Milk production data for the lactating rat can be incorporated into a PBPK model using the following TABLE function in ACSL:

```
TABLE KtransC,1,8/24,216,384,456,504,552,
720,960,1.18e-02,1.14e-02,1.01e-02,6.40e-03,4.17e-
03,3.69e-03,0.0,0.0/.
```

The data in this statement are for production rate of milk (L/h) divided by the weight of the litter—BWL [202]. This rate of milk production would be multiplied by the BW of the litter (which will also be changing over the course of lactation) and used together with the milk chemical concentration to describe neonatal dose from suckling [99]. In this formula, KtransC is the rate of milk production/milk transfer scaled by BWL. The values 1 and 8 preceding the brackets indicate the size of the data matrix (1 × 8). The first eight values inside of the brackets denote the time postbirth in hours, and the final eight numbers denote the measured values for milk production adjusted by litter weight.

Maternal kinetic parameters during lactation: During lactation, the maternal system is returning to its prepregnancy state with regard to many of the kinetic parameters (e.g., GFR and serum-binding proteins). These parameters may be determined by fitting the model output to data collected in the tissues of lactating animals. This approach is feasible in laboratory animals, but not in the human. The parameters may also be set for the naive animal, based on kinetic data, and then altered using published data on protein activity, protein concentration, or glomerular filtration during lactation. This method has been used successfully in the rodent and human and is more useful for extrapolating to populations where tissue data are not available.

Kinetic parameters in the neonate: Important kinetic parameters that show highly dynamic behavior in the neonate include glomerular filtration and metabolic enzyme activity. In both the human and rodents, changing enzyme expression and activity lead to significant alterations in chemical kinetic profiles. Likewise, the increasing GFRs associated with maturation of the kidneys lead to increased clearance for chemicals eliminated in the urine [99,163,182,183]. The most comprehensive analysis of the effect of metabolism, glomerular filtration, and physiological changes after birth on chemical kinetics was for the human [183]. This study incorporated age- and gender-specific differences in physiological and biochemical processes into a predictive PBPK model spanning the time from birth to adulthood. The resulting life-stage model was then exercised for several environmental chemicals with a variety of physicochemical, biochemical, and mode-of-action properties. The most important age-dependent pharmacokinetic factor was determined to be the reduced activity of many metabolic enzyme systems in the infant.

Example PBPK Model of Lactation: Perchlorate

Perchlorate (ClO_4^-), the soluble anion of the solid rocket fuel ammonium perchlorate, is a thyroid iodide uptake inhibitor and drinking water contaminant. Since ClO_4^- has a similar size and shape to that of iodide (I^-), it binds to sodium–iodide symporter (NIS) at the membrane of the thyroid follicle and reduces the amount of iodide available for hormone synthesis. The presence of NIS in the mammary gland [204] also leads to inhibition of iodide uptake in milk together with accumulation and transfer of the ClO_4^- anion, into the milk. Because ClO_4^- mimics I^-, both anions were described using a similar model structure (Figure 6.17). A full description of the model and explanation of model elements are available elsewhere [99]. This example will focus on the description of transfer of the free anions, ClO_4^- and I^-, between the lactating and suckling rat.

Two anion symporters in the mammary gland move I^- (and ClO_4^-) into the mammary gland via an active uptake mechanism: NIS and a second Pendrin transporter [204–208]. These two transport processes provide active anion uptake (1) from the mammary blood to the mammary gland and (2) from the mammary gland to the milk. Active uptake equations used Michaelis–Menten kinetics. In this case, the mammary gland accounted for the transfer of the two anions and the inhibition of I^- in the milk since both transporters are possible sites of competition for the anions (Figure 6.18).

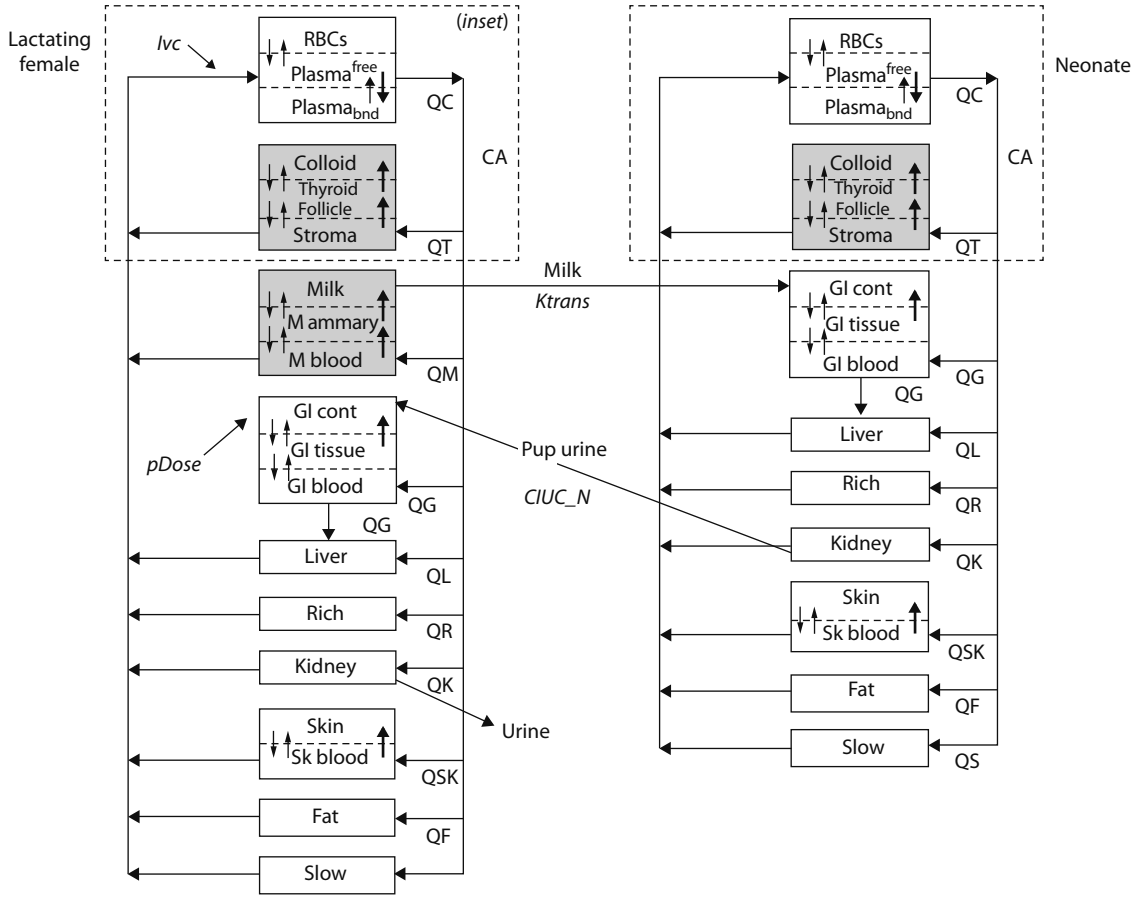


FIGURE 6.17 Schematic of perchlorate PBPK model for lactating dam (left) and neonate (right). Model structure for sister iodine model is identical with the exceptions of the inset area outlined with the dashed line. In the plasma and thyroid, some descriptions of iodine incorporation into hormones were included, which is described elsewhere. (From Clewell, R.A. et al., *Toxicol. Sci.*, 74, 416, 2003.)

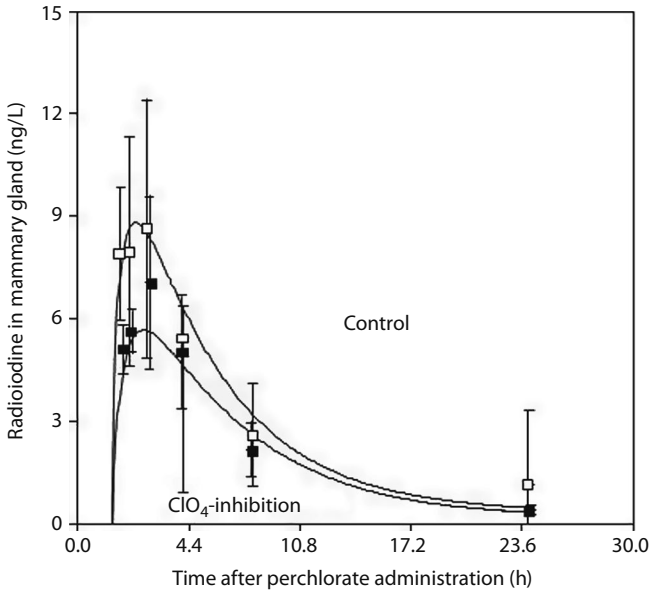


FIGURE 6.18 Radioiodide concentration in milk with and without ClO_4^- [162]. The top simulation and data indicate the control group. The lower simulation and data indicate the inhibition group. (Data from Potter, G.D. et al., *J. Biol. Chem.*, 234, 350, 1959.)

Clearly, it is not always necessary to include such detailed description of the mammary gland.

This model included transfer between pup urine and the maternal GI. This process reflects the ingestion of pup urine by the dam during grooming. In studies using radiolabeled iodine, as much as 60% of the dose administered to the neonate was transferred to the dam during grooming [209]. The importance of including these processes will vary depending on the relative amount of chemical eliminated via milk, which is determined by the pharmacokinetic properties of the compound [210]. For chemicals that are poorly transferred in the milk, the neonatal dose will present a negligible fraction of the dose, and ingestion of pup urine will not present a significant source of chemical to the dam. For chemicals that are efficiently transferred in milk, or where the chemical is administered to the neonate directly, this transfer from the pup to the dam may be important.

Comparing Susceptibility to Chemical Exposure across Life Stages

PBPK models for the developmental period can be used to determine which populations are likely to receive the greatest level of exposure to a particular chemical. The (ClO_4^- and I^-) models were developed for the naive adult, pregnant

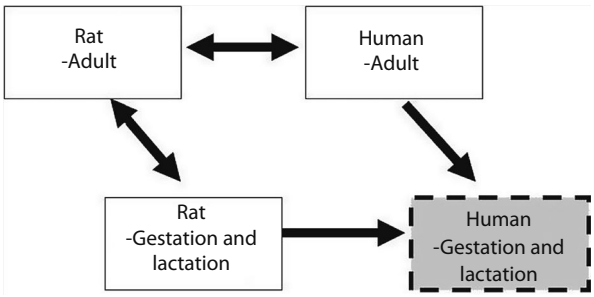


FIGURE 6.19 Parallelogram approach to estimating human developmental model parameters from rat perinatal models and rat and human adult models. Calculations are described in detail elsewhere. (From Clewell, R.A. et al., *J. Toxicol. Environ. Health A*, 70, 408, 2007.)

and fetal, and lactating and neonatal rat. These models were then used to extrapolate to human exposure using the *parallelogram approach* (Figure 6.19) [163]. Using the human models, together with descriptions of bottle-fed infants and extending the model from weaning to adulthood, it was possible to compare ClO_4^- exposure across all relevant life stages in the human (Figure 6.20). Using the published water ingestion rates for infants [211], a bottle-fed infant (1 month postpartum) would have the highest serum perchlorate levels, with an average serum AUC five times greater than that of a nonpregnant adult. The serum AUCs in fetus and breast-fed infant are approximately 3.5-fold higher than the adult. Thus, the fetus and the infant (bottle- and breast-fed) are the populations expected to have the greatest exposure to perchlorate (per kg BW). Relative exposure for several other chemicals across life stages has also been explored in the human [183,190]. The application of these life-stage PBPK models is an important tool in identifying susceptible populations for consideration in chemical risk assessments.

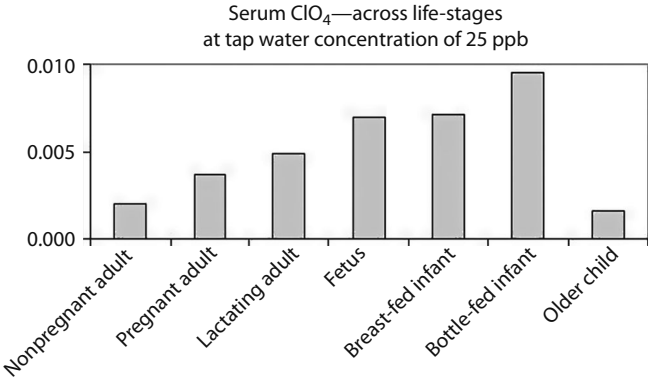


FIGURE 6.20 Predicted serum perchlorate area under the curve (AUC) in the adult, fetus, breast-fed infant, bottle-fed infant, and older child. Simulations in adults, fetus (gestation week 38), and breast-fed infant (postnatal month 1) were performed at daily maternal water intake of 0.029 L/kg-day (~2 L) and a drinking water perchlorate dose of 0.0007 mg/kg-day (25 ppb for ~70 kg adult). Simulations in the bottle-fed infant (postnatal month 1) and older child (7 years) assume daily water intake at 1 L/day at 25 ppb ClO_4^-.

CONCLUDING REMARKS

The desire to increase the biological basis of chemical risk assessments has driven the development of new methodologies such as PBPK modeling. The development and the application of PBPK models in turn demand well-formulated statements about the chemical mode of action. The requirement for an explicit mechanistic hypothesis gives PBPK models their power but at the same time serves as the greatest impediment to their acceptance by regulators. PBPK models also serve to make other uncertainties in the risk assessment more visible, such as cross-species and interindividual variation. In some cases, the increased visibility given to these uncertainties has led to improvements in the default risk assessment process. By replacing poorly characterized uncertainties (in the default approach) with definable model uncertainties, PBPK models have spurred the development and application of the sophisticated uncertainty analysis techniques, such as Monte Carlo analysis and hierarchical Bayesian analysis, that are now used to provide a better understanding of the range of risk estimates across a population consistent with the information available on a given chemical. It is crucial that this parallel development of biologically motivated descriptions of dosimetry and tissue response and methods for their quantitative evaluation continues as the emphasis inexorably shifts from modeling of pharmacokinetics to modeling of pharmacodynamics.

The growing popularity of the PBPK modeling approach represents a movement from simpler kinetic models toward more biologically realistic descriptions of the determinants that regulate disposition of chemicals in the body. To a large extent, the application of these PBPK models to study the time courses of compounds in the body is simply an integrated systems approach to understanding the biological processes that regulate the delivery of chemicals to target sites. Many PBPK models integrate information across multiple levels of organization, especially when describing interactions of compounds with molecular targets, such as reversible binding of ligands to specific receptors, as in the case of methotrexate binding to dihydrofolate reductase [50,212]. In such cases, the PBPK models integrate molecular-, cellular-, organ-, and organism-level processes to account for the time courses of compounds, metabolites, and bound complexes within organs and tissues in the body. The system under scrutiny in PBPK models is more the integrated physiological system, appropriate enough for a discipline defined as physiologically based modeling.

The main goal of PBPK models is quite simple—to predict the target tissue dose of compounds and their metabolites at target tissues and, in some cases, to describe interactions in target tissues. PBPK models once developed are extensible. They can be used to extrapolate to various other conditions because of their biological fidelity. While the goal in applying these models is to predict dosimetry, it is important to remember that the overall goal of using PBPK modeling in risk assessment is broader than simply estimating tissue

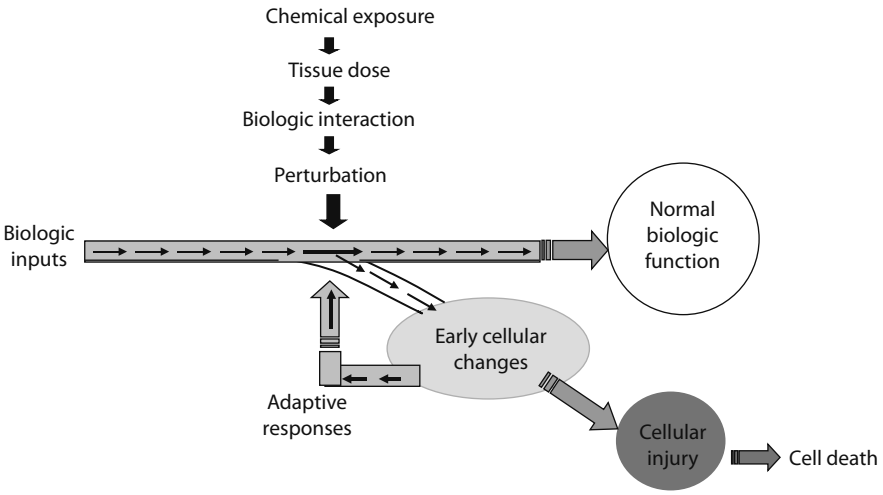


FIGURE 6.21 Diagram of the approach for understanding the pharmacological and toxicological effects of compounds in terms of their interaction with the biological system. The horizontal axis represents the biological component of the interaction, and the vertical axis represents the chemical component. Drugs can both restore altered biological function (efficacy) and produce altered function (toxicity).

dose, regardless of the level of detail provided in the interactions of compounds with tissue constituents. The goal in the larger context is to understand the relationship between dose delivered to target tissues and the biological sequelae of the exposure of target tissues to compounds. The specific steps that lead from these dose metrics to tissue-, organ-, and organism-level responses have usually been considered part of the pharmacodynamic process. Another inexorable development will be the expansion of these quantitative modeling approaches into the pharmacodynamic arena [5]. This latter area will represent a systems biology approach for describing perturbations of biological systems by compounds and the exposure/dose conditions under which these perturbations become sufficiently large to pose significant health risks.

The systems biology approach (Figure 6.21) focuses on normal biological function and the perturbations associated with exposure to compounds. Perturbations of biological processes by compounds lead to either adverse responses (chemical toxicity) or restoration of normal function to a compromised tissue (drug efficacy). The effects of compounds, whether for good or ill, can best be described by PBPK approaches linked through models of responses of cellular signaling networks. Toxicity and efficacy are then defined by an intersection of compound action with the biological system [213]. Toxicology and pharmacology are disciplines at the interface of chemistry/pharmacokinetics (primarily embedded in the vertical component) and biology/pharmacodynamics (primarily captured by the horizontal chain). Clearly, the main differences in the next generation of systems approaches in pharmacokinetic and pharmacodynamic modeling will be the increasingly detailed descriptions of biology afforded by new technologies and the expansion of modeling tools available for describing the effects of compounds on biological signaling processes.

QUESTIONS

6.1 What are the major criteria for deciding which tissues/organs to include in a PBPK model for a chemical?

6.2 Draw a box and arrow representation of a PBPK model to account for subcutaneous dosing of a volatile compound that is metabolized to a nephrotoxic metabolite in the kidney.

6.3 Write the mass balance equations for the compartments in the model from question 6.2.

6.4 Drug–drug interactions can alter the persistence and responses to individual drugs. Write equations for two different compounds metabolized by a common enzyme in liver. How would these equations change if the compounds shared a common enzyme for phase I metabolism and clearance?

6.5 Describe four different situations where it would be necessary to include changes in BW and volumes of specific tissues/organs over time.

6.6 Use the model code in the coding example to calculate the ventilatory air flow, tissue blood flows, and tissue volumes for a 500 g rat. Repeat for a 25 g mouse.

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7 Toxicopanomics

Applications of Genomics, Transcriptomics, Proteomics, and Lipidomics in Predictive Mechanistic Toxicology

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CONTENTS

TOXICITY TESTING IN THE TWENTY-FIRST-CENTURY PARADIGM SHIFT

Toxicity testing is at a turning point now that long-range strategic planning is in progress to update and improve testing procedures for potential stressors. The report by the U.S. National Research Council (NRC)[1] envisions a shift away from traditional toxicity testing and toward a focused effort to explore and understand the signaling pathways perturbed by biologically active substances or their metabolites that have the potential to cause adverse health effects in humans. The identification of these *toxicity pathways* is imperative in order to understand the mode of action (MOA) of a given stimulus and for grouping together different stimuli based on the toxicity pathways they perturb. The first component of the vision focuses on pathway identification, which is preferably derived from studies performed in human cells or cell lines using omics assays. The second component of the vision involves *targeted testing* of the identified pathways in whole animals and clinical samples to further explain toxicity pathway data. This two-component toxicity-testing paradigm, combined with chemical characterization and dose–response extrapolation, delivers a much broader understanding of the potential toxicity associated with a biologically active substance.[2–5]

Systems biology plays an important role in this paradigm, consolidating large amounts of information that can be probed to reveal key cellular pathways perturbed by various stimuli. Programs such as Next Generation (NexGen) Risk Assessment from the Environmental Protection Agency (EPA)[6] encourage the community to incorporate systems biology data into toxicity testing as articulated in a statement of goals for the near future, created at the Prototypes Workshop held in 2011.[7] Current systems biology methodology should be reviewed and further developed using an iterative approach. Stimulus-specific signature pathways need to be developed by the careful clarification of already identified key pathways. Systems data may also provide a means to better correlate in vitro and in vivo findings. However, the consistency of data obtained from in vitro and in vivo methodology across species needs to be assessed, and appropriate scaling methods should be developed for in vitro to in vivo correlation.

The U.S. EPA commissioned the NRC to develop a vision for toxicity testing in the twenty-first century (Tox-21c).[1,8] The fundamental idea underpinning this request is to base the new toxicology primarily on pathways of toxicity (PoT).[9] The committee suggests that there is a finite number of distinct pathways resulting in toxicity, and if these can be successfully identified and mapped, then new assay test batteries can be developed. Tox-21c has identified the promise of new technologies and the need for large-scale efforts.

There are several research initiatives that revolve around the 3Rs (replacement, refinement, and reduction) principle aiming to establish research solutions, which will reduce and eventually replace animal testing in product safety assessment. For instance, the six research projects within the SEURAT-1 Initiative (safety evaluation ultimately replacing animal testing)[10] represent collaborations between over 70 European universities, public research institutes, and companies. The collaboration focuses on the development of the technology building blocks that will replace current repeated-dose systemic toxicity testing in animals. Work on nonstandard methods is also ongoing under the auspice of the Organisation for Economic Co-operation and Development (OECD). The quantitative structure–activity relationship (QSAR) toolbox aims to categorize chemicals based on their intrinsic properties, thus enabling robust hazard testing for an entire category of chemicals with supposedly similar structure–activity relationship by extrapolation of testing results from only some members in a particular category.[11–13] AXLR8 is a central European Commission-funded body mediating information exchange between the leading European and global research teams that work on improving the capacity and speed of safety testing. The essential goals also include the identification of more human-relevant testing methods as well as an effort to drive the shift toward a toxicity pathway-based standard.[14] Furthermore, the European Partnership for Alternative Approaches to Animal Testing (EPAA) aims to influence European regulatory testing and decision-making so the 3Rs can be efficiently applied to reduce redundant and unnecessary animal testing.[15]

NETWORK-BASED APPROACH TO QUANTIFY THE IMPACT OF BIOLOGICALLY ACTIVE SUBSTANCES

Systems biology approaches yield large molecular datasets organized into meaningful biological networks that can lead to mechanistic insights and understanding of potential toxic effects. We have developed a five-step approach to assess the biological impact of a stimulus, which combines systems-wide experimental data with a computational process. The intensity of the biological impact of a stimulus is expressed by a quantitative biological impact factor (BIF). The main goal of our five-step approach is to adopt a toxicological MOA framework to describe how any substance may adversely affect human health and to use this knowledge to develop complementary theoretical, computational, and experimental (in vitro) models that can be used for safety assessment.[16]

To produce systems-wide data, several well-chosen experimental systems are exposed to stimuli in a time- and dose-dependent manner. Instead of merely extracting differentially expressed gene or protein lists, the data are analyzed in the context of biological network models. Because the network models used for this approach are built with cause-and-effect

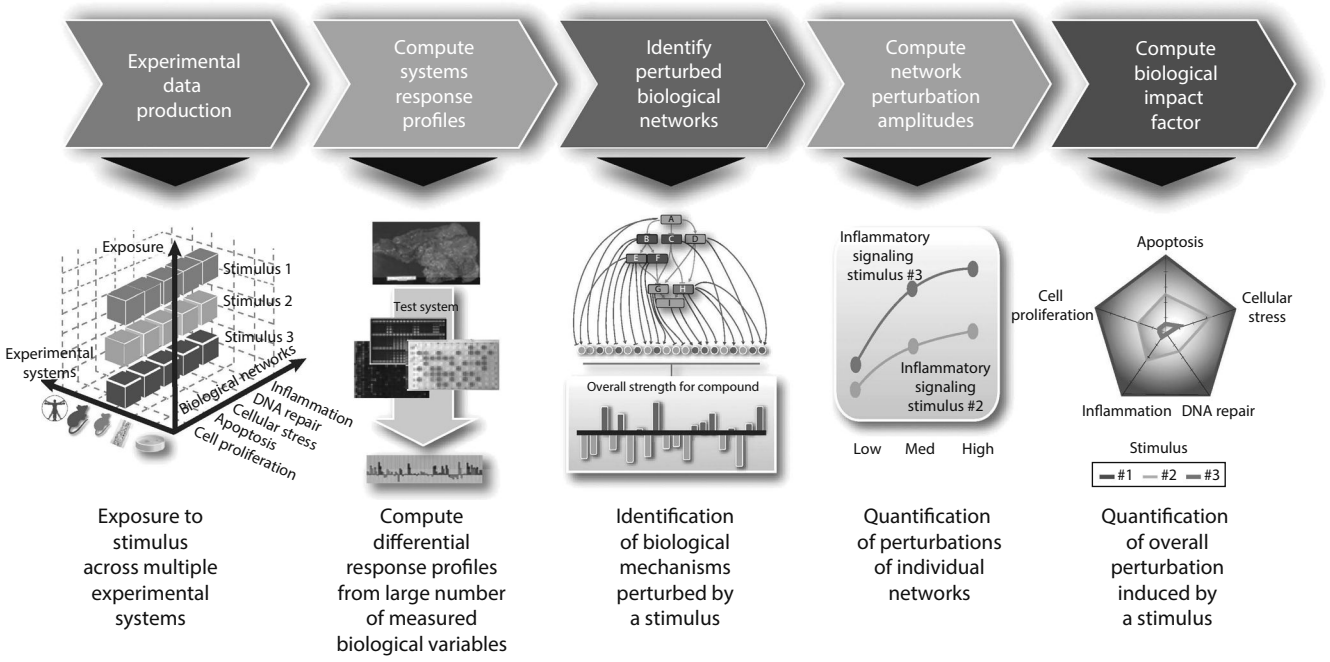

FIGURE 7.1 A network-based approach to quantifying the impact of biologically active substances. The five arrows illustrate the following steps: (1) design and conduct of a systems biology experiment, (2) calculation of systems response profiles, (3) building and identification of the relevant biological networks, (4) computing the NPA, and (5) computing the BIF.

relationships to describe biological processes, analyses of these networks can provide a more detailed molecular understanding of the observed biological perturbations. The approach thus provides the means for a mechanism-based quantitative comparison of the biological impact of a stimulus between individuals and species and can potentially exhibit predictive power through deterministic scoring algorithms. The five steps of our strategy are described in the following and comprise (Figure 7.1) (1) the design and execution of systems biology experiments, (2) the extraction of systems response profiles from the data generated in step 1, (3) the identification and building of relevant biological network models, (4) the computation of network perturbation amplitudes (NPA) scores, and finally (5) the aggregation of the NPA scores into a BIF.

DESIGN OF A SYSTEMS BIOLOGY EXPERIMENT

The success of the five-step strategy greatly depends on the quality of the design of the experiments to produce the systems biology data. To maximize this quality, a number of factors need to be taken into consideration. First, a clear scientific question has to be formulated, and the key assumptions that are made should be enunciated. These should drive the selection of the most adequate exposure modalities and biological test systems to address the scientific question. Second, it is crucial to select the most adequate measurement methods and ensure an optimal combination of dose- and time-dependent sampling schedules. Finally, a well-planned experiment is also statistically powered to enable proper interpretations of the results.

Ideally, the results from a well-designed experiment should do at least one of the following: (1) determine the reactivity (molecular bioactivity) profile of stimuli in order to associate them with likely molecular targets in a cell- and tissue-specific context; these profiles can in turn be linked to possible MOA, (2) capture specific MOA in order to discriminate stimuli that are likely or unlikely to induce toxicity in humans, (3) identify stimuli that are associated with a specific MOA and rank them with respect to their toxicokinetic potency, and (4) identify stimuli associated with a specific MOA and estimate threshold values for lethality that can be used to derive an in vivo effect level, as a function of dose dynamics.[10]

A thorough experimental design phase also enables the computational biologist to assess the experimental feasibility in concert with the experimental scientists who provide input on the kind of experimental information that is essential to a quantitative analysis.[17] Our strategy relies on three equally important dimensions in the experimental design space: (1) the experimental system, (2) the exposure regimen, and (3) the relevant biological networks, which will be measured and related to particular biological endpoints relevant to an adverse/toxic effect.

The experimental system is subjected to a standardized time- and dose-dependent exposure regimen (Figure 7.2a). A zero dose is used to represent control conditions. The treatment design consists of multiple biological replicas to ensure statistical power during the analysis. Additionally, the statistical design associated with the execution of the experiment is a crucial component to assess the experimental error by handling variation, eliminate any systematic bias, and account for

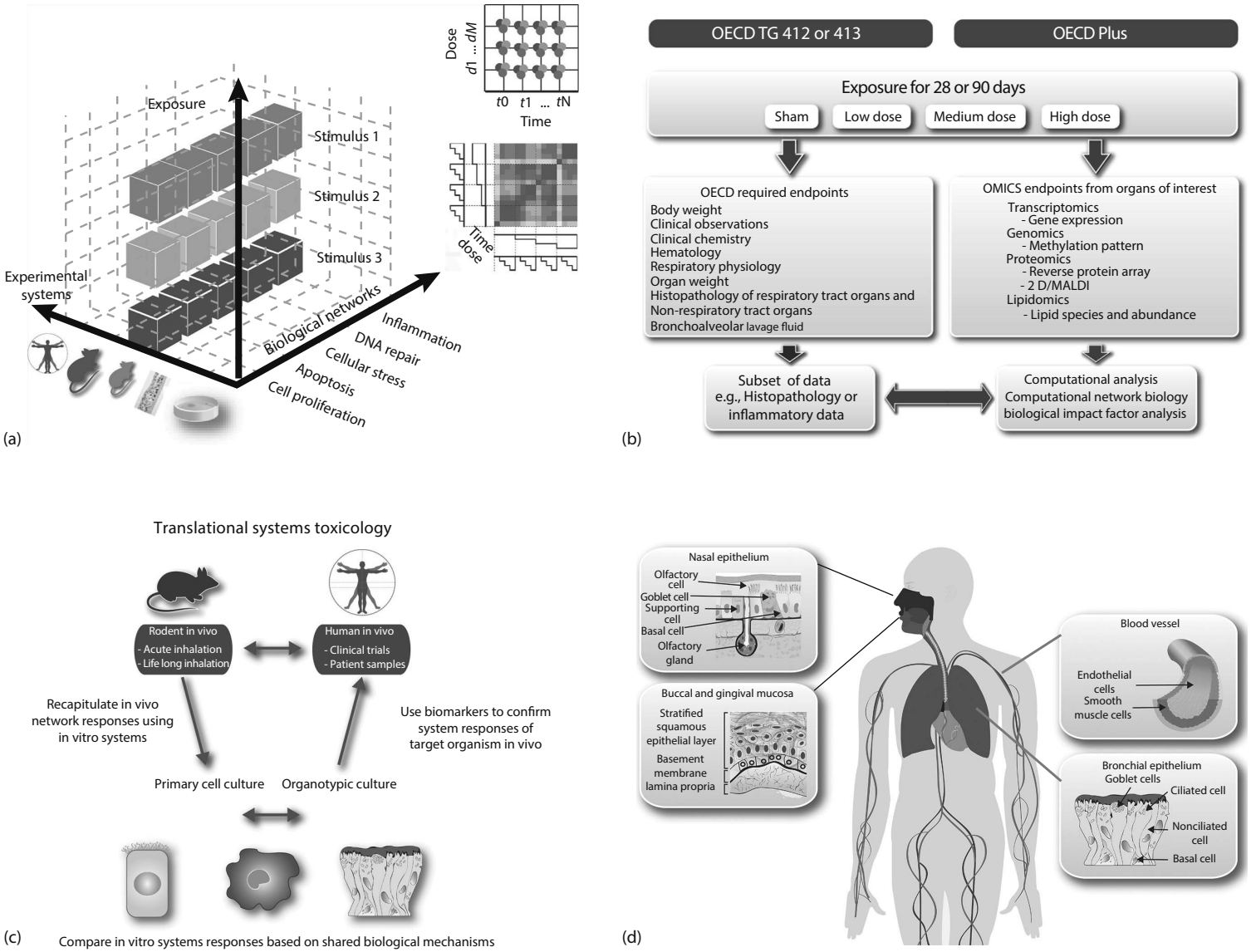

FIGURE 7.2 The experimental systems. (a) The cubic experimental design space with its three equally important components: (1) the experimental system, (2) the exposure regimen, and (3) the biological networks accompanied by the standardized time- and dose-dependent exposure regimen and a comparability heat map between two stimuli. (b) Comparison of the endpoints in the classical and OECD Plus concept. (c) Principle of translational systems toxicology from experimental animal models and in vitro models to human context. (d) Example of human tissues recapitulated through organotypic in vitro experimental models.

extraneous confounding variables including those linked to the used technology and the extraction/isolation steps. This means also that a sufficient number of biological replicas need to be included and processed individually throughout the entire workflow.

Experimental Systems

The data collected from longitudinal clinical studies are clearly the most relevant when assessing the potential human health risks associated with exposure to active substances. However, the adverse effects and disease(s) related to a stimulus usually take decades to manifest, and it is often difficult or even impossible to obtain datasets from such longitudinal human studies. Toxicity testing thus relies largely on animal models, as well as models based on cellular and organotypical (3D) in vitro cultures. While in vitro and in vivo models are known to have many shortcomings, however when appropriately used, they can provide mechanistic insights into the biological perturbations caused by a stimulus. By taking a systematic approach, we believe that the gaps between in vivo/in vitro systems and in vivo human biology can be reduced, potentially resulting in the identification of specific biomarkers for use in human studies. Eventually, these mechanisms provide the link from the initial exposure to a stimulus and the downstream onset of disease.

In Vivo Systems

Description of the 28-Day or 90-Day OECD Plus Concept

Traditional toxicological assessment approaches for experimental inhalation toxicology follow *OECD Guidelines for the Testing of Chemicals*. This is a collection of about 100 of the most relevant internationally agreed-upon testing methods for assessing, identifying, and characterizing potential hazards of new and existing chemical substances, chemical preparations, and chemical mixtures used by government, industry, and independent laboratories.[18] In particular, test guidelines (TG) 412 and 413 have been designed to fully characterize test article toxicity by the inhalation route for a limited duration (i.e., 28 or 90 days, respectively) and to provide robust data for inhalation risk assessments.[19,20]

The OECD TG 412 and 413 describe the methods to assess the inhalation toxicity of an inhalable material such as a gas, dust, mist, vapor, volatile substance, aerosol, or particulate suspension, using repeated exposures for a 28-day or 90-day inhalation period. Studies conducted in accordance with OECD guidelines have shown the assays to be suitable for the testing of cigarette prototypes and ingredients.[21–26] The study designs include the assessment of comparator products and a sham-exposed control group and/or vehicle controls as needed at three or more concentration levels. Exposure assessment, in-life observations (e.g., body weight, feed consumption, ophthalmoscopy), clinical pathology (blood and urine), gross pathology, and organ weights, as well as histopathology with particular attention to the respiratory tract, other relevant target organs, and gross lesions, are evaluated. The guidelines allow for the inclusion of satellite (reversibility) groups, bronchoalveolar lavage (BAL), neurologic tests, and additional clinical pathology and histopathological evaluations in order to better characterize the toxicity of a test article.

However, experience demonstrates that toxicological markers are only detectable after substantial damage has already occurred and therefore are considered to lack sensitivity.[27] Recently, the combined approach of omics and conventional toxicology has been shown to be a useful tool for mechanistic investigation and for the identification of putative biomarkers.[27,28]

In relation to chemical hazard/risk assessment, omics technology provides tools for improving the understanding of mechanisms of toxicity, reducing uncertainty in grouping of chemicals, and providing alternative methods for screening chemicals. OECD assists internationally collaborative efforts on molecular screening and toxicogenomics with the aim of defining needs and possibilities for their application in a regulatory context. Yet, their concepts have not been systematically applied across in vivo studies.[29]

In Figure 7.2b, a concept of combining the conventional toxicological assessment approach suggested in the OECD with additional molecular biological endpoints is presented. In brief, in a toxicity study employing additional animals for the purpose of obtaining *omics*, endpoints are employed. These animals undergo the same exposure conditions at the same time as the animals in the OECD study. Organs (e.g., lung, liver) and body fluids (e.g., blood, urine) of interest are collected from the exposed animals. Omics data (e.g., transcriptomics or proteomics) are generated, computationally analyzed and subsequently correlated with the endpoints described by the OECD guidelines. The omics data are obtained from the exact same tissue sites where a phenotypic change is expected to occur based on the corresponding classical endpoints as suggested by the OECD guidelines. In some cases, it is instructive to perform laser capture microdissection to excise and analyze a specific cell type of interest (i.e., lung parenchyma) prior to ribonucleic acid (RNA) or protein extraction: this facilitates the identification of the toxicity mechanisms at cell, tissue, and organ level.

These standardized toxicology testing guidelines do however not involve animal models designed to reproduce key features of the human diseases known to result from chronic exposure to stimuli such as environmental toxicants or cigarette smoke (CS). To test products in the context of such diseases, one would therefore need to leverage existing, or develop new, animal models, which reproduce at least some key features of the human diseases as a consequence of exposure to the toxicant(s). For instance, CS causes well-documented changes in lipid metabolism, and the resultant proatherogenic lipid profile is thought to be responsible for

the accelerated growth rate of atherosclerotic plaques and the increased incidence of cardiovascular disease in smokers.[30] Plaque development, a key feature of human cardiovascular disease, can be tested in the apolipoprotein E-deficient knockout mouse ($ApoE^{-/-}$),[31] which is a well-established model of human atherosclerosis.[32] The systemic proinflammatory status of $ApoE^{-/-}$ mice makes this strain a strong candidate model to extend the scope of smoking-related disease end points. For example, ApoE-deficient mouse is a suitable model to detect smoke-related COPD paramenters such as emphysema. COPD is defined as a progressive, irreversible airflow limitation caused by the combined effects of emphysema, chronic bronchitis, and narrowing of the small airways.[34] In the Western world, CS is the main etiological factor in the pathogenesis of this disease.[35] Published literature describes several rodent models mimicking at least one feature of COPD.[36–39] Different strains of mice have been reported to show different susceptibilities for the development of smoking-induced COPD.[40–42] The C57BL/6 mouse strain (the genetic background of the ApoE-deficient mice) and the A/J mouse strain are widely used as rodent models of CS-induced COPD.[43–47] Both mice strains develop smoke-induced emphysema that can be observed by parameters such as lung function as well as histopathology and gene expression of the lung and airway tissue.

In Vitro Systems

Animal models are usually used to assess the toxicological impact of certain stimuli on living beings. They provide a comprehensive view of how a substance interacts with a whole organism. Nevertheless, toxicity testing on animals presents considerable challenges. It is very expensive, time-consuming, and ethically controversial. More importantly, there are significant concerns regarding the poor predictability of animal studies for human outcomes. The actual effort to *replace, reduce, and refine* animal experiments (as stated by [48]) is based on the use of more appropriate cell-based assays that could provide more relevant data on the effects of short- and long-term exposure to toxicants. Figure 7.2c represents the different experimental systems and how they are connected following the principle of translational systems toxicology.[49] One example of in vitro, and in vivo, as well as interspecies, extrapolation has been illustrated by Kienhuis et al.,[50] where the assessment of acetaminophen-induced hepatotoxicity was conducted in the rat and in human in vitro cellular systems and additionally, between rat in vitro and in vivo systems at the level of gene expression.

To increase the relevance of in vitro assays and to further mimic physiologically relevant cellular functions, primary culture of target organ-specific cells obtained from human donor has been further investigated as a potential surrogate. Primary cells show more preserved cell-type-specific functions than immortalized cell lines.[51–53] In addition, the use of human primary cells also offers the advantage of testing the response to an exposure on cells from different donors allowing an evaluation of the importance of human genetic variability. To reproduce the tissue-specific architecture, the biochemical and mechanical cues, and the cell–cell interactions of the microenvironment in tissues, a number of in vitro models have been further developed as 3D tissue reconstruction models. The first consideration in assessing toxicity is to investigate the possible routes of exposure and therefore the target organs that, under normal circumstances, protect the body from environmental stressors. In the list of 3D models already developed (Table 7.1), the skin[54] and the respiratory tract[55] are already covered and used to assess how stimuli penetrate these tissues to impact the human organism.

When normal human bronchial epithelial (NHBE) cells are cultured at the air–liquid interface, they can differentiate into an organotypic pseudostratified bronchial epithelium-like tissue with all the morphological properties that define the bronchial epithelium in vivo.[56] Cells are polarized, develop tight junctions, and show typical aspects of ciliated and nonciliated cells, goblet cells, and basal cells as seen in vivo. In addition, cells forming the 3D airway cellular model express a comparable gene expression profile to tracheal and bronchial epithelial cells that have been collected in vivo by brushing human airways.[57] Furthermore, the gene expression profiles of a human 3D airway model (obtained from MatTek™) exposed to whole CS or air have been compared via computational methods to the in vivo smoking gene signature of bronchial epithelial cells obtained from a large group of smokers and nonsmokers.[58] The results of this study show that the human organotypic bronchial epithelium-like tissue culture exposed to whole CS at the air–liquid interface presents similar biological perturbations as the ones observed in smokers' airway epithelium. Similarly, a recent study on drug efficacy has shown that 3D cultures of endometrial cancer cells were better reflecting the effect of some drugs tested in patients than when cultured in two dimensions.[59] Experimental evidence demonstrating the relevance of 3D human organotypic cultures is accumulating, and more work will be required to further validate the use of such experimental models for toxicological assessment. Figure 7.2d illustrates the organotypic culture systems that are included in our strategy in testing tobacco products.

The establishment of co-culture systems in both two and three dimensions is also underway and promises to more closely recapitulate key biological functions. The presence of different cell types in the same in vitro model provides an important additional step to re-create essential physiological steps, which involve more than just one cellular player (such as the release of growth factors, modifications of extracellular matrix (ECM), cell–cell interactions). Finally, the design of complex flow or perfused systems has been reported in both large-scale bioreactors as well as smaller-scale metabolism toxicity models. One example recently published is the impressive reconstitution of organ-level lung functions on a chip.[60] The authors developed a mechanically active microdevice that reconstitutes the alveolar–capillary interface of the human lung and mimics the cyclic mechanical strain of breathing. The model also includes the presence of a microvascular channel.

TABLE 7.1
Example of Human In Vitro 3D Experimental Models Available Commercially or Already Developed and Described in the Literature

3D Human Reconstructed Models or Tissue Explants	References	Potential Applications
Skin epidermis	[10,215–217]	In product development, toxicological assessment, safety assessment, and drug discovery/development in cosmetics, personal care, household products, chemicals, pharmaceutical and biotech testing
Respiratory tract Tracheal/ bronchial epithelia	[10,15,216]	In product development, toxicological assessment, safety assessment, and drug discovery/development (e.g., intrabronchial drug delivery assessment) in personal care, household products, chemicals, pharmaceutical and biotech testing
Keratinocyte eye cornea	[10,216]	In toxicological assessment and safety assessment and drug discovery/development in the pharmaceutical industry (e.g., ocular irritation assay and corneal permeability, metabolism, and differential display)
Vaginal cervical mucosa	[10,216]	In toxicological assessment and safety assessment and drug/product discovery/development in pharmaceutical industry (e.g., vaginal irritation assay, bacterial or viral adhesion screening for antibiotics or products, in vitro candidosis research, vaginal permeability and metabolism)
Buccal and gingival mucosa	[10,216]	In toxicological assessment and safety assessment and drug/product discovery/development in pharmaceutical, personal care industry (e.g., oral irritation assay, oral anti-inflammatory assay, in vitro candidosis research, oral permeability and metabolism)
Nasal epithelia	[15]	In product development, toxicological assessment, safety assessment, and drug discovery/development (e.g., intranasal drug delivery assessment) in personal care, household products, chemicals, pharmaceutical and biotech testing
Blood–brain barrier	[218]	Assessment of drug transport and metabolism across the human blood–brain barrier to detect potential neurotoxicity effects
Placental barrier	[219]	Assessment of drug transport and metabolism across the human placental barrier to detect potential fetotoxicity effects
Lymph node	[220]	In drug discovery/development, organ research, and tissue regeneration
Liver	[124,221]	In product development, toxicological assessment, safety assessment, and drug discovery/development in household products, chemicals, pharmaceutical and biotech testing, as well as in organ research and tissue regeneration

Note: All of these models are using primary normal human cells.

Responding to the need for more reliable human in vitro models of in vivo responses, the National Institutes of Health (NIH), the U.S. Food and Drug Administration (FDA), the Defense Advanced Research Projects Agency (DARPA), and the Defense Threat Reduction Agency (DTRA) are sponsoring a variety of projects focused on developing 3D organotypic organ models as well as miniaturized human multi-micro-organ bioreactor systems.[61] Researchers in Germany are also developing a *human on a chip*, consisting of functional human organ units—human *microorganoids*—with feed supplies and waste reservoirs. These miniaturized dynamic 3D multitissue cultures are envisaged to be used for repeated-dose toxicity testing in lieu of 28-day or 90-day OECD rat studies.[62]

Exposures

Ideally, the exposure matrix will have been well characterized chemically, even in the case of a complex mixture. The goal is to re-create an exposure regimen, that is, the dose, frequency and timing, and overall duration of exposure that most realistically mimics the human situation while being technically feasible. It is therefore imperative to define a set of standard exposure regimens to be applied systematically to the selected experimental systems. Such exposure regimens should be validated and verified, at least in part, during the experiment to ensure that the expected exposure is actually taking place and the stimuli are indeed delivered to the target site. Furthermore, appropriate biological assays and measurements should be used to obtain time- and dose-dependent data in order to capture both early and late events and ensure that a representative dose range is covered.

If exposure information is available or can be estimated for the substance to be tested, then it should be considered in the study design. This step will minimize the chance of unrealistically high doses being used that could overwhelm the homeostatic mechanism of the experimental system, and thus fail to reflect the pathways that might be functional at more realistic doses.[63]

Exposure of In Vivo Systems

Methodologies to assess toxicity due to exposure to gases, aerosols, or combinations thereof require the generation of atmospheres that laboratory animals (mainly rodents) can be exposed to. The compositions of the atmospheres depend on the objective of the particular study but must be conducted under well-controlled conditions. Inhalation experiments use

TABLE 7.2
Summary of Different Exposure Modes in Experimental Models

Experimental Systems	Mode of Exposure	Stimuli
2D submersed Monolayer cell Co-culture of monolayer and suspension cells Permeable filter support[125]	Homogenous/heterogenous (apical vs. basal exposure)	Any soluble stimuli added to the medium
3D air–liquid interface[222]	Apical side exposure, at the air interface, or basal side exposure, at the liquid interface	Inhaled compounds (solids, liquids, gases, nanomaterials), whole CS
3D submersed multicellular spheroids (single or co-culture) Spontaneous cell aggregates[222] Liquid overlay[223] 3D hanging drop[224] 3D spinner flask/rotating wall vessels[225,226]	For cell aggregates, gradient of diffusion of gas/soluble nutrients/chemical agents	Any soluble stimuli added to the medium
3D co-culture on a scaffold[227,228]	For various cell types, exposed to the soluble nutrients/chemical agents	Any soluble stimuli added to the medium
Bioreactors[229]	For various cell types, perfusion of the cells, medium flow, gradient of diffusion of gas/exposed to the soluble nutrients/chemical agents. Heterogenous	Any soluble stimuli added to the medium
Laboratory animals	Parenteral (subcutaneous, intraperitoneal, intravascular, intradermal, intralesional, ocular, intranasal, intratracheal, intracranial, intrathoracic, intracardiac injections, osmotic pumps, and slow-release subcutaneous implants) Inhalation (nose-only, whole-body) Topical (skin, mucous membrane), irradiation Enteral (additives to the drinking water or food, intragastric via gavage)	

either whole-body exposure or nose-only exposure chambers.[64–66] Whenever possible, nose-only rather than whole-body modes of exposure should be used in inhalation studies that are designed to reduce exposure through nonrespiratory routes. The exception is for large animal numbers, chronic inhalation studies, or study types that require virtual continuous exposure.[65] Other routes of administration to expose laboratory animals are given in Table 7.2.

Exposure of In Vitro Systems

As mentioned in the previous paragraph, in vitro experimental systems are either made of cells in submersed cultures or cultures with an air–liquid interface. Thus, the exposure mode will be chosen depending on the selected in vitro systems and the type of stimulus to test (Table 7.2).

Regardless of the methods used, the toxicological principles underlying dose selection, frequency and timing of exposures, and duration of study need to be fully considered in the experimental design.

TECHNOLOGY PLATFORMS TO MEASURE MOLECULAR CHANGES

Genomics and Transcriptomics

Genomics is the field of research that focuses on the genome, that is, the mapping of the entire DNA sequence in a given organism and tracks any alterations associated with disease or resulting from exposure to harmful substances. The term *mutagenicity* refers to the induction of transmissible changes in the amount or structure of the genetic material of cells or organism. Such changes are permanent and will be inherited to the daughter cell upon division. Genomic changes include point mutations, copy number changes, as well as large-scale changes that affect the chromosomal structure. Deletions and duplications in a chromosome, referred to as copy number variations (CNVs), are a significant determinant of individual differences as well as contributors to many diseases. Recurrent CNVs are the result of homologous recombination between repeated sequences, and nonrecurrent CNVs can occur anywhere in the genome through nonhomologous recombination events.[67] Genetic changes may also lead to survival and growth advantages of the cell carrying them, frequently observed during malignant transformations. Even though evaluation of DNA damage is often included in the toxicity assessment, it is also important to further identify the affected genes when DNA damage leads to genomic changes.

Epigenomics: Reversible gene expression changes that are transmissible both mitotically and meiotically, but do not involve DNA sequence modifications are referred to as epigenetics. According to a long-standing model, hypermethylation of CG islands in the promoter regions of target genes inhibits the initiation of transcription, presumably by blocking the transcriptional regulator binding sites.[68]

Eukaryotic cell DNA is wrapped around eight histone proteins forming the nucleosome, the basic unit of chromatin. The chromatin structure is highly dynamic and undergoes covalent modifications that contribute to inactive heterochromatin or active euchromatin regulating the gene expression.[69]

While epigenetics serves an important purpose for various cellular functions during development, aberrations in methylation patterns or the histone code on individual genes as well as globally can signal system perturbation caused by a stimulus. It is clear that exposure to exogenous stimuli causes dose-dependent epigenetic changes and that certain malignancies resulting from chemical compounds stem from changes in the epigenetic landscape.[70–72]

Transcriptomics or gene expression profiling is used to study changes in gene expression taking into account the entire transcriptome, that is, all mRNAs in a cell population. Currently, transcriptomic analysis is probably the most well-established approach for identifying perturbed pathways and gaining mechanistic insight on the response to a stimulus. Transcriptomic research uses omics techniques, such as microarray and RNA sequencing.

Microarray technology is based on the inherent property of a DNA molecule to form a stable duplex (hybridization) from two complementary strands. One of the first applications of using short-labeled DNA probes to specifically bind other DNA molecules (derived from biological samples via hybridization) was described by Southern about 40 years ago.[73] An adaptation of this technique that used binding of labeled DNA probes to RNA molecules in order to detect RNA transcripts was correspondingly called northern blotting. It was the first molecular biology technique used to study gene expression and was essential for the development of microarrays. In northern blotting, the RNA isolated from biological samples is subjected to a size fractionation by electrophoresis and is then placed (blotted) on a membrane. DNA probes carrying the radioactive or chemiluminescent labels corresponding to the genes of interest are then applied. The concentration of the expressed RNA transcript is quantified by either radioactivity or chemiluminescence measurements of the bound labeled DNA probes.

cDNA microarrays: Although many journals still consider Northern blotting to be the *gold standard* for gene expression measurements, it suffers from drawbacks such as the low throughput (number of samples), low sensitivity, and limited number of genes that can be interrogated simultaneously. These limitations have been overcome by the introduction of cDNA microarrays, where individual fragmented, or full, cDNA clone sequences are printed on glass slides. In this approach, contrary to northern blotting, it is now the RNA transcripts that are isolated from biological material, labeled, and hybridized on a glass slide containing the cDNA probes (called a dual-channel array). In a typical experiment, the RNA is isolated from two samples, labeled with different fluorescent dyes (Cy3 and Cy5) and hybridized on the same array. Comparative image analysis of the two fluorescent colors from the same array allows for a medium throughput and for sensitive measurements of differential gene expression between the two samples.[74]

Oligonucleotide microarrays: The next major technological advance arrived with the invention of high-density array printing by Affymetrix. It is now possible to design an array of 25 oligomer probes that covers the whole collection of RNA transcripts (transcriptome) of a single organism. More than one million probes can be placed on the array in a particular order including mismatch and other quality control (QC) probes. In a typical experiment, each RNA sample is individually hybridized to a microarray, and comparisons are made between the samples at the data analysis stage. Up to 48 microarrays per run can be processed on an array scanner resulting in a high throughput. Initially, the microarrays were designed from the available 3′-expressed sequence tag (ESTs), thus covering mostly the 3′-end of expressed genes. With the progress in full cDNA sequencing and RNA sequencing by Next Generation Technologies, current microarrays can evenly cover each gene or each exon of the gene (so-called exon array). The exon arrays are now available for human, mouse, and rat.[75] The use of exon arrays has improved the analysis of alternatively spliced RNA transcripts as well as the accuracy of the overall gene expression measurements. Similar arrays have been developed by Roche NimbleGen and Illumina Inc.

Affymetrix GeneTitan: The idea of combining three major components—a hybridization oven, a fluidics station, and a scanner—into a single system has been realized by Affymetrix in the GeneTitan.[76] It supports gene expression studies in 16-, 24-, and 96-format of the lower-cost GeneChip® Array Plates and is able to process two plates per day and up to 750 samples per week. The gene expression profiling GeneChip® high-throughput (HT) perfect match (PM) array plates for human, mouse, and rat are listed among the available products of the new platform. However, as the processing of the array plates is completely different from that of the cartridge arrays, the results are not guaranteed to be similar especially for the expression measurements of low-expressed genes.[77]

Trends in gene expression: Next generation sequencing (NGS) technologies are emerging as the tools that will likely supersede microarray technologies in the near future because of their advantages in accuracy, throughput, and flexibility of gene expression measurements. For instance, gene expression studies can be made with organisms for which gene chip technology is not available such as model systems for environmental toxicology. The NGS protocols are designed to provide exact transcript counts and results that closely approach those of quantitative polymerase chain reaction (PCR). Increasing output and the number of multiplexing primers would allow for the processing of thousands of transcriptome samples in a single run. NGS methods require no prior knowledge of the probes and are directly applicable as soon as an RNA sample is available, although knowledge of the genome reduces the errors in annotation of expressed genes.

In the last decade, several parallel sequencing techniques have emerged based on a number of technologies, such as pyrosequencing, fluorescence-based sequencing by synthesis and sequencing by ligation, ion semiconductor, or single molecule real-time sequencing. The sequencers using these technologies offer read lengths and throughputs ranging from about 150,000 single-end long reads (~8 kb) to 6 billion 2 × 100 bp paired-end reads.[78] Although these technologies are continuously improving, with typical reads becoming longer and with higher accuracy, the characteristics of the offers from the leading sequencer providers vary as is summarized in Table 7.3.

Independent of the chosen technology, HT sequencing has applications in genomics, transcriptomics, and epigenomics. In a systems biology approach, the interpretation of the obtained results should be carried out in conjunction with the results of other analysis methods.

Deep sequencing of a genome (DNA-seq) is mainly used for de novo and resequencing assembly of whole genomes. However, it also enables a wide range of analyses that are gaining popularity as a result of the increased number of available annotated reference genomes. For example, by comparing an appropriate resequenced genome assembly to a reference genome, structural variations, CNVs, single-nucleotide polymorphisms (SNPs), or small insertions or deletions can be identified. If genomic regions of interest are known, targeted resequencing can be used to reduce cost, complexity, and time.

Gene expression profiling is also possible with deep sequencing of a transcriptome (RNA-seq). Unlike array-based methods, an advantage of RNA-seq is that it does not require prior gene identification or annotation. RNA-seq can therefore also be used for the discovery of novel transcripts and gene fusions.[79,80] In addition, array-based methods, which use target-specific probes, require the use of different arrays to study different biological aspects of a sample, such as gene expression, transcript expression, transcription start site utilization, or alternative splicing. On the contrary, depending on the methods used the same RNA-seq data can provide qualitative and quantitative measurements at the gene, transcript, transcription start site, or alternative splicing levels.[81]

Whole-genome DNA methylation can be investigated either at a single nucleotide level using shotgun bisulfite sequencing (MethylC-seq) and reduced representation bisulfite sequencing (RRBS) or at the level of a few tens of nucleotides with methylated DNA immunoprecipitation (MeDIP-seq), methylated DNA capture by affinity purification (MethylCap-seq), methylated DNA binding domain sequencing (MBD-seq), or methylation-sensitive restriction enzyme sequencing (MRE-seq).[82,83] MethylC-seq and RRBS require the sequencing of both the untreated and the bisulfite-treated genomes in order to compare them and identify methylated cytosines. With MeDIP-seq, MethylCap-seq, and MBD-seq, methylated genomic DNA fragments are enriched before sequencing. On the contrary, MRE-seq enriches unmethylated genomic DNA fragments. These methods reduce the amount of necessary sequencing, but do not provide single nucleotide accuracy.

Histone modifications can be studied using chromatin immunoprecipitation (ChIP) with antibodies specific for a given histone modification. After precipitation, the DNA bound to the precipitated chromatin can be hybridized on a microarray chip (ChIP-chip) or sequenced (ChIP-seq) on an HT platform. ChIP-chip will require a large number of arrays to cover whole mammalian genomes, while ChIP-seq provides the whole-genome coverage even at a low sequencing depth.[84]

With the development of platforms based on HT sequencing, genomic, transcriptomic, and epigenomic analyses are freed from the requirement for prior knowledge inherent to array-based platforms. The platforms thus move us closer to measuring what is in the sample rather than what is on the chip. Furthermore, as HT sequencing directly measures the DNA present in a sample, it can capture several modifications and alterations simultaneously. The data generated by these platforms are therefore more flexible in the way they can be analyzed and as a result can provide a wider spectrum of genomic information. Computational resources and

TABLE 7.3
Comparison of the Leading Sequencing Technology Providers (as of End-2013)

Provider	Technology	Detection	Number of Reads	Read Length	Advantages	Disadvantages
Roche 454 GS FLX+	Pyrosequencing	Luciferase light emission	1 million	700–1000	Relatively long reads	Prone to errors with homopolymers
Applied Biosystems SOLiD 5500xl	Sequencing by ligation	Fluorescent labeling of 5′-dimers	2 billion	50–75	Very high accuracy	Short read length
Illumina HiSeq-2500	Sequencing by synthesis	Four different fluorescent dyes	Up to 6 billion	100–150	Highest throughput	Long running time
Ion Torrent Proton	Ion semiconductor	pH change	165 million	200	Short running time	Prone to errors with homopolymers
Pacific Biosciences pacBio RS II	Single molecule real-time fluorescence	Fluorescence	150,000	~8000	Longest reads	Low number of reads, high error rate

methodologies for the analysis of large-scale epigenomic datasets have been developed in recent years; however, as noted by Huss,[84] "custom tools are needed to optimally analyze ChIP-seq data on histone modification and BS-seq data on DNA methylation." Furthermore, reaching the full potential of whole-genome epigenomic studies is still hindered by the lack of guidelines and repositories for data submission. Such provisions would speed up research and allow for advanced applications.[86]

Proteomics

The proteome represents the full complement of the proteins produced by an organism,[87] and proteomics is a systematic approach to characterizing all proteins in a cell population. The data produced by the proteomic technologies hold significant potential for drug discovery, diagnostics, and molecular medicine by complementing the genome-centric view of biological networks with protein-specific data. Proteomic data can be equally valuable in toxicity testing since the proteome responds readily to various stimuli, including those that can lead to adverse health effects in humans. Toxicoproteomics sets out to develop tools that can identify potentially damaging changes in the proteome thereby further increasing the understanding of a stimulus on the mechanisms of toxicity. Proteomic technologies can be divided into those used for the discovery and identification of potential biomarkers and those used in targeted approaches for biomarkers quantitation and verification.

Biomarker Discovery and Identifications Approaches

Gel-Based Approaches

2D polyacrylamide gel electrophoresis (2DGE) can be used to assess perturbations on the proteome. This technique relies on the separation of proteins based on their pH (charge) as well as their size and has the capability to separate and visualize up to 2000 proteins in one run. The first dimension, which is known as isoelectric focusing (IEF), separates the proteins at the pH where they exhibit neutral charge (isoelectric point), and the second dimension further separates the proteins by their mass. State-of-the-art image acquisition and analysis software such as SamSpots from nonlinear dynamics allow simultaneous comparison of control and treated samples to identify the differentially regulated proteins by their relative intensity and volume in a label-free approach. The difference in gel electrophoresis (DIGE) is the labeling of proteins with fluorescent cyanine dyes (Cy2, Cy3, and Cy5) of different samples or treatments. The characteristics of these dyes allow the analysis of up to three pools of protein samples or conditions simultaneously on a single 2D gel to detect differential variances in proteins between samples.[88] The most challenging aspect has been in the development of algorithms that can deal with the warping phenomenon. Investigators now account for gel warping by running several gels per sample and analyzing gels by principal component analysis (PCA) to determine which should be excluded from further analysis.[88] Figure 7.3c describes the workflow for the 2DGE for biomarkers identifications.

Even though 2DGE is a powerful tool to identify many proteins when using well-established protocols, the approach has serious limitations. The major limitation is that not all proteins can be separated by IEF. Membrane, basic, small (<10 kDa), and large (>100 kDa) proteins cannot be separated on the IEF and hence, cannot be detected in the 2DGE and require a separate approach based on membrane purification and 1D gel electrophoresis (1DGE). The second limitation is that less abundant proteins are often masked by the abundant proteins in the mixture.[89,90]

Gel-Free Liquid Chromatography Mass Spectrometry Approaches

Protein fractionation is crucial for simplifying the mixture before analysis with mass spectrometry (MS). Liquid chromatography (LC) is the most commonly used method for protein fractionations. The LC approach takes advantage of the differences in the physiochemical properties of proteins and peptides, that is, size, charge, and hydrophobicity. The use of 2D-LC fractionates proteins on two columns with different physiochemical properties to achieve the maximum separation of proteins and peptides in the complex mixture.[91]

MS is widely considered to be the central technology platform for toxicoproteomics. MS has brought many advantages to the advancement of toxicoproteomics including unsurpassed sensitivity and improved speed and the ability to produce HT datasets. Due to the high accuracy of MS, peptides in the femtomolar to attomolar range can be detected in tissues and biological matrices with an accuracy level of less than 10 parts per million (ppm). This is greatly beneficial in comparative analysis where simultaneous comparisons between control and treated samples are key to increase our understanding of how stimuli affect the proteome and the subsequent identification of potential biomarkers.[91]

There are two approaches that are widely used for protein identification and differential protein quantification. The first approach is a label-free approach where the proteins or peptides of each sample are separated by LC and subsequently analyzed by MS. The main advantages of this approach are the following: (1) comparison of multiple samples is possible (no restriction in sample number), (2) it covers a broad dynamic range of concentrations, and (3) no further sample treatment is required. This approach is however error-prone and requires large computational power to perform the data analysis. The second approach is a label-based approach. One of the most common techniques is the use of isotope tags for relative quantification (iTRAQ). The main advantages of iTRAQ are the following: (1) simultaneous comparison of up to eight samples, (2) reduction of sample number as samples are pooled before MS analysis, and (3) low probability of introducing experimental errors due to pooling. The limitations of the technique are the limited dynamic range and the fact that the protein profiles have

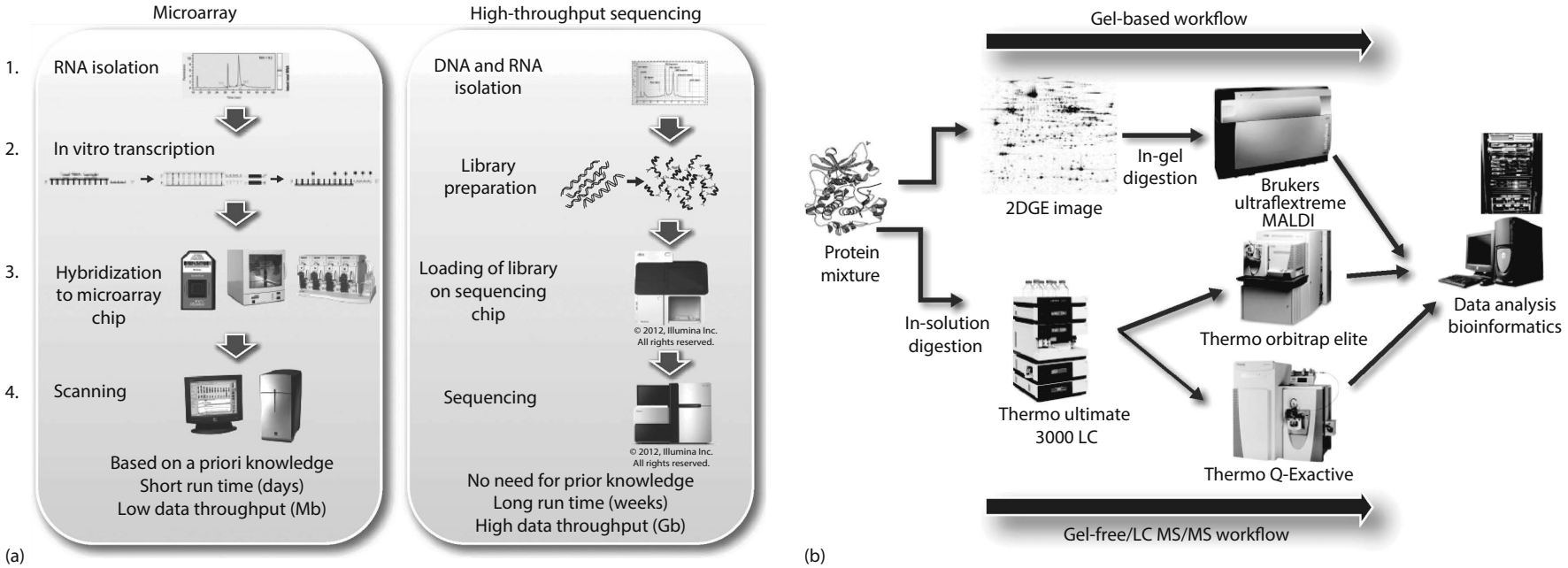

FIGURE 7.3 The technology platforms. (a) Microarray and HT sequencing data generation workflows for nucleic acids-based analysis. (b) Gel-based and gel-free proteomics workflows demonstrated for protein biomarkers identifications.

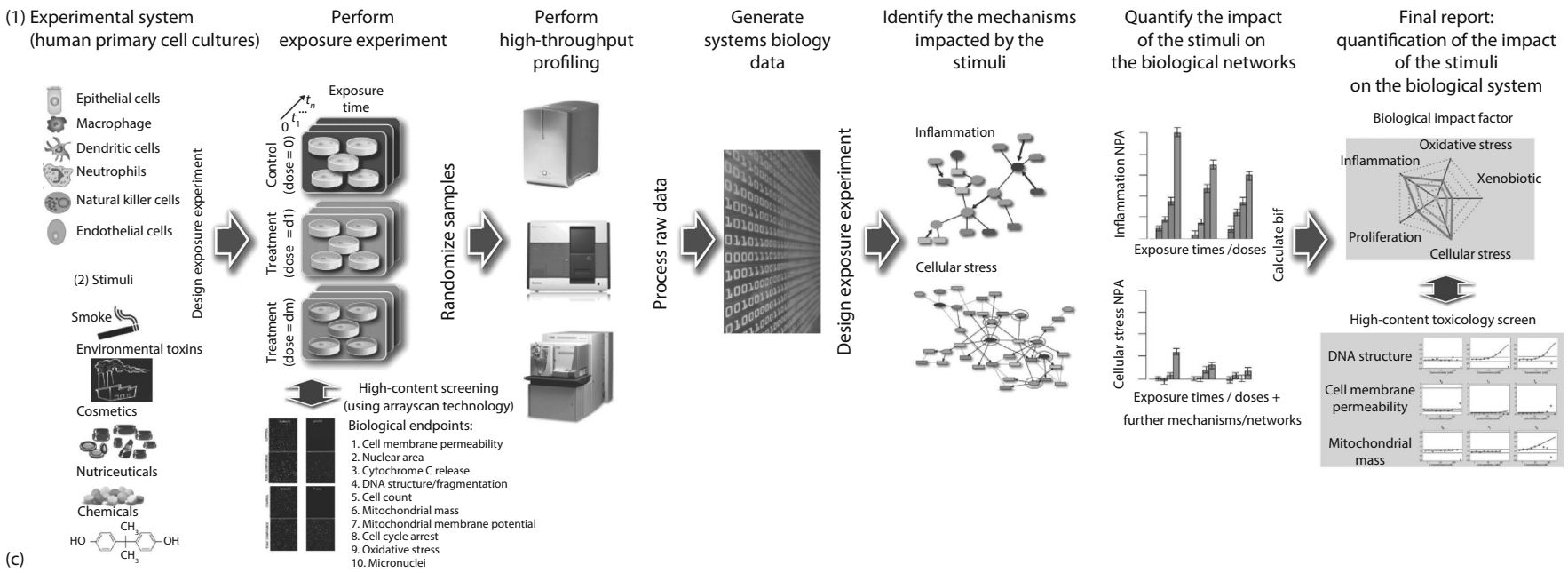

FIGURE 7.3 (continued) The technology platforms. (c) Schema representing the different steps of generating HT measurements in toxicological impact assessment. To test a specific stimulus, the chosen in vitro system is exposed following an experimental plan with defined dose ranges, exposure durations, and relevant time points to capture the different endpoints of interest. Obtained HT profiles are then analyzed to identify and quantify the time- and dose-dependent biological perturbations triggered by the exposure. These results can be further complemented with additional measurements (e.g., cell membrane permeability, cell count, cytochrome *c* release) using HCS technique.

to be similar.[92] Figure 7.3c illustrates the gel-free LC MS/MS workflow for biomarker discovery.

The major advantages of the gel-free approach are the following: (1) lower sample volumes can be analyzed, (2) less abundant proteins can be detected, (3) HT in sample analysis and data generations is possible, and (4) different classes of proteins can be analyzed. The major disadvantage is that posttranslational modifications (PTMs) of proteins and peptides cannot be detected or analyzed.[91]

Peptidomic Approaches

Peptides are generated by more than 400 proteases acting at different intracellular sites and, upon secretion, in the extracellular domain. Peptidomics complements proteomics in shedding light on the protein degradation processes, which are often part of disease mechanisms. The advantages of peptidomics are the following: (1) MS instrumentations offer good sensitivity to detect low amounts of peptides present in the samples, (2) peptide approaches can complement gel-based and gel-free approaches for protein biomarkers identifications, and (3) the biological function of individual peptides can be elucidated. The application of peptidomics is however limited by the number of peptides that can be captured, which can lead to an incomplete picture of the peptides involved in the studied biological networks.[93]

Biomarker Quantification Approaches

MS-Based Approaches

As systems biology requires accurate quantifications of a specified set of peptides/proteins across multiple samples, targeted approaches have been developed for biomarker quantification. Selected reaction monitoring (SRM) was developed to reliably deliver precise quantitative data for defined sets of proteins, across multiple samples using the unique properties of MS. SRM measures peptides produced by the enzymatic digestion of the proteome as surrogates to their corresponding proteins.

An SRM-based proteomic experiment starts with the selection of a target list of proteins, based on previous experiments, scientific literature, or prior knowledge. This step is followed by the following: (1) the selection of target peptides (at least 2) that optimally represent the protein, (2) the selection of a set of suitable SRM transitions for each target peptide, (3) the detection of the selected peptide in a sample, (4) the optimization of other SRM assay parameters if some of the peptides cannot be detected, and (5) the application of the assays to the detection and quantification of the proteins/peptides.

The major advantages of the SRM technique are the following: (1) multiplexed as 10s to 100s of protein can be monitored during the same run, (2) absolute and relative quantification is possible, (3) the method is highly reproducible, and (4) the method yields absolute molecular specificity. The limitations of the technique include the following: (1) only a limited number of measurable proteins can be included in the same run (the system is not able to monitor thousands of proteins per run or analysis), and (2) the sensitivity cannot reach the entire proteome space of all organisms (limit of detection is at the attomolar level).[94]

Antibody-Based Approaches

Enzyme-linked immunosorbent assay (ELISA) is one of the oldest antibody-based measurement platforms used to detect and quantify target proteins in a sample. Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support either nonspecifically or specifically (via capture by another antibody specific to the same antigen, in a *sandwich* ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.[94] While this is a very robust and well-established method, it does not allow for HT analysis and multiplexing. Two more recent approaches address these needs.

Reverse protein array (RPA) technology (for instance, proposed by Zeptosens) represents an example of a key measurement platform for systems biology-based risk assessment. RPA is an HT technology that allows the systematic analysis of up to 192 different lysates on a ZeptoCHIP, which currently can be probed with 200 antibodies validated by Zeptosens. The method has the sensitivity to detect posttranslational modifications such as phosphorylation. Using this technology, it has been shown, for example, that cancer progression is associated with increased phosphorylation of Akt, suppression of apoptosis pathways, and with decreased phosphorylation of extracellular-signal-regulated kinase (ERK).[96] The RPA data can lead to the expansion and refinement of previously identified disease networks. Phosphorylation changes outside of the networks implicated by systems response profiles (SRPs) from the gene expression data will lead to the expansion and rearrangement of network topologies, potentially elucidating novel biologic processes. Information pertaining to proteomic changes will also enable the dissection of a larger disease network by focusing attention on its modulated subnetworks. For example, while transcriptomic evidence may not clearly distinguish the effects of different epidermal growth factor (EGF)-family receptors, phosphoproteomic measurements of the receptors and their substrates can potentially identify specific control networks upstream of the observed gene expression changes. A major advantage of the technique is the ability to multiplex measurements and thereby facilitate the HT analysis of samples. A limitation of the technique is that each antibody must be thoroughly validated by western blot to make sure there is no cross-reactivity with other

proteins that may influence the measurement. Furthermore, lot-to-lot variations of the antibodies can also result in inaccurate measurements.[97]

Luminex is another antibody-based measurement platform, which allows multiplexing and exhibits high sensitivity and specificity. The principle behind this technology is the ability to measure multiple analytes simultaneously in a single-reaction well. With Luminex technology, molecular reactions take place on the surface of microsphere sets that have been color-coded using a blend of two dyes with different fluorescent intensities. The advanced optics captures the color signals, which are processed digitally in real time to quantify each reaction.[98]

Lipidomics

Defects in lipid homeostasis contribute to several pathophysiological conditions ranging from metabolic diseases to cancer. Lipids exert important functions as essential constituents of all cellular membranes, as bioactive compounds, and as energy stores. Current MS-based lipidomic techniques are capable of detecting and quantifying hundreds of molecular lipid species in various tissue and biofluid samples in an HT manner. Reproducible and precise results from large sample sets can be obtained by automated sample preparation and lipid extraction in a 96-well format.[99] The total lipid extracts can then be analyzed by multiple MS platforms, by either detecting the lipid extracts directly by shotgun lipidomics[100] or after LC-based separation[101] to detect low abundant lipid species. Optimally, using both approaches, hundreds of molecular lipids from several lipid classes of high and low abundance can be relatively quickly identified and accurately quantified from small sample volumes.

Profound changes in the cellular/tissue lipid composition occur in response to intracellular and environmental factors. Lipidomics is thus a powerful tool to monitor the overall status of cells and tissues and, due to its sensitivity, has great potential to identify and detect potential biomarkers indicating toxicity at an early stage. As an example, oxidized lipids, including eicosanoids, have been suggested to be major mediators of toxicity,[102] which can be readily measured in biofluids. Indeed, urinary leukotrienes have been associated with certain forms of cancer, including prostate and kidney cancer.[103,104] Lipids from necrotic tissues have also been demonstrated to be present in urine.[105] Moreover, urinary leukotriene T4 levels correlate with lung function in asthmatic patients.[106,107] It is highly likely that lipidomics will yield useful biomarkers indicating dysfunctionality or toxicity of other organs, such as the liver.

The utility of lipidomics as a tool to establish the MOA for active substances is highlighted in the case of Ximelagatran, an oral direct thrombin inhibitor, which was withdrawn from the market. The drug showed a potential risk of severe liver injury following its chronic use despite the fact that no signs of liver toxicity were observed in clinical trials. In a recent study, increased membrane fluidity induced by ximelagatran in hepatocytes was associated with a decreased phosphatidylcholine/phosphatidylethanolamine molar ratio after short exposure to the drug,[108] providing a potential explanation and measure for liver toxicity. It is important to note that these effects were observed after short exposure using low compound concentrations, underlining the sensitivity of the method. Moreover, the observed membrane fluidity effects were selective to primary human hepatocytes, with no similar effect seen in hepatocytes derived from rat. Since rat and human hepatocytes have strikingly different lipid contents, this may indicate that the response depends on the composition of the target membrane.

High-Content Screening

Molecular changes should be as often as possible correlated with cellular- or tissue-level changes. While histopathology is the main approach to gather such data, phenotypic assessment of cells in culture can be performed using high-content screening (HCS) methods.[109] This technique is based on the visual detection of a panel of functional biomarkers providing precise temporal, spatial, and contextual information that defines the biological status of the cells. The capture of the different biomarkers can be done either on fixed cells labeled with multiplexed, fluorescence-based reagents after stimulation/treatment or directly on living cells during the time of the exposure. The panel of parameters that could be simultaneously measured is broad and could reflect changes in the level of expression of cellular products (e.g., phosphorylated protein) and/or changes in the morphology (visual appearance) of the cell. Readouts can be adapted to the type of cells used in the assay and address key cellular mechanisms such as (1) apoptosis and autophagy, (2) cell proliferation, (3) cell viability, (4) DNA damage, (5) mitochondrial health, (6) mitotic index, (7) cytotoxicity and oxidative stress, (8) nascent protein synthesis, and (9) phospholipidosis and steatosis. Toxicity in cells exposed to stimuli is often the result of a combination of multiple mechanisms that are more likely to be captured with a panel of parameters easily and simultaneously obtained through an HCS approach. In addition, these mechanistic endpoints can be detected below the point of high cytotoxicity allowing the analysis of additional HT measurements such as transcriptomic or proteomic profiling (Figure 7.3c). HCS technology can be performed using automated digital microscopy (e.g., ArrayScan) or flow cytometry, both combined with computational systems required for data analysis and storage. There is a large choice of HCS instruments on the market, and all of them offer the ability to capture, store, and analyze images automatically. Some HCS instruments also integrate into large robotic cell/medium handling platforms that allow a rapid and unbiased assessment of a large number of experiments. This technology is thus largely used for explorative screening, in particular, in the systematic phenotypic assessment of in vitro models during or following exposure with different doses of stimuli. Figure 7.3c illustrates how HCS

can be combined with transcriptomic analysis and thereby not only enhance the identification of categories of stimuli with similar properties associated with a particular cellular event but also help to uncover toxicological mechanisms. Furthermore, the systems biology approach will establish the biological relevance of the pathways identified, and with the NPA scoring algorithm (discussed in the following), one will be able to determine their quantitative contribution to the phenotypic changes.[110]

Data and Information Management

Next-generation technologies for biological systems research challenge the researchers with unprecedented amounts of data. Consequently, the management and processing approaches of the accumulated data are continuously being developed. A plethora of open-source software solutions (e.g., caArray,[111] MARS,[112] BASE,[113] EMMA,[114] MIMAS,[115,116] TM[117]) are readily available for the storage and management of raw and preprocessed data, as well as experimental information. It is to be noted that an essential component of a systems biology data integration platform is the capability to capture detailed information describing the experiment, thus enabling reproducibility and proper analysis and interpretation. The Institute of Medicine (IOM) specified experimental transparency as one of the major requirements for the *Evolution of Translational Omics*.[118] A satisfactory solution has been provided for microarray data by the minimum information about microarray experiment (MIAME)-based[119] exchange format MAGE-TAB,[120] which has become the standard for datasets deposited in public microarray databases such as ArrayExpress[121] or Gene Expression Omnibus (GEO).[122] A similar standard exists for proteomics experiments, namely, the minimum information about a proteomics experiment (MIAPE).[123]

A large component of MAGE-TAB is, aside from the data model, the ability to control terminology by employing ontologies. An ontology is a formal representation of knowledge within a domain and typically consists of classes, the properties of those classes, and relationships between them. Due to their ability to account for semantic differences in terminologies, ontologies provide a common terminology over a domain that is necessary to enable interoperability and ensure formalization of the community view. The scientific community extensively uses ontologies including (1) the experimental factor ontology supporting research activities across the in silico, in vitro, and in vivo exposure domain,[124] (2) the National Cancer Institute (NCI) Thesaurus developed in collaboration with FDA where more than 10,000 FDA terms and codes were integrated,[125] and (3) the environmental exposure ontology that aims to link exposure science to diverse environmental health disciplines, including toxicology, epidemiology, disease surveillance, and epigenetics.[126]

An assessment of ontology development initiatives with a focus on their use in predictive toxicology has shown their suitability for the twenty-first-century mechanistic-based toxicology that supports the 3Rs principle.[127] CCNet's ToxWiz ontology[128] proves suitable for application to toxicology investigations. OpenTox[129] provides a semantic web framework that can be utilized to integrate various ontologies into software applications and linked resources. OpenTox supports the creation and curation of OpenToxipedia, a community-based predictive toxicology knowledge resource.[130]

Considering these primarily independent ontology development initiatives, there is a need for a broader international effort to coordinate activities and to ultimately provide the community with a globally accepted and useful open-source toxicology ontology, much like that demonstrated by the Gene Ontology.[131]

The advent of computational biology workflow management systems (e.g., Galaxy,[132–134] GenePattern,[135] and Taverna[136]) provides interoperative workflows, facilitates a reproducible data analysis process, and simplifies the exchange of methodologies. CaArray and caGrid[137] from the National Cancer Institute Biomedical Informatics Grid (NCI caBIG®) are prominent examples of a systems biology data integration environment that includes knowledge management and workflow systems. The infrastructure allows integration with commercial data analysis and biological pathway inferencing systems as well as advanced programming interface (API) that are highly flexible and allow efficient access. Data mining capabilities can be efficiently provided by BioMart,[138] an open-source biological query framework.

ArrayTrack,[139] an integrative solution for data management, analysis, and interpretation of pharmacogenomic and toxicogenomic studies is proposed by the FDA. ArrayTrack may also be used to facilitate FDA genomic data submissions.[140]

Improving the reproducibility of microarray experiments has been a focal point of the community for several years as illustrated by initiatives such as the Microarray Quality Control Phase I (MAQC-I) project.[141,142] Technological progress in the field of bioanalytics has also led to fairly robust protocols with experimental scientists having to ensure best laboratory practices at every step. Insufficient control over quality introduces variability in the data and is detrimental to the signal-to-noise ratio.

CALCULATE SYSTEMS RESPONSE PROFILES

Experimental Workflow

Figure 7.4 shows a typical workflow for a study to measure gene expression in an in vitro or in vivo experiment. The statistical design associated with the execution of the experiment is a crucial component to assess the experimental error by handling variation, eliminate any systematic bias, and account for extraneous confounding variables linked to the technology used, the extraction/isolation steps, and others.

It is particularly important to randomize the sample grouping during the intermediate steps of organ dissection

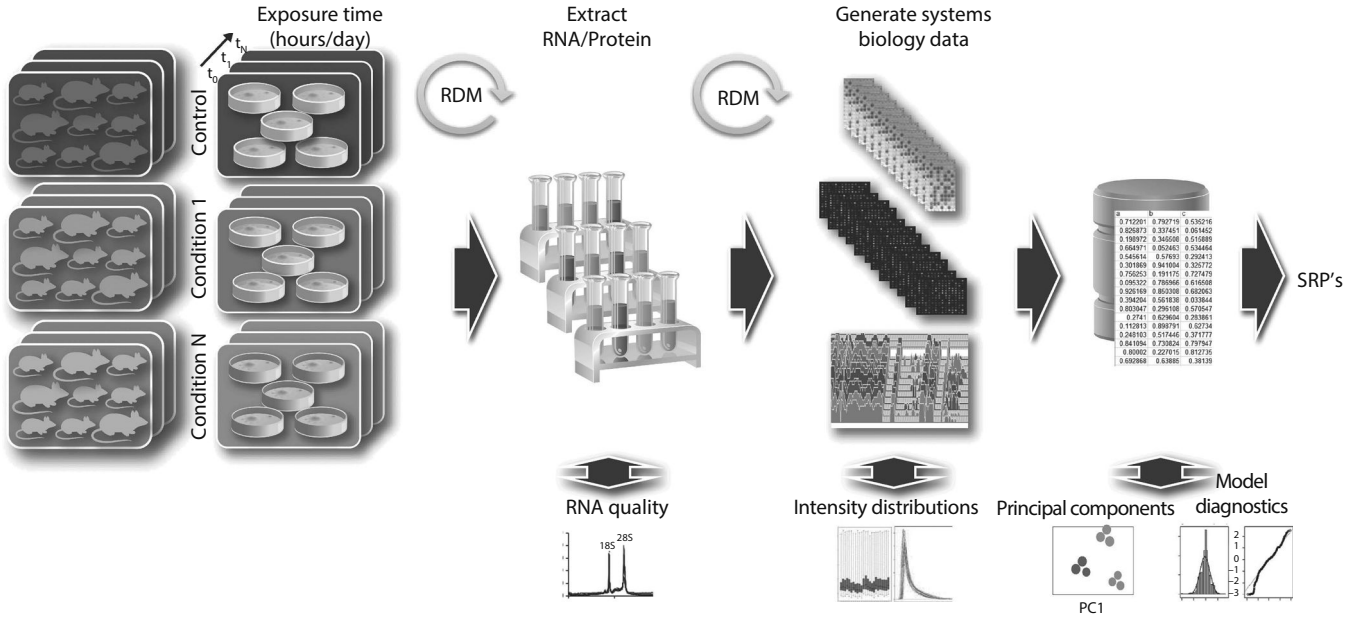

FIGURE 7.4 The workflow for computing SRPs for an experiment conducted in vitro or in vivo. The molecular species of interest are isolated from samples derived from an experiment conducted in vivo or in vitro with appropriate dose–time exposure regimen and several biological replicas. The systems biology data is generated and used for the calculation of the SRPs. Each step involves sample randomization (RDM) to avoid batch effects and QCs for each biological replicate.

and RNA extraction to avoid batch effects that can mask the actual treatment-induced signals.[143] When measuring gene expression with widespread DNA microarrays, single-channel technology (such as Affymetrix GeneChip®) circumvents the additional complications incurred by having to account for both channels when using dual-channel arrays. Finally, as indicated on the bottom of Figure 7.4, each biological sample must be controlled for quality. Once the experiment has been successfully completed, and all QCs deemed satisfactory, the generated data are ready for input into the next stage of the process where calculations of the SRPs are made.

From Experimental Data to Systems Response Profiles

The quality-controlled measurements generated by omics technologies constitute an SRP for each exposure in a given experimental system. The SRP expresses the degree to which each individual molecular entity is changed as a consequence of the exposure to the stimulus and is the result of rigorous QCs and statistical analysis as described earlier; the effects of interest are solely guided by the treatment design. In this way, different data types (transcriptomics for messenger RNAs and microRNAs, proteomics/phosphoproteomics, lipidomics, etc.) can be integrated and coanalyzed to provide the most accurate possible quantitative representation of the biology. Several processing steps are necessary in computational systems biology ranging from the raw data normalization, the design of the appropriate statistical models, to the acquisition of rich experimental meta-information in a standardized format. The following section discusses various aspects of the SRP computational workflow.

The experimental studies used to generate the data are assumed to be optimized for quality and relevance, with all measuring devices adjusted and validated according to manufacturers' instructions. The goal of the processing workflow is twofold. First, it transforms the input data (*raw data*) into the appropriate SRPs, accompanied by measures of their statistical significance. Second, it computes quality metrics at the various processing stages, in order to confirm the appropriateness of both data and applied transformation methods. Figure 7.4 gives a schematic view of the workflow. Its components are explained in the following paragraphs.

Raw data normalization: The input raw data contain intensity measurements performed directly on the images of the array (*probe level* for Affymetrix GeneChip®). As such, they do not yet provide the intensity values of actual genes (*probe set level* for Affymetrix GeneChip®). In addition, between-array comparisons might contain array-specific biases due to independent measurements of each array. The goal of raw data normalization is to generate mRNA-based intensities that can be compared across all arrays in the experiment. An efficient method to achieve this task is the robust multichip average (RMA) algorithm, based on the reasonable assumption that probe intensity distributions are identical across all arrays.[144] The first step consists of background subtraction where the effects of parasite hybridization on the microarray probes are reduced. An improvement of this step has led to the GeneChip® robust multichip average (GCRMA) algorithm, which explicitly takes into account the nucleotide content of the probes in the evaluation of the background contributions.[145] The next step is the actual normalization based on the quantile normalization algorithm that exploits

the earlier assumption of identical probe intensity distributions across all arrays. The last step is the summarization, which computes an estimate of the actual mRNA abundance based on the intensities of the multiple matching probes and uses the median polish algorithm.[146]

SRP calculation: Normalized data constitutes the input of the actual SRP calculation, together with the experimental design details that contain the relationships between all measured samples. For the sake of clarity, the simplest case of a pairwise comparison between one group of *treated* samples and one equally sized group of *control* samples is considered (see section *Design Experiment for Data Production*). More complex designs are common, and they can be handled in a similar manner to the pairwise comparison, as long as linear models are used appropriately in the calculation.[147] In a pairwise comparison context, the SRP measures the effect of the applied treatment at the gene level by comparing against a group of control samples that did not undergo the treatment. Specifically for a given gene, the response consists of the difference between mean log2 intensities of the group of treated samples and mean log2 intensities of the group of control samples. This quantity is usually referred to as differential gene expression. The associated statistical significance is provided by the *t*-statistic taking into account the expression variance within each group. In the case of microarray experiments, the number of samples is often small, so that variances are difficult to be estimated accurately. A solution to this problem is provided by the moderated *t*-statistic, which improves the specificity of the SRP statistical significance by using empirical Bayes methods[147] or the significance analysis of microarrays (SAM).[148] The SRP specificity is further increased by applying multiple testing corrections, for example, the Benjamini–Hochberg correction,[149] to account for the fact that thousands of genes are measured on the microarray. At the end of the process, the SRP is characterized by the differential expression values of all genes measured on the microarray, complemented by their statistical significance, usually in terms of *P*-values or false discovery rates (FDR).

QCs: The data quality is controlled at three different levels, and specific features are examined in each case. At the raw data level, several within-array metrics are computed that allow the detection of possible hybridization in homogeneities on the microarray as well as sensitivity issues in the intensity range.[150] Normalized data are used to perform between-array comparisons and thereby identify possible outliers. Multivariate approaches like PCA are used to verify the consistency of the data, typically by showing that in reduced-dimensional space, the samples belonging to the same treatment group are closer to one another than to samples from different treatment groups. Normalized data can also be corrected for possible (nonconfounding) experimental batch effects, if this information is available.[151] This operation reduces the fraction of data variance that is not due to the test treatment and therefore increases the statistical significance of the downstream-calculated SRPs. Several algorithms have been developed for performing this task such as the combat method that uses an empirical Bayes approach. At the SRP level, the assumptions underlying the statistical models used in calculations must be, at least partly, verified. This information is derived from the so-called diagnostics plots. For the *t*-test described earlier, MA-plots, volcano plots, QQ-plots, histograms of the residuals, and so on are used to visualize this information.

Several studies have shown that the reproducibility of published gene expression studies is not 100%.[152] In order to ensure optimal acceptance by both the scientific community and regulatory authorities, particular attention must be given to aspects of the process that can improve reproducibility. These are discussed in the following paragraphs.

Technical standardization: To facilitate a reimplementation of the processing pipeline, it is beneficial to use the free and open-source software, such as Bioconductor,[153] which has become a standard in computational biology. The algorithms described earlier are all available as Bioconductor R packages thereby enabling the full SRP pipeline to be run in the R environment. An additional capability of Bioconductor is its software versioning and archiving policy put in place in the repository,[90] which significantly contributes to the reproducibility of the processing pipeline.

BUILDING OF MOLECULAR NETWORKS AND QUANTIFYING THEIR ACTIVATION

Network Model Basics and Computational Aspects

While classical toxicogenomic studies use differentially regulated gene lists,[154] we are using network models to gain insight into the effects of a given stimulus. This approach provides a more detailed molecular understanding of biological network perturbations by extracting mechanistic information from HT datasets. The networks are built using biological expression language (BEL), a semantic programming language that allows for the representation of biological processes in a computable format.[22] BEL-encoded causal relationships describing the pathways of interest are extracted from the Selventa Knowledgebase,[155] a comprehensive repository containing over 1.5 million nodes (biological concepts and entities) and over 7.5 million edges (connections between nodes). The knowledge base is built by manually curating scientific literature and extracting causal molecular relationships from primary publications.

The nodes in the networks are biological entities—protein abundances, mRNA expressions, and protein activities (e.g., kinase or phosphatase activities, transcriptional complex activities)—or processes (e.g., apoptosis). For many nodes, there is also literature-derived and BEL-encoded knowledge about the ability of that node to regulate the expression of particular genes. For example, the cytokine tumor necrosis factor (TNF) may be represented in a network model as increasing the activity of its receptor, TNF-R1. In addition, there is inherent knowledge about the

genes that are regulated by TNF. This knowledge is collected from experiments where the genes whose expression levels change following exposure to TNF. The number of downstream genes can vary from few to several hundred depending on the node. These node–gene expression relationships are exploited by reverse causal reasoning (RCR), an algorithm that operates on molecular profiling data to infer the activity of an upstream node from differentially expressed genes.[155–158] RCR relies on the presence of nodes in the model that are computable for this particular algorithm, in this case, nodes where causal downstream knowledge about the ability of that node to influence the expression of a particular gene. Specifically, it relies on two metrics to compute potential upstream causal explanations for changes observed in the data: richness and concordance. Richness is calculated as a *P*-value based on a hypergeometric probability distribution and determines how significant the signal is (e.g., is there a predicted modulation in MAPK13 activity). Concordance is calculated as a *P*-value based on a binomial distribution and determines confidence in the directionality of the prediction (e.g., is the predicted activity of MAPK13 increased or decreased). Generally, predictions are only considered further if they meet criteria for both statistics and then are interrogated for biological plausibility given the context of the experiment under investigation. The BEL framework is an open-source technology for managing, publishing, and using structured life-science knowledge. It can be downloaded from the web (http://www.openbel.org/), along with a corpus of knowledge and tools that enable model investigation (KAM Navigator) or RCR on gene expression data (Whistle).

Model Building

Building network models is a multistep, iterative process shown in Figure 7.5a. The construction of network models starts with the careful selection of model boundaries, that is, the selection of appropriate tissue/cell context and biological processes to be included in the model. This is unlike other common approaches for building pathway or connectivity maps where connections are often represented out of tissue or disease context. Once the boundaries have been defined, the scientific literature is carefully reviewed to extract cause-and-effect relationships that comprise the literature model's nodes and edges (causal relationships between the nodes).[159]

Following the assembly of the literature-based network model scaffold, RCR on molecular profiling data is performed to ensure that the network accurately reflects the biological processes of interest. Multiple datasets are used to verify the network content, ideally from experiments where the experimental exposure perturbs the biological mechanisms captured by the network model under construction. RCR predictions are computed for all nodes with downstream genes in the Selventa Knowledgebase[155] from the differentially expressed genes in a dataset. Any predicted nodes that are not present in the literature model are investigated in detail by a scientist for relevance and mechanistic connection to the biological processes being modeled. Examples of this investigation process include the following interrogations: Determining whether an inferred change in the activity state of a biological mechanism or pathway is consistent with what would be expected in the context of the experiment under investigation and examining whether the inferred mechanism is known to be expressed in the tissue under study. This results in a more comprehensive, integrated model that includes nodes derived from existing literature as well as nodes derived from experimental datasets underscoring the value of combined use of molecular profiling data and prior biological knowledge of cause-and-effect relationships.

In the last steps of the model building, the integrated model is carefully reviewed by subject matter experts edge by edge and amended to include all relevant biology as well as exclude any inaccurate causal relationships and entities from the model. To verify that the model captures the desired biology, parts of the final model are validated with relevant datasets. The nodes that are predicted to change by RCR from the transcriptomic data are mapped to the model and evaluated against any phenotypic observations in the experiment. If the predicted nodes highlight relevant signaling pathways, the model is considered to capture the expected biology by linking the differential gene expression with observed phenotypes. Moreover, any new nodes that do not *fit* the current knowledge can be experimentally verified in the appropriate context to gain new biological insight.

Sometimes, public experimental data from an appropriate experiment are limited. In such cases, a focused experiment can be designed with relevant biological endpoints and systems biology data to validate parts of a network. To validate the cell proliferation network model, we used a well-controlled cell culture experiment where NHBE cells in culture were allowed to reenter the cell cycle after G1-arrest induced by exposure to a small molecule cyclin dependent kinase (CDK)4/6 inhibitor, PD-0332991. While this compound is highly specific for the cell cycle-dependent kinases CDK4 and CDK6, no activity has been found against a panel of 36 additional protein kinases.[159] PD-0332991-mediated inhibition of CDK4 and CDK6 has been shown to specifically reduce the phosphorylation of retinoblastoma (RB1) protein at Ser780/Ser795, thereby inducing an exclusive G1 arrest.[160] The cultures subsequently released from cell cycle arrest provide a systems biology dataset representing the reentry to cell cycle concomitant with a direct cell proliferation measurement in a human lung in vitro system and the means to experimentally validate the computational model. We chose the NHBE cells since they have been frequently used to assess lung epithelium responses, including cell cycle progression under various conditions,[161–164] and can be synchronized in vitro in G0/G1.[163] We confirmed the reentry to cell cycle by labeling of S-phase cells at 2, 4, 6, and 8 h after CDKI washout; an unperturbed proliferating control group receiving

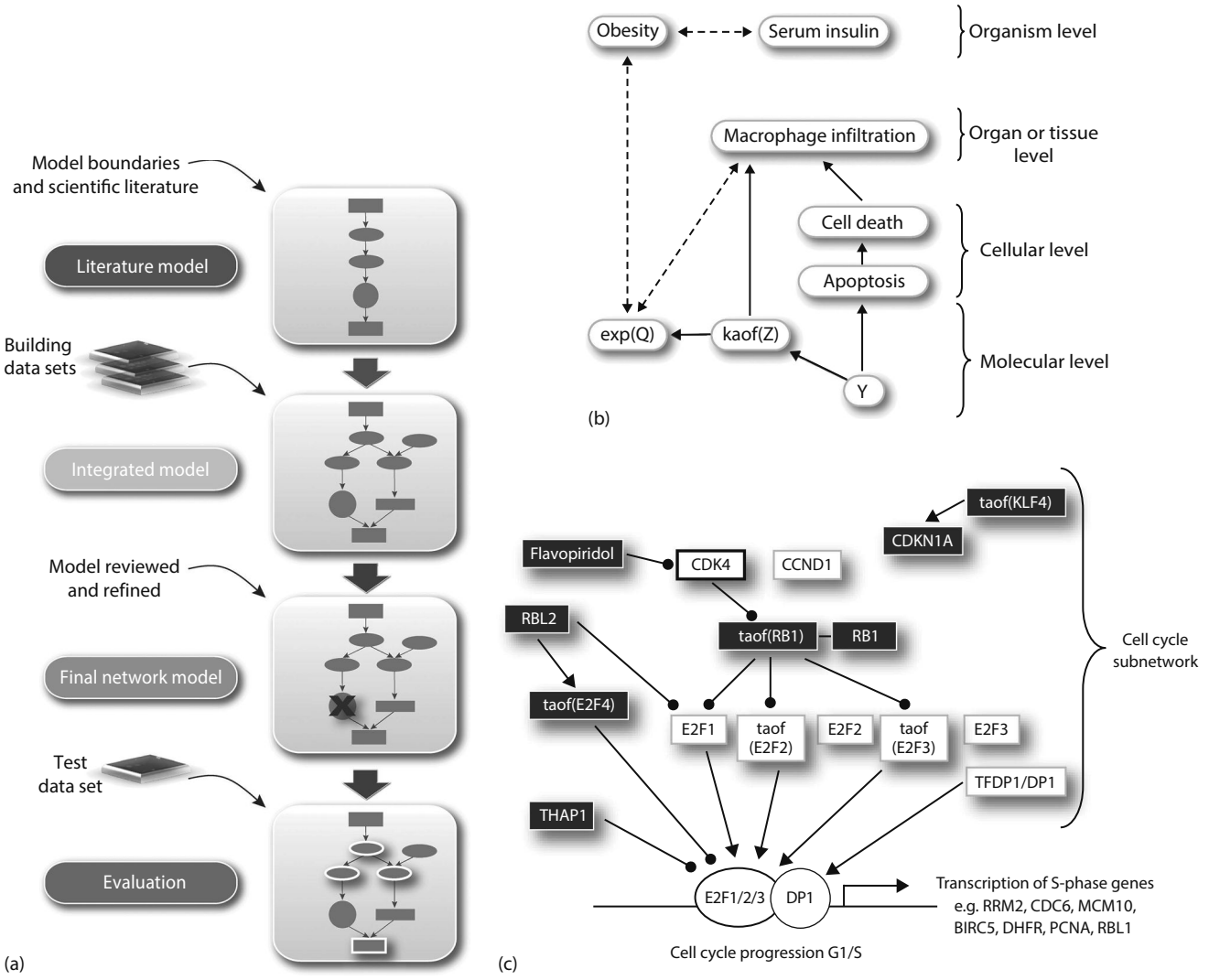

FIGURE 7.5 Model building using the BEL. (a) The network is built in steps starting with the literature analysis and extraction of causal life-science relationships. The integrated model is obtained by enhancing the literature model with molecular profiling data and RCR. The integrated model is carefully reviewed and finally validated with a relevant dataset. The *X* in the second last step indicates the decision to remove some nodes from the model as a result of model review. The highlighted nodes in the last step indicate the nodes that were specifically validated with relevant data. (b) The network model can represent knowledge from molecular, cellular, and organ level to an entire organism. (c) Transcriptomic data and RCR from a tailor-made validation experiment show the relevant pathways that are activated when cells reenter the cell cycle. The light boxes represent the nodes that are predicted increased, and the dark boxes represent the nodes that are downregulated upon removal of the CDK inhibitor.

only growth medium was also included. To validate the cell proliferation network, we applied RCR to the cell proliferation datasets described earlier (E-MTAB-1272). We used the differentially expressed gene list to compute RCR for more than two thousand biological entities, included in the Selventa Knowledgebase.

Within the cell cycle subnetwork, 26 nodes were predicted to be regulated. In agreement with the experimental setup, the node for CDK4 was predicted to be increased upon inhibitor washout. Several connections between the key players were identified as shown in Figure 7.5c. Remarkably, the node highlighting the activity of flavopiridol, a cyclin-dependent kinase inhibitor under clinical development, was also inhibited (Figure 7.5c). This underscores the utility of network models using relevant experimental data in verifying the MOA of active substances. A comprehensive validation of the cell proliferation network has been published along with valuable mechanistic information on proliferative processes in the context of the lung.[165]

Properties of Network Models

The causal network models resulting from the three-step building process described earlier provide many advantages over differentially expressed gene lists. The networks are highly dynamic, they can be modified to specific species and tissue contexts by the definition of appropriate boundaries, and they are easily updated as new knowledge

becomes available. They also capture a wide range of biology that exists in a system outside of mRNAs, including but not limited to genomics, transcriptomics, proteomics, phenotypic or clinical observations, chemicals, lipidomics, methylation states or other modifications (e.g., phosphorylation), and miRNAs. Owing to the preserved topology, the causal relationships within the network can be easily traced to a measurable entity thus providing a link between the upstream node and the SRPs. By mechanistically interpreting the SRPs, the networks provide the basis for the next two steps of the five-step strategy, the NPAs and BIF calculations. In addition to RCR, these are two more examples of analytics that rely on networks built on the computable BEL language.

The application of RCR on transcriptomic data in this way implies a critical divergence from more commonly known pathway techniques such as *Kyoto Encyclopedia of Genes and Genomes* (KEGG)[166] and ingenuity pathway analysis (IPA) for model building.[167] These methods identify differentially expressed genes whose protein product activities act in a pathway of interest, thereby relying on the *forward assumption*, where gene expression changes are only indicative of the abundance of the protein or the predicted activity of the encoded protein. The caveat remains that changes in gene expression do not always correlate with changes in protein activity. Furthermore, in these cases, it is not possible to make any inferences about genes whose biological function is entirely unknown. RCR does not rely on these assumptions and can take into account the multiple states of regulation that may exist for a protein before action due to the fact that BEL can capture the life cycle from mRNA to protein abundance to interactions with other proteins to various activity states.

Current Network Models

To date, we have built six complete biological network models that capture mechanistic detail of various biological processes. The proliferation network focuses on diverse biological areas that lead to the regulation of normal lung cell proliferation.[159] The cell stress network reflects the biology underlying the physiological cellular response to endogenous and exogenous stressors, including oxidative stress, hypoxia, shear stress, endoplasmic reticulum stress, and xenobiotic stress, elicited in response to common pulmonary and cardiovascular stressors.[168] We have also constructed network models that cover inflammatory processes in both pulmonary[169] and cardiovascular tissues as well as a network model for DNA damage response and the four main cellular fates induced by stress.[170] The final network model constructed describes angiogenesis and the processes involved in respiratory tissue remodeling and repair.[171] The main focus of these six networks is on the biological processes that can operate in nondiseased mammalian pulmonary and cardiovascular tissues. The detailed listing of the biological processes included in these six networks is described in Table 7.4. All network models are available for the scientific community through publications in peer-reviewed journals. The network models are provided in extensible graph markup and modeling language (XGMML) format so that they can be visualized and analyzed using freely available open-source network viewing software such as Cytoscape. Providing the network model in a format compatible with open-source access provides both a high degree of scientific transparency about the contents of the network models and provides the scientific research community with a unique set of network models to adapt for their own use.

The networks themselves are not static entities. Their content is based on prior knowledge, whether gained through literature curation or through integration with relevant predictions illuminated by RCR. However, it is necessary to update them as new knowledge becomes available. This relies on updates to the knowledgebase itself, and processes are being put in place to optimize sustainable knowledge augmentation solutions. Manual efforts are viable options, but can be time-consuming and expensive, and therefore assisting or replacing such a process with natural language processing (NLP) is under development. The NLP tools developed toward this end can also be made open source to benefit the BEL community at large.

The networks are also amenable to the investigation of interspecies translatability. Additional analytics have been developed that can interpret omics data based on a priori knowledge in order to facilitate comparison of specific biology across experimental conditions in different species. One of these conditions is the special case where data from an experiment performed in one species can be compared with data from another in order to determine areas of shared and unique biology. The Selventa Knowledgebase captures causal information about mouse, rat, and human relationships. If a causal relationship between two nodes has been captured for one species (e.g., A increases B), and homologs for A and B (e.g., A′ and B′) exist in a second species, then this same relationship is assumed to exist in the second species. In this way, networks that have been developed based largely on relationships describing human biology can be *homologized* in the Selventa platform to either mouse or rat. One main advantage of this property is that an area of biology that may be less comprehensively studied in humans, perhaps for technical or ethical reasons, can still be represented in the network.

We believe that our network approach is well positioned to meet twenty-first-century toxicity-testing needs. To progress, pathway definition, pathway annotation, and a central repository of the annotated pathways are needed. Moreover, new methods for pathway identification need to be established and evaluated. Finally, the pathways need to be continually refined as new knowledge emerges, and a peer-review process will be necessary to enable the scientific community to reliably develop new and updated versions of the pathway biology with emergent knowledge.

Our network building efforts are a good first step toward these goals. By organizing gene lists into a biologically meaningful context, it is more feasible to analyze omics data as well as identify potential toxicity pathways. These networks could provide a consistent way to group stimuli based on their MOA and could become the standard in toxicity testing.

TABLE 7.4
Biological Network Models and Biological Processes

Network (Nodes/Edges/References)	Subnetwork/Biological Processes
Cell proliferation (429/848/1597)	Cell cycle
	Calcium
	Cell interaction
	Clock
	Epigenetics
	Growth factor
	Hedgehog
	Hox
	JAK STAT
	Mapk
	Notch
	Nuclear receptors
	PGE2
	Wnt
	mTor
Cellular stress (730/1280/428)	Drug metabolism response
	Endoplasmic reticulum stress
	Hypoxic stress
	NFE2L2 signaling
	Osmotic stress
	Oxidative stress
Inflammatory (lung) (1320/1619/1327)	Mucus hypersecretion
	Epithelial cell barrier defense
	Epithelial proinflammatory signaling
	Neutrophil response
	Macrophage-mediated recruitment
	Neutrophil chemotaxis
	Tissue damage
	Macrophage activation
	Macrophage differentiation
	Th1 differentiation
	Th1 response
	Th2 differentiation
	Th2 response
	Th17 differentiation
	Th17 response
	Treg response
	Tc response
	NK cell activation
	Mast cell activation
	Dendritic cell activation
	Dendritic cell migration to tissue
	Dendritic cell migration to lymph node
	Megakaryocyte differentiation
	Microvascular endothelium activation
Inflammatory (cardiovascular) (611/1072/1344)	Platelet activation/aggregation
	Endothelial activation/dysfunction
	Monocyte/macrophage interaction
	Early plaque formation
	Vulnerable plaque rupture

(*continued*)

TABLE 7.4 (continued)
Biological Network Models and Biological Processes

Network (Nodes/Edges/References)	Subnetwork/Biological Processes
DNA damage and cell fates (1052/1538/1231)	Apoptosis—caspase cascade
	Apoptosis—endoplasmic reticulum (ER) stress-induced apoptosis
	Apoptosis—MAPK signaling
	Apoptosis—NFkB signaling
	Apoptosis—PKC signaling
	Apoptosis—proapoptotic mitochondrial signaling
	Apoptosis—prosurvival mitochondrial signaling
	Apoptosis—TNFR1/Fas signaling
	Apoptosis—TP53 TS
	DNA damage—components affecting TP53 activity
	DNA damage—components affecting TP73 activity
	DNA damage—components affecting TP63 activity
	DNA damage—DNA damage to G1/S checkpoint
	DNA damage—DNA damage to G2/M checkpoint
	DNA damage—double-strand break response
	DNA damage—inhibition of DNA repair
	DNA damage—nucleotide excision repair (NER)/XP pathway
	DNA damage—single-strand break response
	DNA damage—TP53 TS
	Necroptosis—Fas activation
	Necroptosis—proinflammatory mediators
	Necroptosis—receptor-interacting serine-threonine kinase/reactive oxygen species (RIPK/ROS)-mediated execution
	Necroptosis—TNFR1 activation
	Autophagy—autophagy-related gene (ARG) induction of autophagy
	Autophagy—autophagy induction
	Autophagy—mammalian target of rapamycin (mTOR) signaling
	Autophagy—protein synthesis
	Senescence—oncogene-induced senescence
	Senescence—regulation by tumor suppressors
	Senescence—replicative senescence
	Senescence—stress-induced premature senescence
	Senescence—transcriptional regulation of the senescence-associated secretory phenotype (SASP)
Tissue repair and angiogenesis (666/1215/1371)	HIF1A regulation and signaling
	Sprouting and tubulogenesis
	Growth factors—cell growth and nutrient support
	Vascular endothelial growth factor (VEGF)-mediated cell growth and nutrient support
	Immune regulation of angiogenesis
	Cell migration and spreading
	Differentiation of progenitor cells
	Immune regulation of tissue repair
	Fibrosis and epithelial–mesenchymal transition

Compute Network Perturbation Amplitudes

Any relevant dataset evaluated against our network models gives a qualitative overview on the biological pathways that are affected by a given stimuli. To enable a quantitative measure of the perturbation of biological networks, we have developed a computational approach that translates gene fold-changes into NPA scores. The NPA has two main components (Figure 7.6):

1. A biological network model describing a biological process or mechanism. As described earlier, the network models are a *cause-and-effect* mechanistic knowledge representation based on all relevant literature and published datasets.
2. An expression dataset resulting from an experiment, whose controlled perturbations are hypothesized to be involved in the biology related to the network.

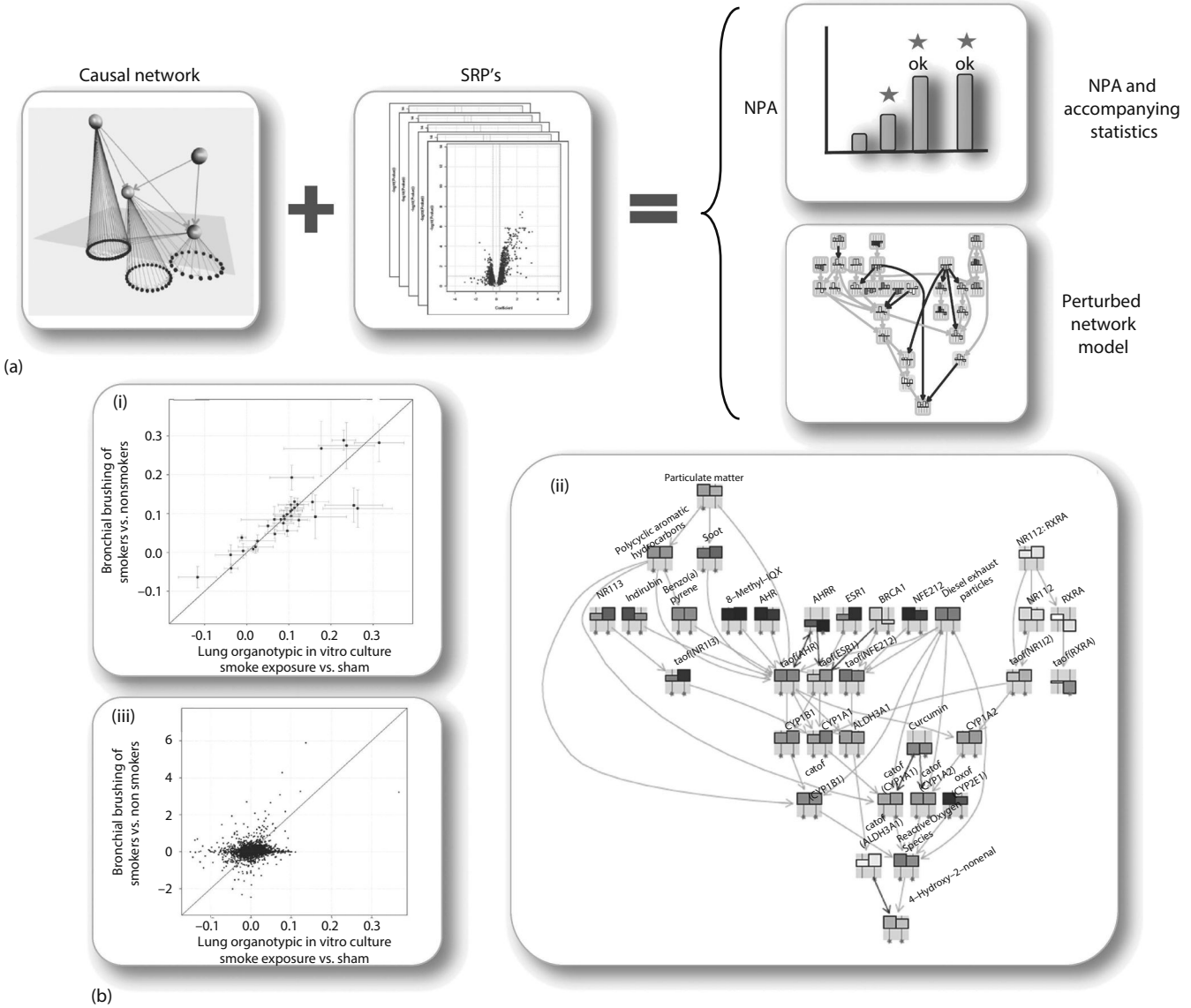

FIGURE 7.6 NPA. (a) The causal networks capture biology in the nodes (big balls) and causal relationships between the nodes. Differential expressions of genes (small black balls) are experimental evidences for the activation of an upstream node. With the use of a network model, the entire SRPs can be mathematically transformed into a small set of numbers, that is, the NPA scores. The NPAs are interpreted with their accompanying statistics. Besides the amplitude itself, backbone differential values will serve as the basis for the interpretation. The light and dark lines indicate positive or negative causal effects within the perturbed network backbone, respectively. (b) Comparison for the whole smoke-exposed and smoke-nonexposed groups in lung organotypic in vitro culture versus bronchial brushing data on the cell stress–xenobiotic response network: (i) NPA backbone differential values together with the 95% confidence intervals; (ii) the same data mapped onto the network backbone; for each node, the left-hand bar represents the perturbation amplitude for smokers as compared to healthy nonsmokers, and the right-hand bar represents the perturbation amplitude for smoke-exposed as compared to control lung organotypic in vitro culture. The intensity of the shading of each bar reflects the absolute value of the backbone differential values. (iii) The gene fold-changes underlying the cell stress–xenobiotic response network.

While RCR has been previously exclusively employed as an exploratory tool for identifying relevant perturbed biology by drawing qualitative mechanistic inferences based on statistical enrichments,[157,172,173] NPA derives a *response profile* at the network level, which then allows a coarse-grained view of the effects of the applied treatment encompassed by the SRPs. Similar to RCR, NPA takes advantage of the measured downstream effects to infer the activity of an upstream node, but also extends this by integrating the topology, the directionality, and the sign of the edges of the network in the computation. This, in turn, can be expressed as a linear combination of the measurable data. As a consequence, the NPA scores are expected to correspond to the resulting changes in the activity of the cellular processes described by the network model.

By this stage in our overall process, the SRPs have been obtained from an appropriately designed experiment, and the application of omics tools has been used to measure gene expression, protein abundance, posttranslational modifications, and lipids. The methodology described hereafter is built on our previously published algorithms.[174] Following the steps depicted in Figure 7.6a, NPA calculation first provides the assessment of the NPAs, and second, enables mechanistic interpretation of the data based on the biology contained in the network model.

Formally: Let $G = (V, E)$ be the complete signed directed graph underlying the biological network. The measurable evidences (the downstream genes) induced by the processes in the model (black nodes in Figure 7.6a) is denoted by V_0. Its complementary, $V \backslash V_0$, is called the backbone of the network model (grey nodes in Figure 7.6a).

The *backbone differential values* are the result of a fitting procedure between the network model and the input gene differential expressions. The more closely the gene differential expressions fit the cause and effects of the network model, the further from zero are the backbone differential values.

Formally: Let $\beta \in l^2(V_0)$ be an SRP for the downstream genes. Assuming that contrasts *propagate* through causal edges according to the signs and direction (linear approximations of the *real* transfer function), one wishes to compute the smoothest function $f \in l^2(V)$ with the boundary condition $f|_{V_0} = \beta$. It leads to the following optimization problem: $\arg\min_{f \in l^2(V)} \Sigma_{x \to y} (f(x) - sign(x \to y) f(y))^2 \cdot w(x \to y)$ such that $f|_{V_0} = \beta$, can be reformulated as

$$\arg\min_{f \in l^2(V)} f^T L f \quad \text{such that} \quad f|_{V_0} = \beta.$$

If $L = diag(d) - (A + A^T)$, where $A_{xy} = \begin{cases} sign(x \to y) \\ w(x, y) \end{cases}$ if $x \to y$, 0 else, if denotes the signed directed Laplacian of the graph and d the node degrees, denoting L_2 in the restriction of L to $l^2(V \backslash V_0) \to l^2(V_0)$ and L_3 to $l^2(V \backslash V_0) \to l^2(V \backslash V_0)$, and differentiating the earlier expression leads to the solution of (*): $f|_{V \backslash V_0} = -L_3^{-1} L_2^T f|_{V_0}$.

Now that values are inferred on the backbone, we define the perturbation amplitude to be the total backbone *energy*:

$$\mathrm{NPA}(G, \beta) \overset{\text{def}}{=} \frac{1}{|\{x \to y\} \quad \text{s.t.} \quad x, y \notin V_0|} \times \sum_{\substack{x \to y \\ \text{s.t.} \quad x, y \notin V_0}} (f(x) + sign(x \to y) f(y))^2.$$

Observe that the NPA score can be rewritten as a quadratic form: $\mathrm{NPA} = f|_{V \backslash V_0}{}^T \, Q f|_{V \backslash V_0}$, where

$$Q = \frac{1}{|\{x \to y\} \quad \text{s.t.} \quad x, y \notin V_0|} \times \left[\left(diag\left(\text{out}|_{l^2(V \backslash V_0)} \right) + diag\left(\text{in}|_{l^2(V \backslash V_0)} \right) - (-A - A^T) \right)|_{l^2(V \backslash V_0)} \right] \in l^2(V \backslash V_0)$$

Confidence intervals can be estimated for $f|_{V \backslash V_0}$ and for the NPA, calling for the central limit theorem, by using the following classical equalities $var(-L_3^{-1} L_2^T g) = (L_3^{-1} L_2^T) \Sigma (L_2 (L_3^{-1})^T)$ and $var(g^T Q g) = 2tr(Q \Sigma Q \Sigma) + 4 \mu^T Q \Sigma Q \mu$; where Σ is the variance covariance matrix of g.

In addition to the confidence intervals accounting for the experimental error (e.g., biological variation between samples in an experimental group), some companion statistics are derived to give a sense of the specificity of the NPA quantity with respect to the biology described in the network. Those additional statistics are vital for the interpretation of an NPA as it is by essence a backward *computation* whereby the experimental data are used to extract an amplitude.

To this end, two permutation tests are derived to assess whether the observed signal is a property inherent to the evidences (genes) or the structure given by the model. The first test is based on reshuffling the gene labels at the low model level (V_0) leading to a first NPA permutation *P*-value (denoted by *O in the figures when <0.05). The second test assesses whether the backbone structure ($V \backslash V_0$) is important for extracting the signal: the edges of the backbone model are randomly permuted, and a second NPA permutation *P*-value (denoted by K* in the figures when <0.05) is derived. The latter describes the importance of the *cause-and-effect* relationships encoded in the backbone of the network while the former is testing if the results are specific to the underlying evidences in the model. The network is considered to be specifically perturbed if both *P*-values are low (typically <0.05). The NPA results must always be interpreted in light of the three companion statistics: the two permutation *P*-values and the confidence interval.

NPA scoring is therefore an integrated approach that combines omics data with a knowledge-driven network model, to provide measurable quantities causally affected

by a targeted biological process. NPA quantifies the activity changes of the biological/toxicological process relative to a nonperturbed control state of the system. By providing a measure of biological network perturbation, together with its description, it allows correlation of molecular events with phenotypes that characterize the network at the cell, tissue, or organ level. While an NPA score, with its accompanying statistics, provides a quantifiable measure of the degree of perturbation of a biological network, the backbone differential values describe how a perturbation propagates through the network topology to further support biological interpretation. This approach differs from Gene Set enrichment analysis, which is based on the enrichment of sets of genes in experimental data and which enables qualitative investigation of experimental data in light of the statistical enrichment of mechanisms represented by each gene set.[175]

To summarize, NPA is a mechanistically driven quantitative measure of the amplitude of perturbation of a network that can enable a quantitative, system-wide understanding of the biological mechanisms leading to diseases or toxic endpoint. Besides being a comparative assessment tool, the NPA can serve to define biologically based dose–responses (toxicity thresholds).

Use case: In order to gain mechanistic insight into the biological effects of acute CS exposure on bronchial epithelial cells, we exposed the lung organotypic in vitro culture to mainstream CS for 7, 14, 21, or 28 min at the air–liquid interface and investigated various biological endpoints (e.g., gene expression and microRNA profiles, matrix metalloproteinase [MMP]-1 release) at multiple postexposure time points (0.5, 2, 4, 24, 48 h). The transcriptomic dataset has been made publically available (E-MTAB-874).

To show the validity of the NPA approach, we have used the aforementioned lung organotypic in vitro culture dataset (E-MTAB-874) and a public dataset of smoker bronchial brushings (GSE7895) to probe the translatability of the network perturbation using a well-defined network model. We and others have shown that the in vitro airway model closely resembles the human situation both morphologically as well as at a molecular level[56,57,176–180]. Coherence between smoking-induced responses, such as the antioxidant response, has been observed between human smoker bronchial epithelium samples and in vitro organotypic cultures. When scoring a network model that captures the signaling involved in *xenobiotic metabolism*, the perturbation amplitude is very similar for several nodes (Figure 7.6bi). The xenobiotic stress response recapitulated by the network is coherent in both systems (Figure 7.6bii), whereas the gene by gene comparison does not compare in an evident manner (Figure 7.6biii).

By extracting quantitative information from differentially expressed lists of genes using the networks, we have successfully reproduced the response of two systems to one stimulus.

Biological Impact Factor Calculations

The final step of the strategy is the computation of a BIF. As indicated by its name, the BIF aims to quantify the biological impact resulting from the exposure of a biological system to one or several stimuli; it represents a holistic score that describes the systems-wide effect of all the processes captured in the underlying network models and their associated NPA scores. The attributed BIF values for stimuli can then be quantitatively compared based on a high-level view of their biological effects. Since it aggregates NPA scores that have themselves already filtered a large fraction of the noise initially contained in the SRPs,[174] the BIF is expected to produce results with increased robustness against technical and biological sources of variability.

In Figure 7.7, the BIF is represented as a starplot in which the multiple axes contain the NPA scores computed for each of the considered biological network models. Computing the surface of the polygon formed by the NPA scores obtained for a given SRP constitutes an intuitive BIF algorithm. Similarly, the fundamental idea behind the BIF is to use the amplitudes of the perturbations induced by the exposure in an appropriate set of biological network models as the input of a simple scoring scheme, which provides a quantitative measure of their global effect. From this point of view, the BIF algorithm is first and foremost intended to detect and display trends in its input dataset. As a consequence, the a priori selection of networks to be included in the BIF calculation, while it must be biologically sound, does not constitute its most critical aspect, since only the significantly perturbed ones will contribute to the BIF results. Ideally, even if the chosen networks do not exhaustively cover the underlying biology, they will still capture a significant portion of the systems response due to the strategy put in place in Step 3.

Having computed the NPA scores for the selected biological network models (Step 4), the relative importance of each network model must be determined. While the BIF deduced from the radar chart in Figure 7.7 weights every axis equally, other choices are possible. Network preference based on a priori qualitative knowledge is not easily translatable into objective and reproducible weights. Data-driven weighting schemes, such as multivariate dimension reduction methods, may be more appropriate.[181] The final step of the BIF calculation consists of aggregating the weighted NPA scores. As illustrated by the surface-based BIF from the radar chart in Figure 7.7, a simple sum of the weighted NPA scores is not necessarily the most meaningful solution. Methods based on more advanced geometric considerations may be more appropriate and is the approach used for the use cases. The aggregation process is also expected to determine the contribution of nodes belonging simultaneously to several network models, such as the highly connected nuclear factor (NF)-κB transcription factor. Additional methods are being developed to avoid overweighting these contributions.

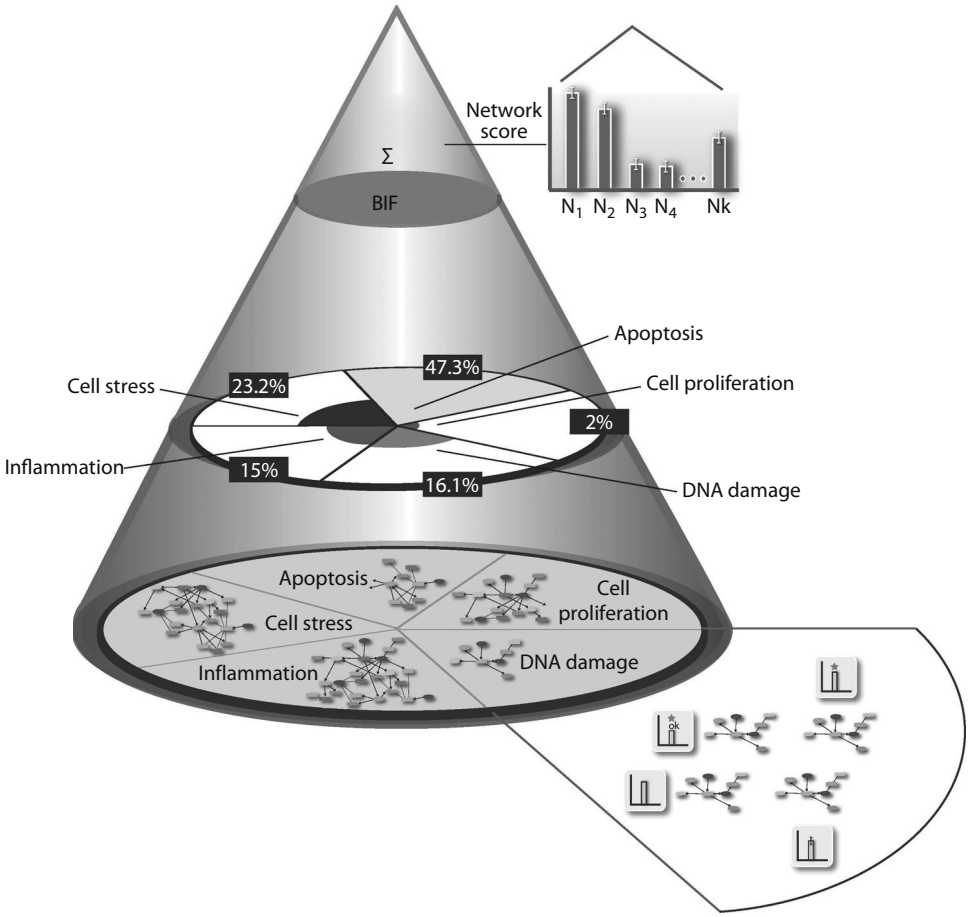

FIGURE 7.7 Calculation of the BIF. The bottom level of the BIF cone illustrates the networks and their individual perturbation amplitudes. The middle level contains a starplot showing the contribution of the mechanisms to the overall BIF (BIF at the top of the cone).

In summary, we have established a well-defined framework (Figure 7.7) that enables transparent information agglomeration such that entire SRPs can be mathematically transformed into a limited set of numbers (NPA scores and then a BIF value). If the validity requirements of the four steps are met, the BIF has the potential to provide a simple but scientifically sound measure of the biological impact of a stimulus on a system, thus serving as a biology-based dose–response model.

Although this aspect has not yet been extensively tested for the BIF, the MAQC-II study has clearly shown that results based on biomarkers involving multiple genes are much less sensitive to the variances inherent in the underlying technologies.[182]

USE CASES FOR A SYSTEMS TOXICOLOGY APPROACH

28-Day Rat OECD Plus Cigarette Smoke Inhalation

We have performed a 28-day rat inhalation study according to the OECD TG 412 by exposing rats for 28 days to filtered air (sham) or to a low, medium, or high concentration of mainstream CS from the reference cigarette 3R4F (n = 10 rats/group). Following CS inhalation, rats mainly develop irritative stress-related adaptive changes in the upper airways and signs of inflammation in the lung,[183] a common disease mechanism related to the pathogenesis of COPD and lung cancer. In order to investigate disease-relevant molecular perturbations at the sites of the histopathological changes, we added five more rats per exposure group.

We measured the free lung cells in bronchoalveolar lavage fluid (BALF) by flow cytometry and measured inflammatory mediators by multianalyte profiling (MAP). We sectioned the respiratory tract at defined sites for histopathology. We separated the respiratory epithelium of main bronchus and lung parenchyma from the left lung by laser capture microdissection. The RNA isolated from the main bronchus, lung parenchyma, and the respiratory nasal epithelium (RNE) was processed and analyzed for gene expression on whole genome Affymetrix microarrays (GeneChip® Rat Genome 230 2.0 Array).

Reflecting an inflammatory response, there was a strong increase in neutrophil and macrophage counts as well as an increased level of inflammatory mediators in BALF

(Figure 7.8a). Histopathological evaluation revealed the usual dose-dependent adaptive changes in the airways as well as inflammatory responses in lung. In Figure 7.8a, the scoring for alveolar macrophages in lung parenchyma and one representative image are presented. Based on gene expression data, the NPA and BIF calculation returned major perturbations of regulatory networks related to inflammation, cell stress, cell proliferation, and senescence (Figure 7.8b). Corresponding to the histological changes, the stress-related responses were more pronounced in the RNE and bronchi, while the inflammatory responses were more pronounced in the lung parenchyma.[183] These global BIF scores for the agglomerated network models can be broken down into NPA scores for the individual subnetworks. As an example, the NPA scores for three subnetworks of the *inflammatory network* (Figure 7.8c) are shown, which relate to the histopathological findings of increased numbers of alveolar macrophages and macrophage nests in the lung parenchyma, as well as to increased levels of inflammatory mediators in BALF (Figure 7.8a). NPA scores for *epithelial proinflammatory signaling*, *macrophage activation*, and *neutrophil response* networks were significantly increased for the lung parenchyma, while they were not activated in the bronchial epithelial tissue where it is known that this kind of inflammatory reaction does not usually occur as a response to CS inhalation (Figure 7.8c).

This example demonstrates that our modular network approach with the *inflammatory network* model can resolve cell-type-specific signaling contributions from transcriptomics data on the level of inflammatory subprocesses. The observed histopathological endpoints correlated with the degree of perturbation of their associated biological networks in vivo, thus providing support for the usefulness of this approach as a powerful tool to investigate disease mechanisms in vivo and to develop a systems biology-based risk assessment.

91-Day Rat Formaldehyde Inhalation

We have also applied our quantitative approach to a public dataset from an experiment exposing rats to several doses (ranging from 0.7 to 15 ppm) and durations of formaldehyde (FA) gas in vivo (GEO accession number GSE23179).[184] FA is a known carcinogen and irritant that causes dose-dependent tumor formation in rat nasal epithelium upon long-term exposure.[185] The transcriptomic data from the nasal epithelium after 5 days and 4 and 13 weeks following exposure was accompanied by multiple measured phenotypic endpoints, such as cell proliferation, necrosis, and inflammation, processes that are all well represented in our biological networks. Based on the list of differentially expressed genes, the original paper concluded that FA exposure of 6 ppm or greater induces changes in cell cycle, DNA repair, and apoptosis, as well as ErbB-, EGFR-, Wnt-, TGF-b-, hedgehog-, and notch-signaling-related pathways.[184]

Even though our networks represent biological processes in the lung context, it is reasonable to assume that the nasal epithelium behaves similarly as the pulmonary epithelium in response to stimuli.[186] This allowed us to assess the biological impact of FA exposure on rat nasal epithelium across multiple biological processes in both a dose- and time-dependent manner. To calculate the BIF for FA-induced perturbation, we used network models that best reflect the phenotypic observations in FA inhalation studies. These included the entire *cell stress*, *proliferation*, and *inflammatory* networks as well as the networks that reflect DNA damage and cellular fates after prolonged stress, that is, *necroptosis*, *autophagy*, *apoptosis*, and *senescence* (see Table 7.4). The overall BIF values for each treatment and time point are shown in Figure 7.9a. There is a monotonic trend with exposure concentration for all time points, and the highest perturbation is achieved in nasal epithelium from rats treated with 15 ppm FA for 5 days (1 week time point) compared with air-exposed rats. The decrease in BIF with increasing exposure duration obviously reflects an overall adaptation of the tissue responses to the repeated exposure. Remarkably, this overall BIF score can be correlated with future tumor incidence resulting from FA exposure. Figure 7.9b shows the tumorigenicity rate in the rate nasal tissue measured in a study conducted by Monticello et al.[187] The dosing procedure was identical to that in the dataset we have used for BIF calculations.[184] Similar to the threshold dose for tumorigenicity, 10 ppm FA has shown elevated BIF at all time points measured, linking the BIF scores for FA exposure to tumorigenesis rates in rat nasal epithelial tissue. Figure 7.9c starplots show the different network-level BIFs for the high-dose group. The most acute effects are on the inflammatory processes, which is a known consequence of necrotic cell death.[188] Necrosis is one of the measured biological endpoints in the nasal epithelium of the exposed animals[184] and a known effect of FA (157).[189] By 13 weeks, perturbation of *DNA damage* and *senescence* networks showed an increased contribution to the overall BIF as compared to the other networks studied. FA exposure is a known carcinogen and inducer of dose-dependent increases in DNA–protein cross-links, in both in vitro and in vivo contexts.[190,191] While there is only very limited information on FA effects on senescence in the relevant context, some evidence points to p53 activation by FA leading to apoptosis or senescence that may be dependent on the cell type.[192] Moreover, FA induces global hypomethylation in cultured lung cells,[193] a phenomenon that has been observed in cells undergoing premature, as well as replicative, senescence.[194] The perturbation of the *proliferation* network is also evident upon FA exposure throughout the time points, consistent with the increased proliferation observed in the nasal epithelium; its contribution to the overall BIF increases toward more chronic exposure.[184] We have further analyzed more specific biological processes within the selected networks. The NPAs for the individual processes analyzed are shown in Figure 7.9d. Several dose

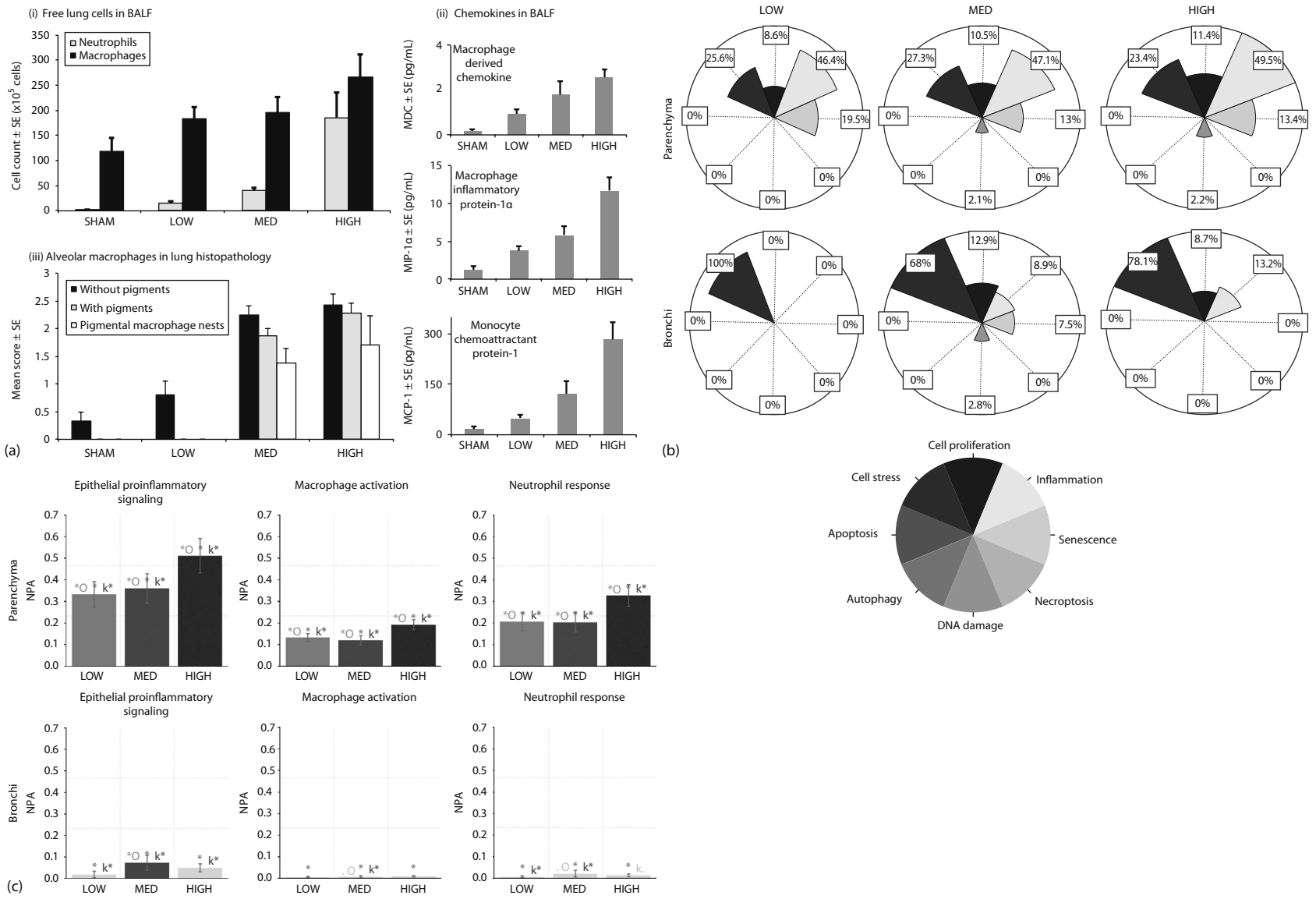

FIGURE 7.8 The 28-day rat CS inhalation study using an OECD Plus design. (a) Inflammatory measurements (i) differential cell count of free lung cells (neutrophils and macrophages) in BALF, (ii) measurement of BALF chemokines, (iii) histopathological findings in lung parenchyma given as mean score of macrophages. (b) Starplots for the smoke increasing dose (LOW, MED, HIGH) groups in parenchyma and bronchi, showing the different BIF at network level, weighted as a function of their contribution to the overall impact factor. The latter is shown in percentages. This shows that inflammation is the most important impacting process in parenchyma, whereas stress is clearly the dominant aspect for the impact of exposure to CS. (c) Quantification of the biological perturbations observed in the *inflammatory* network using NPA scoring method. Three subnetworks (*epithelial proinflammatory*, *macrophage activation* and *neutrophil response*) are shown from parenchyma and bronchi tissues.

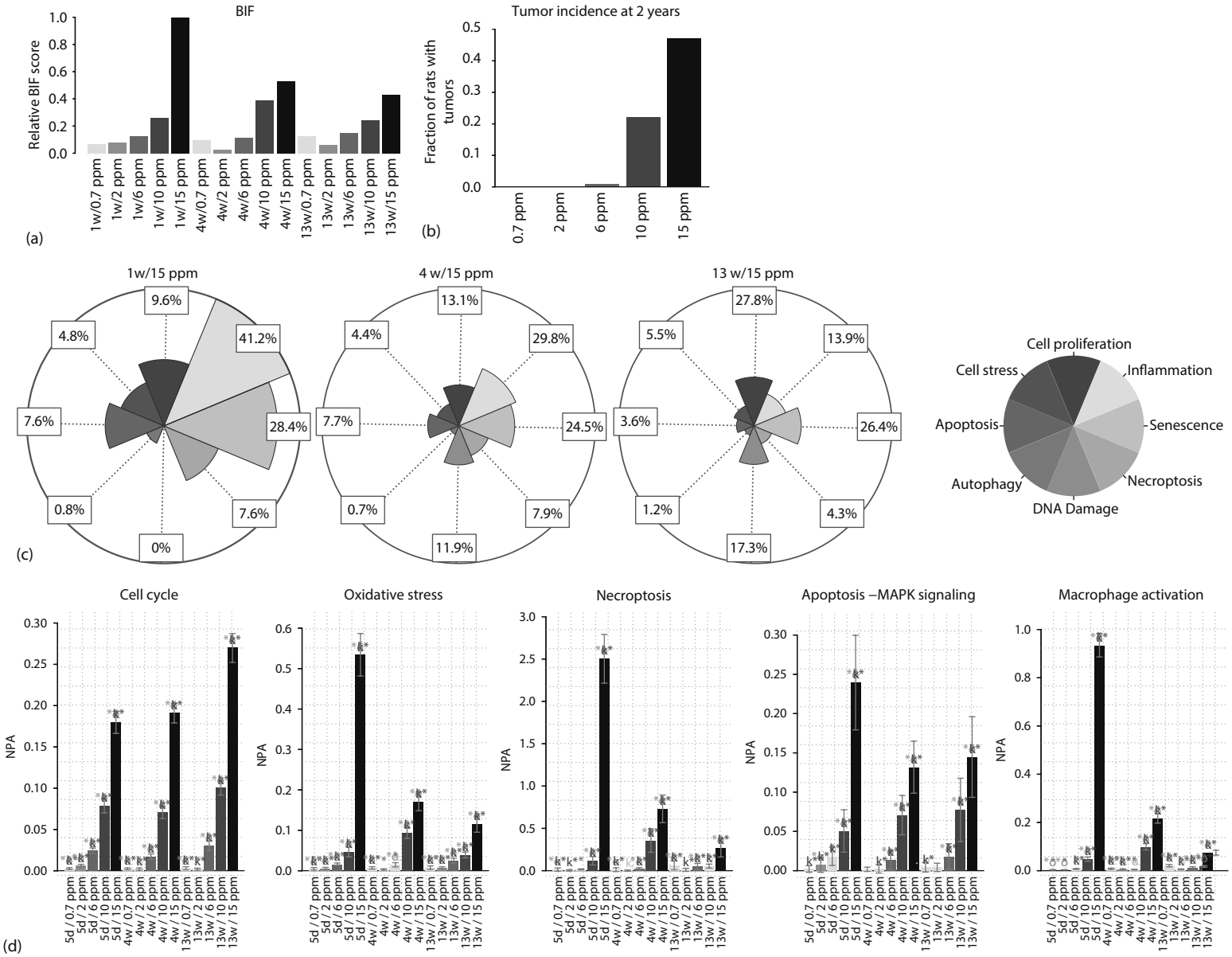

FIGURE 7.9 BIF for rat nasal epithelium exposed to FA. (a) Barplot showing the BIF values relative to the maximum response group (5 days/15 ppm). (b) The tumor incidence at 2 years is reported as the fraction of rats with squamous cell carcinoma in the nasal epithelium. These measurements were taken from a study with an identical dosing procedure as the one in A. (c) Starplots for the high-dose groups, showing the different network-level BIFs, weighted as a function of their contribution to the overall impact factor (shown in percentages). (d) Subnetwork NPA with companion statistics.

versus time response shapes are observed, for example, the perturbation amplitude on the *cell cycle* network is increased, whereas a decrease is seen on *oxidative stress* and *necroptosis* over time.

As shown by the earlier two use cases, our methodology not only identifies the biological processes that are perturbed following exposure to a stimulus, but it can also add a quantitative measure to these perturbations.

The most direct application of the BIF to toxicity testing is the explicit comparison between different stimuli. The BIF scores provide quantitative measures of the impact caused by each stimulus, and this allows the true ranking of the stimuli based on their capacity to perturb biological processes, that is, their toxicokinetic potency. This relative approach is also useful in situations where one of the stimuli is well characterized in terms of perturbed biological networks and long-term risk, while the others are less well studied. In this case, the BIF provides an explicit way to group the stimuli based on the pathways they perturb (and the magnitude of the perturbation) and consequently, to establish the MOA for the less well-characterized stimuli.

BIF can also be calibrated with a quantitative measure of health impact, when a disease-related phenotype is available alongside the measured SRPs as shown by the earlier rat FA exposure use case. If the calibration is done in a robust manner, it opens up broader perspectives in the context of personalized health and safety assessment. Even in the absence of an explicit disease phenotype, if the mechanistic characterization of early biological effect is strongly indicative of the long-term disease outcome, for example, increased cellular proliferation preceding the appearance of precancerous lesions, a BIF can be amenable to calibration and thus be used to encompass information relevant to disease risk. From this perspective, the perturbations of the biological networks are expected to collectively serve as prospective biomarkers for disease risk, similar to compound metabolites detected in body fluids.[195,196] In light of the recognized limited utility of epidemiologic studies to link short-term effects with long-term diseases, BIF can be used to calculate potential long-term risk by quantifying the short-term perturbation caused by stimuli, such as drugs, diets, or environmental conditions.

As emphasized throughout this chapter, since the BIF is supported by mechanistic information contained in all the underlying networks, it can be viewed as a *quantitative mechanistic metabiomarker* of the effects associated with exposure to test stimuli. The methodology enables the knowledge-driven paradigm for risk assessment—as set forward by the European Commission for toxicity testing.[2,4,197,198]

IMPROVER

With the unprecedented amounts of data accompanying various HT technologies, new computational approaches are being developed to facilitate robust analysis and interpretation of these large datasets. Questions arise as to how we can best manage the uncertainties inherent in the application of systems biology information to safety testing. Specifically, how can one ensure the validity of systems biology-based approaches and the resulting information?

With a goal to maintain scrutiny in data analysis and interpretation, we have recently proposed a systems biology verification process and a methodology for verifying the output of research processes in industry.[199,200] The industrial methodology for process verification in research (IMPROVER) decomposes a research workflow into building blocks that represent small pieces of a complex research program. The methodology further aims to identify the building blocks that are not accurate or robust (Figure 7.10). The core idea is that every building block can be verified by a challenge; some internally and some using the *wisdom of crowds*. An example of an internal challenge might be to ensure a sufficiently low noise level by comparing measurements with reference dataset of known quality or to verify that the datasets upon which the toxicity pathways are based on are carefully designed using appropriate dose/exposure levels and that comparisons within the experimental groups including controls are valid. In the external challenge, the question may be solved by external participants, who develop classification methods using public training data and then predict against an unpublished and hidden gold standard. The concurrent realization of the intellectual power that can be harnessed through community collaboration has led to the emergence of crowdsourcing models such as CASP,[201] BioCreative,[202,203] CAPRI,[204] and DREAM.[205–208] The approach has many obvious advantages over the self-assessment of methods. More importantly, it gathers and focuses the community around an important and relevant scientific problem and allows the comparison of the performance of different methods used to solve it. Additional value is added through the discovery of complementary methods to solve a problem, recognizing that the combination of solutions often outperforms the best performing single method.[209] At its best, crowdsourcing could allow the establishment of a state-of-the-art technology in the field and thus improve the quality of an industrial research program. The first IMPROVER challenge, the diagnostic signature challenge was launched and completed in 2012, and the challenge outcome has been shared at a dedicated conference as well as being published in proceedings.[210]

Similar challenges could be built around toxicity testing to find and verify stimulus-specific signatures and MOA for biologically active substances, which show a concordant temporal and dose–response relationship. Developing a community of researchers with similar interests in the twenty-first-century toxicology will foster faster advances to assess and verify the validity of the systems biology-based results (e.g., networks) than are possible with disjointed efforts. By using independent approaches and methods, it is possible to uncover the processes, where the output (biological

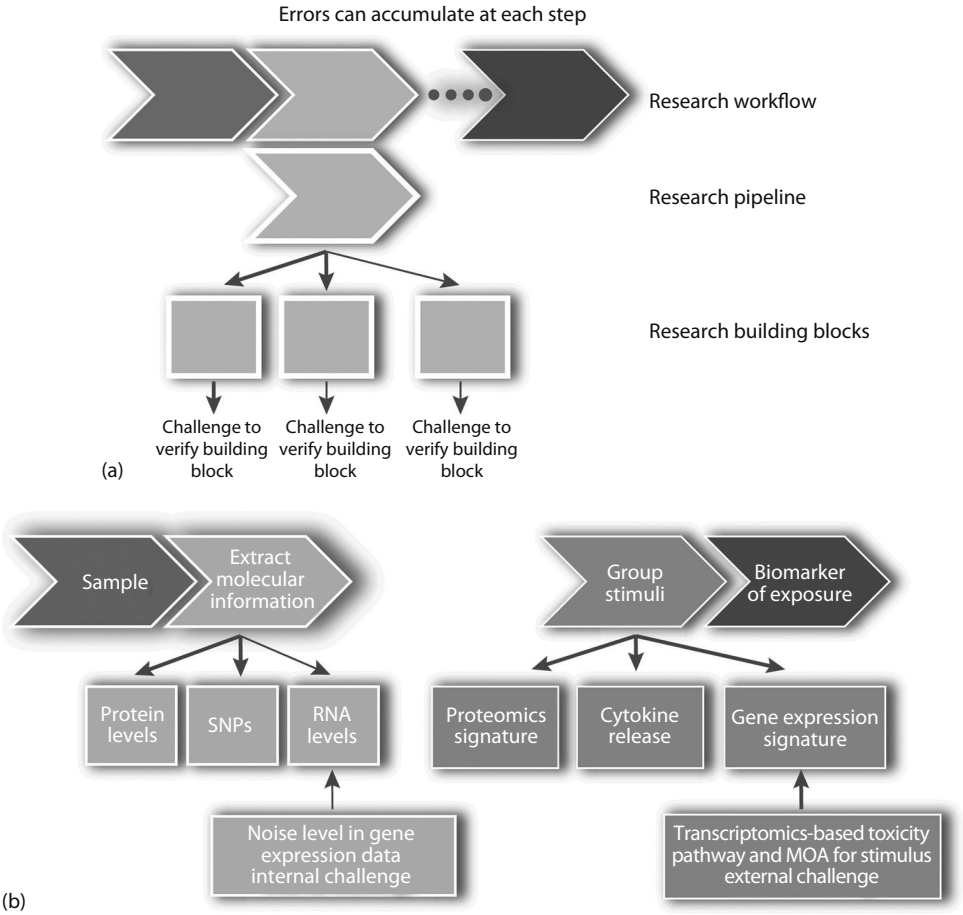

FIGURE 7.10 The basic principles of IMPROVER. (a) The methodology decomposes a research workflow into building blocks that can each be verified by a challenge. The challenge can be internal, within an organization, or external, where the larger scientific community is engaged to verify the robustness of a crucial step in a workflow. (b) A concrete example of the concept to verify the MOA and toxicity pathways for a given stimulus using transcriptomics data.

plausibility) points toward a coherent and complete set of relevant results. This set of results can be further used to assess the safety and biological impact of a substance or a treatment modality on a particular test system. Additionally, because the crowdsourcing approach directly addresses the issue of consistency of findings, which is important for the acceptance of omics approaches in toxicology, success stories will be an important measure of progress as well as critical to maintaining engagement and enthusiasm within the community.

CONCLUSION

Our five-step strategy described earlier tightly integrates experimental and computational approaches in a systems biology pipeline that allows the tandem analysis of biological network perturbations in the context of traditional toxicology data. Network- and mechanism-based impact assessment uses reverse-engineering principles to infer the activity states of signaling mechanisms from observed changes in gene expression. This allows for the determination of common mechanisms that are perturbed even if the underlying differentially regulated genes are not the same. This enables the causal analysis of the components and interactions that are important in a complex system, and thus increases our understanding of the essential features of a complex system. Moreover, the mechanism-based species translation aspects that the new approaches allow to be tackled are of immense potential value. Indeed, they have the potential to increase the predictive value of animal model systems and, more importantly, provide a solid foundation for the development of the 3Rs principle in toxicity assessment.[127]

Clearly, in addition to the biological network models and algorithms, an intelligent experimental design, state-of-the-art technology, and a well-defined workflow are necessary to accomplish the various aims of the five-step strategy. In combination, they build a robust methodology to identify toxicity pathways and MOA for stimuli that have the potential to induce adverse health effects in humans and provide a quantitative mechanistic framework for comparative product testing.

While the twenty-first-century toxicology paradigm shift is occurring, toxicologists and regulators need to closely follow the development of the approaches and the tools that will

be involved in the transformation toward the new era in safety assessment. However, care should be taken not only to evaluate the new methods against traditional toxicology; in addition the new methods should be used to assess how decisions may be supported by new versus traditional data. Evidence is steadily accumulating that the integration of systems biology data, along with conventional histopathology and clinical chemistry, can allow for better and more informed decision-making than traditional safety assessment alone. The focus should not be on measuring large numbers of endpoints, but rather on understanding these data. As such, systems biology could be positioned at the center of these efforts in the future.

To address these challenges, international participation and explicit quality assessments are important to establish common guidelines that can be enforced to reach the goals set by Tox-21c. It is our view that the robustness and reliability of new toxicity testing pipelines can be achieved with the help of systems biology verification methodologies, such as the IMPROVER.

The approaches and technologies outlined in this chapter may eventually also be applied in the field of ecotoxicology and ecological risk assessment where omics methodologies are starting to emerge to characterize molecular and cellular consequences of stressor exposure.[211,212]

Taking a *systems* approach to human biology that relies on mechanisms and pathways has inherent value, contributing both to improved toxicity testing and to the fundamental molecular elucidation of human disease. The *Holy Grail* of biologically based dose–response modeling has been sought for some time. It is believed that systems biology will underpin significant progress toward this lofty goal.

ATTRIBUTION STATEMENT

Selventa and Philip Morris International (PMI) authors performed the building of the six molecular networks together, and codeveloped the initial algorithm to develop the NPAs. The algorithm to score networks based on inferred values of backbone nodes computed using values of downstream gene expression data supported by O+K statistics was developed by Florian Martin (PMI).

PMI performed the 28-day OECD Plus cigarette smoke inhalation study[213] as well as the lung organotypic in vitro culture experiment (E-MTAB-1842 [ArrayExpress] and GSE50254 [GEO]).

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QUESTIONS

7.1 Which international initiatives work on validation of alternative methods to replace in vivo toxicology testing?
7.2 What are some of the questions that a well-designed exposure experiment is expected to answer?
7.3 What is the most important recent technological development for the genomics of common disease?
7.4 What is an ontology and what purpose does it serve?
7.5 What is a systems response profile?
7.6 How are NPAs computed?
7.7 How can the BIF be used to rank different stimuli?
7.8 How can crowdsourcing help to verify a research pipeline?

KEYWORDS

Systems toxicology, Biological impact assessment, OECD Plus studies, Toxicity pathways

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8 Toxicologic Assessment of Pharmaceutical and Biotechnology Products

Michael A. Dorato, Carl L. McMillian, and Tracy M. Williams

CONTENTS

GENERAL OVERVIEW OF DRUG DEVELOPMENT

The World Health Organization (WHO) Scientific Group has defined a drug as "any substance or product that is used or intended to be used to modify or explore physiological systems or pathological states for the benefit of the recipient" [160]. The drug discovery/development process covers a wide range of therapeutic areas and treatment regimens and is a risky, multifaceted, expensive undertaking. The goal is to develop a new product with therapeutic benefits (efficacy) and few side effects (toxicity) [4]. The drug discovery/development process for a new chemical entity (NCE) starts at the chemist's computer with in silico generation and testing of theoretical molecules, followed by synthesis of molecules of interest, and then testing through various in vitro and in vivo pharmacology and toxicology models, including pharmacologic profiling (the determination of pharmacologic effects other than the desired therapeutic effect,

i.e., off-target-of-interest effects), based on the proposed clinical plan for the first human dose (FHD).

The principal aim of nonclinical safety testing is to understand the safety/efficacy of the drug candidate well enough to make a judgment that the risk/benefit profile is adequate to progress a potential drug candidate toward clinical trials [116]. Provided the efficacy pharmacology and initial toxicology profiles are acceptable, clinical safety, pharmacokinetic, and pharmacodynamic studies (phase I studies) are initiated. As the human clinical trials progress through phase II (proof of concept and safety studies) and phase III (pivotal registration studies), the drug candidate moves through nonclinical subchronic studies, chronic and developmental toxicology studies, and oncogenic evaluations. Zbinden [250] has provided a summary of the biological parameters that should be evaluated for new drug candidates (Table 8.1).

Accelerating the development of safe and effective drugs is not a new topic to the pharmaceutical industry [46]. The technical risks in new drug development programs are enormous. Drug development is complicated by the requirement to simultaneously address complex issues related to potency, selectivity, reversibility, solubility, duration, metabolic stability, permeability, toxicity, physical stability, patentability, and manufacturability for each drug candidate. The risk of failure related to one or more of these aspects has been reviewed by Chien [43], where it was reported that <0.02% of NCEs result in marketed drug products and even fewer (i.e., 0.002%) return a profit to support continued drug research (Figure 8.1). Very little of what enters the drug development pipeline ever enters the marketplace [74]. It has been estimated through many sources that the cost of developing an NCE could exceed $2–$3 billion [73–75,176].

By its nature, a drug must modify a biological process (i.e., alter or adjust a physiologic system in some way) [52]. Toxicology is a critical part of drug discovery/development, early- and late-phase drug development, and the role of toxicology in those processes has been extensively reviewed [72,77,97,106,156]. The purpose of toxicology testing

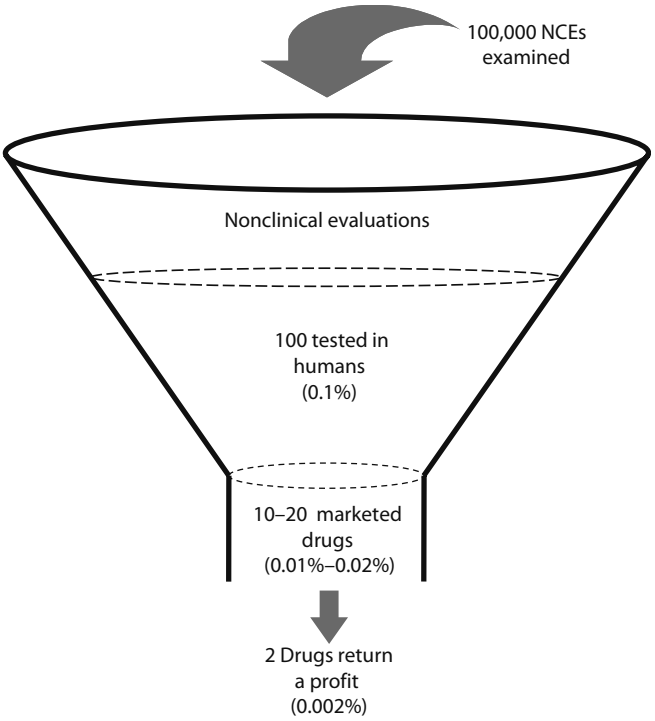

FIGURE 8.1 Attrition rate of new drug candidates. (Data from Chien, R.E., ed., *Issues in Pharmaceutical Economics, Lexington Books*, Lanham, MD, 1979; DiMasi, J.A. et al., *J. Health Econ*., 10, 107, 1991; DiMasi, J.A. et al., *J. Health Econ*., 22, 151, 2003.)

programs is to characterize the toxicity of the drug candidate under various testing conditions (e.g., dose, treatment duration, route) in order to understand potentially harmful effects [53]. A general approach to developing a toxicity profile for a pharmaceutical agent is given in Figure 8.2 and will be discussed more extensively in the following.

During early drug discovery, the input of the toxicologist is often solicited in order to understand the inherent risk associated with a particular pharmacologic target. Understanding the likely on-target (associated with the intended pharmacology) risks through literature reviews, target distribution, and manipulation evaluation (e.g., knockout mouse models) enables a tailored safety assessment early in the process to define an appropriate margin of safety (MOS), which is typically viewed as the ratio of the highest dose associated with no toxicity or manageable toxicity to the efficacious dose in the appropriate test system. In this construct, the highest dose associated with no toxicity is the no-observed-effect level (NOEL); the highest dose associated with manageable toxicity is the no-observed-adverse-effect level (NOAEL). Off-target toxicity, which is associated with responses other than the intended pharmacologic target and inherently driven by the chemical structures being evaluated, is often the focus of applied predictive toxicology tools early in order to influence the selection of a chemical series, with understood or manageable risk, to move into lead optimization.

During the early discovery process of identification of a lead chemical series, toxicologists employ rapid, quantitative in silico and in vitro screening methods, to help identify,

TABLE 8.1
Biological Properties Considered in the Development of Drug Candidates

Acute toxicity
Cumulative toxicity
Absorption from various routes
Elimination $t1/2$ and accumulation in deep compartments
Penetration of barriers
Milk excretion
Teratogenicity
Mutagenicity
Carcinogenicity
Sensitization
Local irritation
Reversibility
Abuse liability

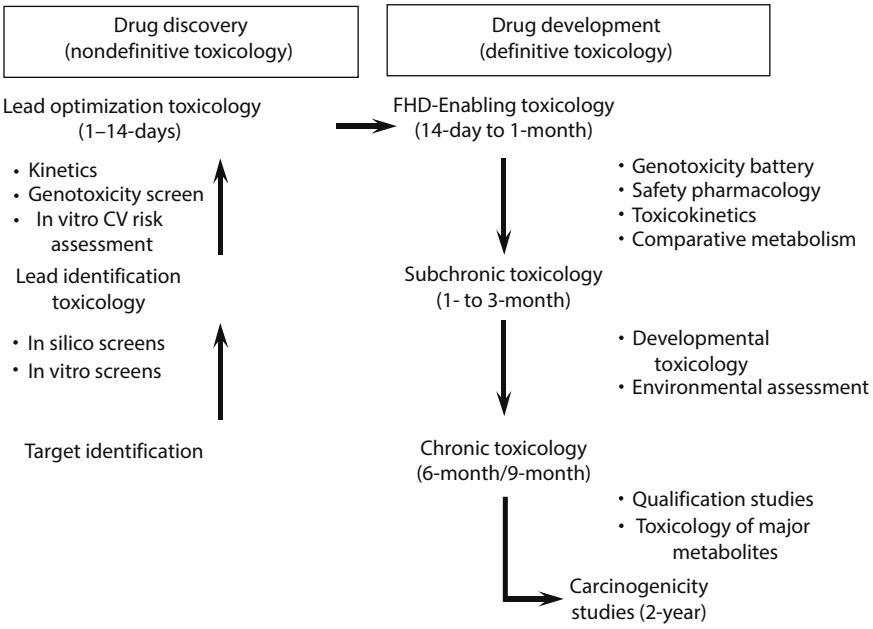

FIGURE 8.2 General approach to developing a toxicity profile for pharmaceutical agents.

out of a myriad of molecules, potential chemical scaffolds worthy of exploring in the hopes of selecting a drug candidate with the best safety profile (Figure 8.3). Computational toxicology tools, even though they have limitations, are advantageous in that quantitative structure–activity relationships can be assessed without the investment of chemical synthesis. Many of these tools are currently commercially available to predict toxicologic responses (e.g., cytotoxicity, cardiovascular, and genotoxicity risk), and this continues to be an area of rapid growth [151,226]. In vitro screening methods, often using high-throughput, medium-throughput, or high-content approaches, allow a great number of compounds to be evaluated, with minimal amounts of compound, in order to rank order compounds for further efficacy and safety investigation [213]. Numerous cell-based models are used to evaluate common toxicities encountered in vivo (e.g., cytotoxicity, phospholipidosis, cardiovascular risk via hERG blockade, and mutagenicity). Predictive toxicology tools continue to be investigated in an attempt to maximize success in lead optimization. The primary goals in the lead optimization toxicology phase of drug discovery/development are to perform in vivo studies to assess target organs, determine the dose-limiting toxicity, compare the adverse events with those measures of efficacy seen in the early pharmacology evaluations (i.e., in vivo pharmacology), and generate a drug candidate with an appropriate MOS for the intended therapeutic indication in humans. The strategies employed across the pharmaceutical industry in maximizing lead optimization [17] and early-stage development [18] have been extensively reviewed. The studies are often

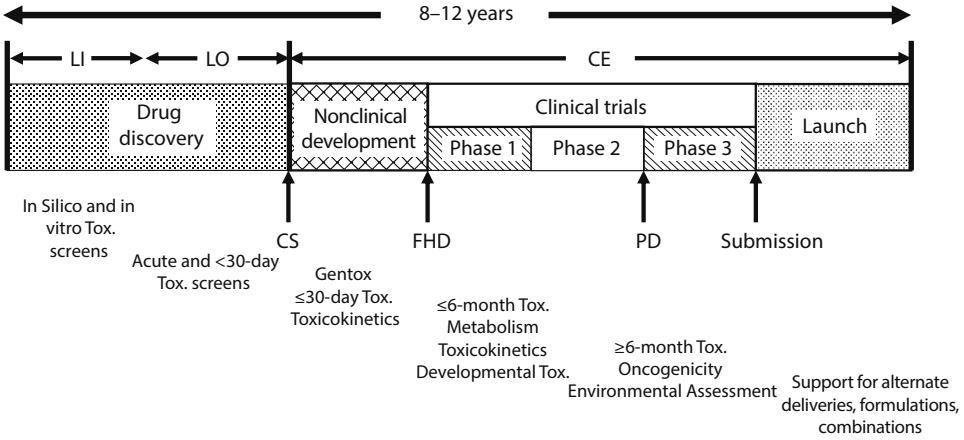

FIGURE 8.3 Duration of toxicology involvement in drug discovery and development showing the major milestones and study types by phase. The goal is to reduce development time while maintaining a focus on nonclinical and clinical safety assessment. CE, Candidate evaluation; CS, Candidate selection; FHD, First human dose; LI, Lead identification; LO, Lead optimization; PD, Product decision.

single- or repeated-dose studies, which are short in duration (<2 weeks), use minimal number of animals, and have abbreviated pathology and toxicokinetic end points. The integration of pharmacology end points improves the evaluation in the lead optimization phase. The lead optimization phase is iterative in that multiple compounds are evaluated in both toxicology and pharmacology studies for safety, efficacy, and pharmacokinetics, in order to declare a drug candidate worthy of advancement to definitive toxicology studies (i.e., those toxicology studies occurring after candidate selection, in the regulatory phases, and used to support first human dosing; nondefinitive toxicology is a general term used to describe those investigative studies conducted prior to the regulatory phase). Sasseville et al. wrote an excellent review on the application of predictive tools and testing paradigms for drug discovery [197].

These early screening procedures used in discovery toxicology, however, are only a prelude to the required comprehensive safety assessments expected from the toxicologist. Regulatory requirements, including guidance documents and good laboratory practices (GLPs), dictate many aspects of the toxicology study protocol and must be followed closely for all definitive (those that support human studies) toxicology studies [84]. The early toxicology studies, conducted in the discovery phase, do not require GLP compliance; however, a process of good research practices (GRPs), ensuring quality, validation, documentation, reproducibility, etc., should be applied.

Prior to initiating clinical trials, physicians need to understand the toxicologic profile produced by the drug candidate in relevant animal models. Prior to the FHD, determination of the relevance of animal models (e.g., metabolism relative to humans) is limited to in vitro evaluations using human and animal tissue preparations. On the basis of the clinicians' needs, the toxicology profile of an NCE is characterized by a number of questions [161]:

- What dose/exposure produces toxic effects in animals?
- What dose/exposure does not produce toxic effects in animals?
- Were the animals relevant models for predicting human toxicity?
- What were the signs and duration of toxic responses?
- Did effects differ following single or multiple dosing?
- Were the toxic responses reversible?
- What were the target organs or systems?
- Was the toxicity expected for this chemical class?
- Are toxic metabolites or reactive intermediates produced?
- Was accommodation to the toxic effects observed?

The answers to these questions form the basis of the toxicology profile supporting initial and continued clinical trials.

The major objectives of toxicologic evaluation change according to the stages of the discovery/development process [220]. The relationships of the studies used to develop a toxicology profile (Figure 8.2) to clinical trial phases are shown in Figure 8.3. The early stages of discovery focus on toxicologic screening (Table 8.2). A sample flow scheme through the early discovery toxicology phase (Figure 8.4) shows the stages of investigation and the integration of in vitro, ex vivo, and in vivo *safety* and efficacy procedures. Definitive toxicology studies are very time consuming and costly; thus, relatively inexpensive, short-term screening procedures are used to eliminate the most toxic compounds [249].

TABLE 8.2
Purpose of Toxicology Evaluations of New Drugs

Phase	Principal Activity	Purpose
Early discovery	Toxicologic screening	Guide chemistry
Lead optimization	Identification of principal target organs	Identification of drug candidates
Before FHD	Characterize safety profile	Regulatory prerequisites for human administration and establish safe clinical starting dose
During clinical trial	Assess toxicologic profile with longer administration	Cumulative effects and mechanisms
Premarketing	Complete routine test program	Regulatory requirements
Postmarketing	Identify special risks due to population or use circumstances	Improve utility and safety

Inherent to these initial approaches to evaluate potential drug toxicities are a number of imperfections: the target systems may not be routinely evaluated; the assay procedures may be inadequate or improperly timed relative to the onset of the response of interest; target-organ exposure may not be evaluated; identification and quantification of adverse events may be inadequate; translation of effects between species may be inadequate; and the test model may not be an appropriate surrogate for human response [253].

There is no simple answer to the often-asked question: "What toxicity profile would cause a company to stop development of a new drug candidate?" [130]. However, the demonstrated toxicity of other compounds in the class, if available, and the gravity of the disease state under study often provide guidance as to what might be an acceptable safety profile for an NCE. An overview of the international pharmaceutical industry's nonclinical testing strategies, in relation to clinical trial phase, is provided in the *Pharmaceutical R&D Compendium* [99].

In addition to the drug substance, the delivery system may also require nonclinical evaluation because it may alter pharmacodynamic (action of the drug on the body) and pharmacokinetic (action of the body on the drug) relationships. The regulatory requirements for the safety assessment of known and novel drug delivery systems have been reviewed by Weissinger [232,233].

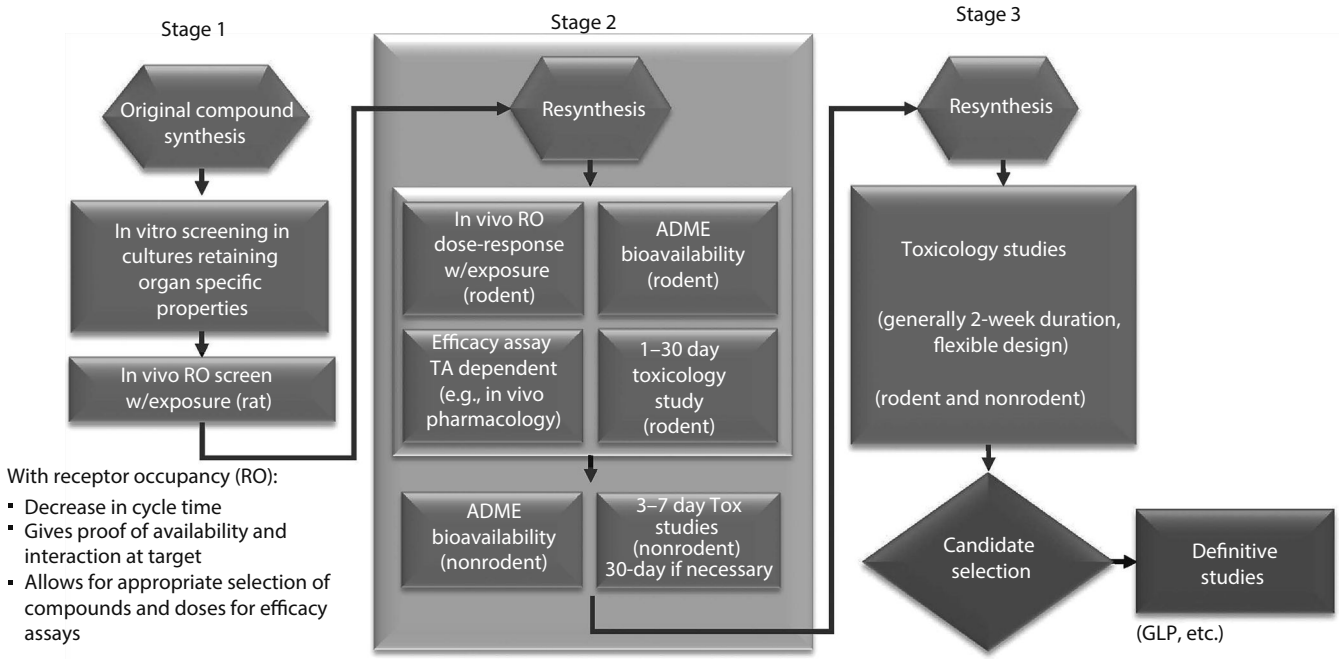

FIGURE 8.4 Drug discovery safety/efficacy flow scheme. ADME is absorption, distribution, metabolism and excretion.

Commercially advantageous forms of genetic manipulation date back to antiquity (e.g., inbreeding, cross-fertilization). The introduction of modern recombinant DNA (rDNA) technology has had a major impact on life science research and has allowed for the large-scale production of protein pharmacologic agents that would have been very difficult to produce by normal chemical synthetic means.

A new biological entity (NBE) is defined as a complex, high molecular weight material that cannot be fully characterized by standard chemical analysis and which may require immunologic, biochemical, or bioassay techniques to measure the quantity present and to assess activity [54]. The development, utility, and relative safety of human insulin [110], human growth hormone [172], and interferon (IFN) [178] have fueled the current interest in the production of biologically active peptides. As the interests of pharmacologists in biotechnology research expand, the difficulties of producing a comprehensive set of safety guidelines increase. Legal definitions of biotechnology products and regulatory guidance for nonclinical assessments have been reviewed by Tsang and Beers [217].

When NBEs were introduced, beginning with insulin, regulatory concepts were not in place to address the problems forthcoming from this new technology [40,114]. The regulatory issues relating to recombinant products are formidable, and the possibility that each biotechnology product might require customized safety testing has been given serious consideration [59,205,214]. Clearly, the immunologic response to foreign proteins may compromise the utility of using traditional animal models in the safety assessment of these agents. Other major regulatory issues include the assurance that recombinant production methods do not result in addition of contaminants and the demonstration to regulators that biosynthetic products are identical to natural substances [108]. In the biosynthetic human insulin (BHI) approval process, meetings between regulatory agency and industry scientists to review the manufacturing process, molecular biology, and purification of the hormone, as well as clinical trial programs, were critical in facilitating eventual approvals. Industry and regulatory agency representatives agreed that the chemistry of an NBE should prove its identity [52]. The identity and purity of rDNA insulin, therefore, received much attention [40]. Anticipation of problems and the communication of concerns were key to the rapid New Drug Application (NDA) approval for BHI (5.5 months). The U.S. Food and Drug Administration (FDA) has strongly recommended that it be involved early in the nonclinical and clinical development plan to facilitate the approval process for both NCEs and NBEs [114].

The U.S. biotechnology policy stated that "the same physical and biological laws govern the response of organisms modified by modern molecular and cellular methods and those produced by classical methods … no conceptual distinction exists between genetic modification of plants and microorganisms by classical methods or by molecular techniques that modify DNA and transfer genes" [6]. Thus, it would not be expected that NBEs per se pose an unusual risk to human health and the environment [6]. The toxicologist should be aware, however, that compounds made via rDNA techniques are not necessarily identical to the natural material, as might be assumed [239]. Dayan [63] suggested that the toxicology profile for an NBE should be defined in terms of chemical identity of the material, extent of prior knowledge, and intended use. The Pharmaceutical Research and

Manufacturers Association (PhRMA) has recommended that nonclinical toxicologic evaluations of NBEs should be decided on a case-by-case basis [215], and regulatory and industry representatives attending the first International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) have also supported this position [147]. The established toxicology information will guide the clinical trial and address possible hazards in the workplace, where humans are exposed to the compound and its precursors and contaminants that are also contained in the bulk material to be tested during the chemical or biological synthetic process.

The role of the toxicologist is usually less routine and requires more innovation in study design when dealing with NBEs than when dealing with NCEs [64]. As is the case with NCEs, however, the principal goals of the toxicologic evaluation of recombinant products are to detect major toxicity, to identify lesser toxicity, to determine the dose relationship of toxic effects and their duration to guide the clinical dose schedule, and to investigate the mechanisms of action related to the toxic response. Cavagnaro [37] has provided a comprehensive overview of the challenges and approaches to nonclinical safety evaluations of NBEs.

The three main areas of concern relative to the toxicity of NBEs are toxicity per se, exaggerated pharmacodynamic effects (anticipated toxicity based on the pharmacologic mechanism of action), and allergic reactions (Figure 8.5) [250,251]. *Intrinsic toxicity* has been defined as undesirable effects having no obvious relationship to the molecule's pharmacodynamic properties. Pharmacodynamic toxicity is defined as an exaggerated pharmacologic response (i.e., hypoglycemic shock from insulin). Immunotoxicity has been related to hypersensitivity, cell transformation, and the production of neutralizing antibodies. The loss of the biological activity of a recombinant therapeutic agent through production of neutralizing antibodies and the development of immune-complex disease in experimental animals are factors that must be given individual attention [215]. It has been suggested that animal models of immunotoxicity are

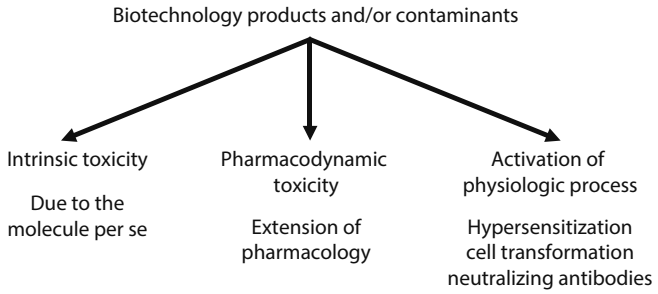

FIGURE 8.5 Main areas of concern with response to biotechnology products. (From Zbinden, G., Biotechnology products intended for human use, toxicological targets and research strategies, in Graham, C.E. and Liss, A.R., eds., *Preclinical Safety of Biotechnology Products Intended for Human Use*, Alan R. Liss, New York, pp. 143–159, 1987; Zbinden, G., *J. Toxicol. Sci*., 14(Suppl. 3), 3, 1989.)

of limited usefulness in that no animal model may be fully suitable for predicting the toxicity of highly species-specific proteins. Friedmann [104] has indicated, however, that the lack of hypersensitivity reactions in response to small peptides in animal experiments may be viewed as an indication of their acceptability in humans. Graham [115] emphasized the use of a case-by-case approach to toxicologic evaluations of NBEs based on their similarity to natural human proteins, immune response in animal models, and production of neutralizing antibodies in nonclinical and clinical studies.

The unique regulatory approval of recombinant insulin most likely resulted in unrealistic expectations in the biotechnology industry regarding the rapidity of review of NBE applications [159]. Two factors will facilitate the regulatory approval of NBEs. As is true in the development requirements for all NCEs, the first factor is therapeutic importance, and the FDA has established a *fast-track* rapid-approval procedure for NBEs that target unsatisfied indications. The second factor is the relationship of the NBE to an established drug. It appears likely that new therapeutic agents derived from biotechnology will have to satisfy all the traditional demands of regulatory agencies [159]. The possibility that subtle changes in chemical structure may exist and may thus influence pharmacokinetics, pharmacodynamics, or immunogenicity is used to support this regulatory position [107].

Questions of safety are not only properly asked about the NBE per se but also about contaminants or residues resulting from the manufacturing or purification processes, antigenic variation, or reversion to the wild type of a living organism [54]. Worker exposure in the production process may be of concern due to relatively high-level, long-term exposure to various end products of the biotechnology process (i.e., live and dead microorganisms and mammalian cells and their derivatives) [240]. This leads to the area where traditional scientific approaches and techniques do not provide a satisfactory toxicologic profile (e.g., transfer of an immortalization factor from a mammalian cell, allergic reactions) [103].

As we have learned with new technologies, every new technology seems *better* the less we know about it. As we develop an understanding of the true potential contributions of new technology over the long term, we realize that the promises of generating meaningful data in the short term were generally overstated. The prime example of this is the expectation that the Ames in vitro genotoxicity assay would replace the 2-year carcinogenicity assays. New focus also takes us in interesting directions; for example, the use of biomarkers (essentially a measured observation or outcome of any biological evaluation) is an old concept that is receiving renewed interest. The development of new procedures to track changes, longitudinally, is of increasing importance in drug discovery/development. If we think broadly enough, the common quote, "The dose alone makes a thing *not* a poison" [218], would have no meaning if Paracelsus (1493–1541) were not thinking about biomarkers. Toxicology studies have always depended on the evaluation of biomarkers, which have traditionally been used in toxicology studies to confirm exposure, to monitor susceptibility to a toxic agent, and to

assess adverse effects [3]. New biomarkers continue to be evaluated and validated for their use in both drug discovery and development as premonitory tools of toxicity as well as for possible translation to clinical trials [213,226,236].

RELEVANCE OF ANIMAL MODELS IN TOXICOLOGIC ASSESSMENT

The suitability of experimental animal data for assessing risk to humans as well as animal welfare concerns [192] are important contemporary issues in toxicology. Animals and humans have much in common anatomically, physiologically, and biochemically [244]. The two main guiding principles of experimental toxicology are that effects produced in animals, when properly qualified, are applicable to humans and that exposure of experimental animals to high doses of a test compound is necessary and valid in determining human risk [153]. Although it is generally agreed that animal assays are not 100% predictive of human effects, they are more predictive than generally thought [120,199]. It has been reported that animal assays are predictive of human toxicity in all but 10% of comparisons [167]. It must be recognized, however, that major differences in response to chemical agents can exist both within and between species [133]. The most serious differences between laboratory animal studies and human clinical trials are related to biochemical and physiological species differences, such as metabolism and genetics (hypersensitivity responses), as well as differences in experimental design, including quantity, route, and duration of drug administration [160]. Humans can be as much as 50 times more sensitive on a milligram-per-kilogram basis than experimental animals [160].

Regulatory agencies and research-based pharmaceutical companies consider laboratory animal toxicology studies as a critical part of the assessment of new drug candidates [102,165]. Confidence in the validity of experimental toxicology is based on the large inventory of chemically induced lesions that occur both in animals and humans. It may be incorrect to assume that what is demonstrated in animal toxicology studies will occur in human clinical trials, but until it is shown that the toxicity expressed is not relevant to humans, that assumption must be made [14]. Also, until our knowledge base expands, animal data must be extrapolated to the human situation using a conservative approach (e.g., use of relatively high doses, assuming that humans are more sensitive than the most sensitive species) [22].

The ultimate goals of the toxicology assessments are to characterize toxicity in animal models to identify potential problems in short- and long-term clinical studies, identify the circumstances under which toxicity occurs, evaluate the extent to which the data warrant extrapolation to humans, recommend safe levels of exposure, and contribute to the decision to test the new drug candidate in humans [12,62,166,185,193,222]. It has been recognized that qualitative extrapolation of drug toxicity from animals to humans is more reliable than estimation of the magnitude of dose producing a similar effect in animals and humans; that is, the pharmacodynamics of an agent are more predictable than its pharmacokinetics [186]. Complicating the ability to extrapolate data from animals to humans are the excessive doses sometimes used, and often required, in animal studies. As a result, adverse effects are described that may be the result of frank intoxication of the animal and are irrelevant in humans. Zbinden [245] has proposed that the ability of animal toxicity studies to predict potential human toxicity is related to the mechanism of drug action. Within limitations, animals and humans respond in ways similar enough, from a pharmacodynamic perspective, for animal toxicity evaluations to serve as useful predictors of human toxicity [60,124,163,166,180]. However, toxicologic evaluations in animals can predict toxic responses in humans only if the response is not unique to humans [102]. Those compounds that are toxic to humans but *relatively* nontoxic to animals (i.e., thalidomide) are of greatest concern. The extrapolation of animal data to humans is likely to become even more complicated as molecular biology techniques continue to allow the more sophisticated characterization of specific human therapeutic targets (human enzymes, receptors) and the ultimate development of drugs specific for these targets.

Table 8.3 lists common undesirable drug effects seen in human studies; 76% of the findings are predictable from animal studies. Predictability is enhanced for those adverse effects that can be directly related to the pharmacologic mechanism of action of the compound. Adverse responses commonly referred to as dose and time related are relatively well predicted from animal studies. It is more difficult to extrapolate effects that are not dose or time related [248].

There is a small element of toxicity that cannot be predicted until large-scale clinical studies are conducted [124]. This may be the result of a very low incidence of occurrence or idiosyncratic responses in a small subset of the patient population; however, considering the increased use of pharmaceutical agents and the relative infrequency of major incidence of human toxicity, the initial laboratory studies are clearly serving a valuable function [12]. A large majority of human drug exposures are relatively free of toxicity and in good accordance with the results of animal toxicity studies [249]. The use of adequate test systems is critical to the predictive (translational) ability of animal toxicity evaluations. Cahn [27] reported that the cardiac effects of calcium antagonists (i.e., ectopic beats, ventricular tachycardia, and ventricular fibrillation) were seen in humans but were not described in long-term animal studies. These effects, however, were demonstrable in animals using appropriate functional evaluations not always included in routine toxicologic testing, and it is these kinds of clinical findings that contribute to the evolution of the science of regulatory toxicology. Oftentimes, toxicologically important end points, such as cardiac, pulmonary, or renal function, are not taken into consideration in the design of *routine* toxicology studies, which are frequently focused on changes in clinical signs and pathology. The toxicologist is challenged to consider potential adverse effects related to the pharmacodynamics of the test compound in the design of appropriate safety studies [180]. Furthermore,

TABLE 8.3
Common Untoward Reactions to Drugs

Clinical Side Effect	Predictable from Animal Studies? (Y/N)	Clinical Side Effect	Predictable from Animal Studies? (Y/N)	Clinical Side Effect	Predictable from Animal Studies? (Y/N)
Drowsiness	Y	Hypertension	Y	Anorexia	Y
Nausea	N	Insomnia	Y	Depression	Y
Dizziness	N	Fatigue	N	Increased appetite	Y
Sedation	Y	Constipation	Y	Tremor	Y
Dry mouth	Y	Tinnitus	N	Perspiration	Y
Nervousness	Y	Weight gain	Y	Dermatitis	Y
Epigastric distress	N	Hypotension	Y	Increased energy	Y
Headache	N	Dryness of nasopharynx	Y	Vertigo	N
Vomiting	Y	Heartburn	N	Palpitation	Y
Weakness	Y	Diarrhea	Y	Blurred vision	Y
Nasal stuffiness	Y	Skin rash	Y	Lethargy	Y

Source: Data from Ronneberger, H. and Hilfenhaus, J., *Arch. Toxicol. Suppl*., 6, 391, 1983; Zbinden, G., *Eur. J. Clin. Pharmacol*., 9, 333, 1976; Zbinden, G., *Pharmacol. Rev*., 30(4), 605, 1978.

many new drug candidates are being targeted toward the aging population, where compromised hepatic or renal function may significantly modify the pharmacokinetics and thus toxicity of the compound. These examples emphasize the importance of using an adequate and appropriate test system to evaluate the toxicity of new drug candidates.

As with standard pharmaceuticals, no animal model is fully appropriate to evaluate the toxicity of highly specific human proteins [115]. Animal testing for biotechnology products is limited to the species showing the same pharmacologic response as humans, without showing signs of immunity [50,107]. This is only feasible when proteins are highly conserved across species. The production of neutralizing antibodies will limit the study duration and thus support for clinical trials. Administration of a highly specific human protein to laboratory animals for a sufficient duration to produce immune-complex disease will do nothing to reveal effects anticipated in clinical trials. Antigenicity of the test material can be a major complicating factor, in that the potential allergic etiology of all lesions developing in animals treated with human proteins must be considered [254]. Alternatively, nonclinical toxicology studies of biotechnology products may be less predictive of allergic responses that may occur in humans following chronic therapy [240]. The appropriate laboratory species for biotechnology product testing should demonstrate similar pharmacodynamics and adverse responses relative to humans. If an animal model demonstrating similar pharmacologic response to humans cannot be selected, species selection based on toxicity likely to be representative of that expected in humans may be acceptable [233]. The FDA does not currently require the study of recombinant proteins exclusively in primate models, but a study in a relevant species is expected. Nonhuman primate models demonstrate many similarities to humans at the molecular level and often turn out to be the most appropriate species for toxicity testing of NBEs [115].

The predictability of animal models debate affects decisions on moving potential drug candidates through the clinical trial phases. Animal models provide predictability, but that statement has many caveats. To keep the matter in perspective, while animal models are not a perfect predictor of either efficacy or safety in the clinical trial phase, the clinical trial phase is not a perfect predictor of efficacy or safety in the marketing phase. A clinical trial study of tens of thousands of patients may not be sufficiently large to show a subtle effect in the marketing of an approved medicine to a population of tens of millions. The increased application of genomics may pave the way for an improved safety risk–benefit assessment, that is, the right drug to the right patient.

An extensive review of in silico, in vitro, and in vivo models used to accelerate drug discovery and development has been published by Atkinson [10].

TOXICOKINETICS

Toxicokinetic analysis has become a standard component of the design and a valuable tool in the interpretation of the nonclinical safety profile of NCEs [61,76]. Various guidances that have been issued in recent years describe objectives and specific recommendations for toxicokinetic evaluation [134,135]. Well-designed toxicokinetic studies provide insight on exposure (typically expressed as drug concentration per unit time or area under the plasma concentration/time curve [AUC]) as well as other pharmacokinetic parameters such as clearance (Cl_T), volume of distribution (Vd), and half-life ($T_{1/2}$). These pharmacokinetic parameters are often further refined in specific studies to assess absorption, distribution, metabolism, and elimination (ADME).

Toxicokinetic parameters guide the assessment of dose proportionality, accumulation potential upon multiple doses, sex differences, and species differences. Figure 8.6 illustrates the relationship of dose vs. exposure after single and multiple doses.

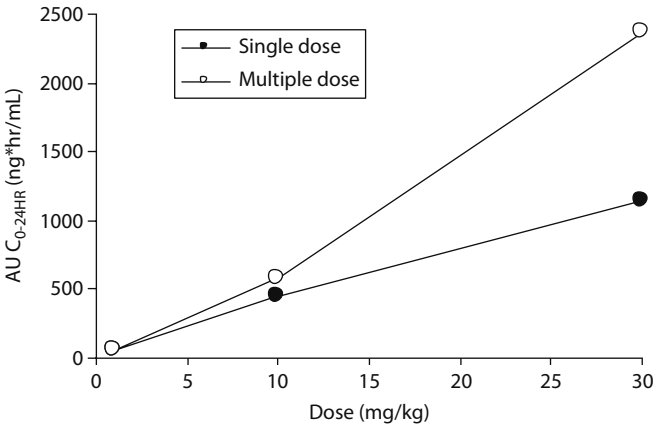

FIGURE 8.6 Relationship of dose vs. exposure after single and multiple doses.

This example shows a linear, predictable increase in exposure across the range of doses examined. It is also noteworthy to point out that the saturation of systemic exposure may occur after oral dosing as a result of absorption-related processes. When saturation is reached, increasing the dose does not result in increases of exposure. Toxicokinetic analysis is useful in determining the dose at which saturation occurs. This may then be used to justify selection of a high dose in a toxicology study.

The majority of toxicokinetic assessments are designed to evaluate parent drug or metabolite concentrations in plasma [16]. These studies are usually an integrated part of the design of various toxicology studies, including acute, subchronic, chronic, reproductive/developmental, and carcinogenicity studies, regardless of route of administration or test species. An understanding of the internal exposure associated with dose-limiting toxicities or premonitory signs and symptoms that precede dose-limiting toxicities or adverse events is fundamental to any toxicokinetic assessment. Such assessments provide the foundation for the establishment of a toxicokinetic/toxicodynamic (TK/TD) relationship of dose, exposure, and adverse events [181,255]. The role of clearance, absorption, or distribution in any observed changes in the relationship aids in the prediction of accumulation, saturation, or decreases in exposure observed after multiple doses.

Similarities in the qualitative profiles of metabolism of xenobiotics across rat, mouse, dog, and nonhuman primates have been reported for decades [34,35]. Quantitative profiles, however, are often quite dissimilar and have been studied extensively; consequently, recent guidance [16,206] has been published to offer additional insight into the appropriate instances in which to quantify metabolites. In addition to the guidance offered, multiple reports have been published, reviewed, and/or debated [109,241]. These offer greater insight into various approaches to investigate and analyze the absolute and relative exposure(s) of metabolites to parent or total drug–related material. Combined, these qualitative and quantitative assessments of an NCE early in the development cycle greatly aid in the selection of the appropriate preclinical species for safety assessment. This early assessment is often coupled with the previously mentioned ADME studies. Increasingly, various in vitro test systems are utilized for the initial assessment of some ADME properties. Specifically, the utilization of cellular and subcellular fractions and tissue slices or segments to assess absorption and metabolism is the most widely employed test system. For example, permeability across Caco-2 cell monolayers has been well established as a model for gut absorption [7].

Human and animal liver samples are utilized to create various in vitro test systems for metabolism. These systems offer the capability to study the rate, extent, and profile of metabolism in a comparative fashion across multiple species in a relatively short time frame. Subcellular fractions of hepatocytes are most commonly used and offer the advantages of speed, robustness, and capacity. In addition to containing the major drug-metabolizing enzymes (CYP450s and flavin-containing monooxygenases) and phase I (oxidative) capability present in subcellular fractions, liver slices and hepatocytes also possess greater capability to complete phase II (conjugative) metabolic transformations [212,214]. The intact cells are also thought to more closely mimic the intact functioning organ.

Both the activities and the abundance of individual isoforms of cytochrome P450s (CYP450s, the primary drug-metabolizing enzymes) have been extensively characterized in experimental animal species and humans. This level of characterization enables these systems to offer the potential for improvement of the selection of preclinical test species as well as decreased utilization of in vivo experiments. Predictions can also be made regarding the correlation of in vitro and in vivo estimates and the likelihood of clinical drug–drug interactions [33,148,171,180,190].

Advances in technology, most notably liquid chromatography–tandem mass spectrometry (LC–MS/MS), have greatly enhanced the ability to quantify both parent drug and metabolite concentrations routinely in the submicrogram-per-milliliter range [1]. This advance is often challenged by the trend to develop NCEs with increasingly higher potencies. The intended goal of toxicology studies is to dose animals in a fashion to obtain higher exposures than those predicted or observed in humans. Plasma concentration exposure assessments are now more widely used and accepted as a better estimate of safety multiples than comparisons of administered dose (e.g., mg/kg or mg/m^2). The assessment of systemic exposure in toxicity studies has been addressed by the ICH, and the objectives of toxicokinetic evaluation and specific recommendations are contained in ICH Topic S3A [134]. ICH M3 offers additional guidance on the assessment of metabolites as an integral part of the overall toxicokinetic assessment of NCEs [145].

TOXICOLOGY GUIDELINES

Drug Development Timelines

Development time (e.g., cycle time) has become an important focus for the pharmaceutical industry. The available data indicate a fourfold increase in drug development time between

the 1960s and the 1980s. From the early 1980s to 1996, however, mean drug development times have been relatively constant at about 10–12 years, with very wide variability [221]. Cycle time, in general, has not changed significantly from 1996 to 2012. The pharmaceutical industry has committed to increasing drug discovery/development efficiency, leading to a decrease in the mean drug development time to deliver innovative pharmaceuticals to patients more quickly and realize increased profitability to maintain aggressive research and development efforts. The FDA has also committed to increasing the development speed of safe and effective drugs through a program called the Critical Path Initiative, described in *Innovation or Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products* [92]. The broad impact of these efforts has yet to be evaluated.

NBEs generally have had a shorter development time (approximately 6–9 years) than NCEs. The NBEs registered to date have generally been well-characterized natural molecules, and the shorter development time is probably related to a better understanding of their actions in humans. The introduction of analogs of natural proteins, some designed to be used at supraphysiologic levels, has led to an increase in development time for NBEs. From 1985 to 2004, the development times for NCEs have reportedly decreased approximately 22%, while the development time for NBEs has reportedly increased approximately 50% [183]. Information on pharmaceutical development cycle time and productivity is continuously evaluated by the Tufts Center for the Study of Drug Development, Tufts University (http://csdd.tufts.edu/).

Regulatory Guidelines for Toxicity Testing

In this section, the toxicology support packages for the registration of NCEs and NBEs are reviewed from slightly different perspectives. More detailed information on the specific studies conducted, their results, and their interpretation are included for the classical agents (omeprazole and zidovudine), because the majority of compounds currently in development would fall into this category and thus require similar testing strategies. Omeprazole was selected for discussion because it had a comprehensive toxicology package at the time of regulatory submission and represents an example of where additional mechanistic studies were critical in the approval process. Zidovudine (AZT) is discussed due to its proposed use in life-threatening disease where no adequate therapy was available. The rapid approval of the drug, in spite of significant toxicology findings and an abbreviated toxicology support package, demonstrates the inherent flexibility in the approval system even with regard to NCEs.

The discussion of specific human NBEs (gonadotropin-releasing hormone [GnRH] analogs, IFN, human insulin) is presented from a more philosophical perspective. Because these agents are naturally occurring and because the major limiting toxicity in animal studies (immunogenicity) is not applicable to clinical trials, the design of the toxicology package posed special issues that were considered on a case-by-case basis. Furthermore, the toxicologic profile was anticipated based on extensive clinical experience with less specific agents (animal-derived insulins) or a broad understanding of the physiological functions of the hormones. The following discussion of the NBEs poses questions, concerns, and general guidelines to be considered in the development of these agents.

Acute, Subchronic, and Chronic Testing

Toxicity testing to support drug development is composed of several major types [53]. Acute (single dose), subchronic (multiple dose; less than 6 months' duration), and chronic (multiple dose; greater than or equal to 6 months' duration) studies are intended to elucidate the target organs for toxicity, demonstrate dose–response relationships, and often are useful in determining potential mechanisms of toxic action. The single- and repeated-dose toxicology studies completed in lead optimization are often leveraged to design, both dose justification and study end points, the definitive toxicology studies described here. For dose selection, consideration of the maximum tolerated dose (MTD), the maximum feasible dose and limit dose based on regulatory guidance, is necessary to ensure the doses maximize the possibility of defining the toxicologic response [24].

A variety of end points are routinely evaluated in subchronic and chronic studies, including body weight, food consumption, hematology, clinical chemistry, urinalysis, and gross and histologic pathology of numerous tissues. A list of common parameters assessed in subchronic and chronic studies is presented in Table 8.4; however, the toxicologist is continually challenged to modify study design to address the anticipated actions of the compound under investigation. This may result in the addition of certain parameters or tissues to be evaluated or a more comprehensive analysis of tissues (i.e., electron microscopic evaluation, immunohistochemistry). Furthermore, previous studies, or knowledge of the toxicity of other agents in the therapeutic class or those that have a similar structure, may suggest alternative assessments, such as the determination of the propensity of the agent to induce hepatic microsomal enzymes, cause phospholipid accumulation, or result in peroxisome proliferation.

An assessment of bioavailability and pharmacokinetics is often an important end point of subchronic and chronic studies. As discussed previously, these data are critical to extrapolate toxicity findings to humans. Often, the toxicokinetic profile of the compound is determined early and late in the study so the potential for drug accumulation can be revealed. Alternatively, drug levels may be lower toward study termination or the metabolite profile may differ due to the induction of drug-metabolizing enzymes. Tissues may also be collected for drug analysis, so levels in affected tissues can be related to the extent of the histopathologic findings. Finally, important dose–response relationships can be established, relative to both parent compound and metabolites, that may be critical in the interpretation of toxicity data.

Subchronic (usually 2-week or 1-month studies) testing is required prior to the FHD. One-month studies in one rodent species (usually rat or mouse) and one nonrodent species (usually dog or primate) generally will support up to 1 month of

TABLE 8.4
Parameters That Might Typically Be Assessed in a Subchronic/Chronic Toxicology Study

Live Phase		
Body weight	Clinical observations	Electrocardiogram (large animal)
Food consumption	Ophthalmology	Physical examination
Efficiency of food utilization (g body weight gained per 100 g feed consumed)		
Hematology		
Erythrocyte count	Mean corpuscular hemoglobin	Thrombocyte count
Hemoglobin	Mean corpuscular hemoglobin concentration	Activated partial thromboplastin time
Packed cell volume	Total leukocyte count	Prothrombin time
Mean corpuscular volume	Leukocyte differential	M/E ratio (bone marrow smears)
Clinical Chemistry		
Glucose nitrogen	Gamma-glutamyltransferase	Cholesterol
Blood urea	Creatinine phosphokinase	Triglycerides
Creatinine	Calcium	Total protein
Total bilirubin	Inorganic phosphorus	Albumin
Alkaline phosphatase	Sodium	Globulin
Aspartate transaminase	Potassium	Albumin/globulin ratio
Alanine transaminase	Chloride	
Urinalysis		
Color	Protein	Ketones
Clarity	Glucose	Bilirubin
Specific gravity	Occult blood	Urobilinogen
pH		
Organ Weights		
Kidneys	Ovaries	Adrenals
Liver	Testes	Thyroids (with parathyroids)
Heart	Prostate	Brain
Histopathology		
Kidney	Stomach	Skin muscle
Urinary bladder	Duodenum	Skeletal
Liver	Jejunum	Bone
Gallbladder	Ileum	Bone marrow
Heart	Cecum	Adrenal
Aorta	Colon	Thyroid
Trachea	Rectum	Parathyroid
Lung	Ovary	Pituitary
Spleen	Uterus	Cerebrum
Lymph node	Cervix	Cerebellum
Thymus	Vagina	Brain stem
Salivary gland	Testis	Spinal cord
Pancreas	Epididymis	Sciatic nerve
Tongue	Prostate	Eye
Esophagus	Mammary gland	Harderian gland
Other		
Blood levels of parent compound/ metabolites	Hepatic microsomal enzyme activity/cytochrome P450 content	Peroxisome proliferation, tissue phospholipid phosphorus concentration

dosing in humans. Where possible, the animal studies should be carried out using the same route of administration anticipated for use in patients. As an aside, it should be acknowledged that clinical studies may initially be conducted by the intravenous route, regardless of the desired ultimate route of administration, particularly for those molecules that are anticipated to show efficacy rapidly, to demonstrate the proof of concept of a new pharmacologic mechanism. Drug developers might thus avoid the time and expense associated with formulation development and maximization of the desired properties of the chemical if it has been demonstrated that the molecule or mechanism is ineffective. These clinical trials require the support of intravenous toxicology assessments. In these circumstances, it is extremely important to evaluate the risk associated with the potential for demonstrating toxicity in the intravenous study that may be irrelevant to the ultimate route of administration.

Phase II and III efficacy testing in patients is supported by longer-term studies. Depending on the proposed duration of human exposure, toxicity studies to support phases II and III may be of 3-, 6-, or 9/12-month duration. Two or more subchronic or chronic studies may be conducted simultaneously (e.g., 3-month and 6-month studies may be initiated at the same time), so patients can be placed on the trial earlier (upon completion of the 3-month study) and maintained on the trial longer (supported by the 6-month study) if the human efficacy and safety data support continued therapy. A potential problem with this approach is that dose selection for the more extended study may be found to be inadequate (doses either too low or too high) based on the findings of the shorter test.

Much discussion has surrounded the utility of 1-year studies. The FDA had been a strong proponent of the 1-year study approach [51], but Japan and the European Union (EU) have suggested that 1-year studies reveal little new information beyond that gained from 6-month studies. These data have been reviewed by Lumley et al. [168], who suggested that for 154 compounds for which short-term (≤6 months) and long-term (>6 months) animal data are available, tests lasting longer than 6 months (excluding carcinogenicity studies) have not provided new substantive safety information. They also pointed out that, although new findings became evident after 6 months of treatment in 9 out of 75 cases, the data did not influence the decision of whether to continue the development of the compound. Parkinson et al. [184] have reported that long-term toxicity studies in dogs provide little new data when compared to 3-month dog studies in conjunction with short- and long-term rodent studies.

The ICH committees have resolved this difference of opinion [138]. We will review the ICH guidelines in the following, but the reader is encouraged to connect with the ICH website, http://ich.org, for an up-to-date review of the current guidelines.

Additional Toxicology Studies to Support Clinical Trials

Other tests conducted prior to initial clinical trials include mutagenicity studies and pharmacologic assessments (e.g., cardiovascular, central nervous system (CNS), and/or gastrointestinal function). A variety of mutagenicity studies are currently employed that assess various types of DNA damage in vitro and in vivo in an attempt to predict the oncogenic potential of the compound under investigation. In pharmacologic screening, the ability of the compound to produce toxicities or *side effects* based on its pharmacologic mechanism of action is assessed; for example, an agent that is shown to bind to β-receptors in vitro might be anticipated to influence cardiac function in subsequent toxicity and clinical testing. Mutagenicity and pharmacology safety studies are often employed as very early screens in the evaluation of potential drug candidates to select one of a group of structurally related compounds that would be least likely to result in carcinogenicity and most likely to demonstrate the specific desired pharmacologic activity. The types and utility of these studies are further described in the following. Finally, special studies might be conducted prior to initial clinical testing to address specific issues, such as irritation testing of an agent proposed for topical use in the patient population.

Reproductive and Developmental Toxicity Studies

The thalidomide incident raised a great deal of concern relative to predictive testing for developmental toxicity, as well as fertility effects in both males and females. Although regulations have differed substantially among countries, worldwide harmonized guidelines for reproductive toxicity testing have been established [140]. The ultimate goal of these studies is to assess reproductive risk to adults, as well as to the developing offspring, at all stages from conception to sexual maturity. Traditionally, animal studies have been conducted in three segments: in adults, treatment premating through mating in the male and premating through either implantation or lactation in the female (segment I); in pregnant animals, treatment during organogenesis (segment II or teratology studies); and in pregnant/lactating animals, treatment from the completion of organogenesis through lactation (segment III, peri- and postnatal study). The current ICH guidance addresses proposed study design approaches and offers that the most probable option for evaluation of reproductive effects includes a combination of three studies that address fertility and early embryonic development; peri- and postnatal development, including maternal function; and embryo fetal development [140].

The harmonized ICH guidelines [140] stress the need for flexibility in testing for reproductive and developmental toxicity and challenge the toxicologist to custom design a combination of studies that will reveal potential effects on all of the parameters considered in the classical segment I, II, and III studies. For treated adults, these include development and maturation of gametes, mating behavior, fertilization, implantation, parturition, and lactation. In the developing organism, where the maternal animal may be exposed to the drug candidate from prior to mating through lactation, assessments of early embryonic development, major organ formation, fetal development and growth, postnatal development and growth (including behavioral assessments), and attainment of full reproductive function are required. These evaluations might be carried out as one comprehensive study with interim assessments, or they might be segmented into several treatment components. Thus, the new guidelines

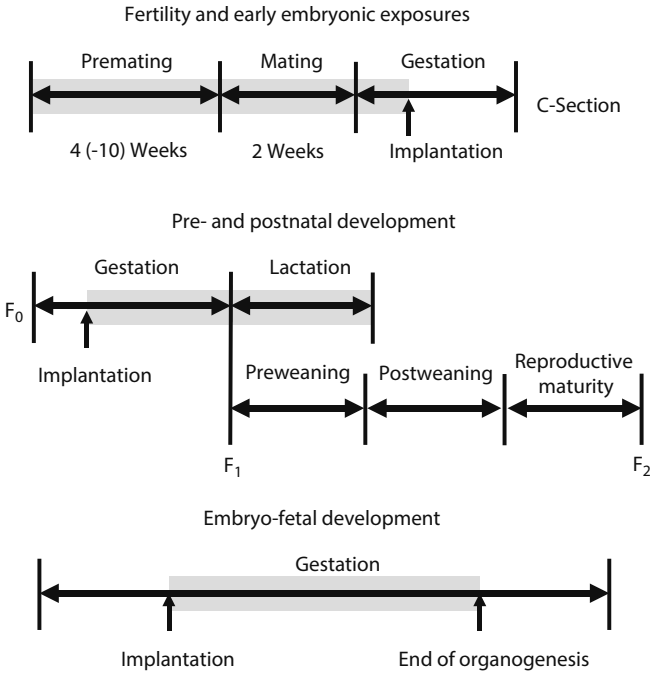

FIGURE 8.7 The three-study design proposed for the assessment of reproductive and developmental toxicity for a standard pharmaceutical. (From ICH, Detection of toxicity to reproduction from medicinal products and toxicity to male fertility, Topic S5(R2), Step 5, ICH Harmonized Tripartite Guideline, *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*, Geneva, Switzerland, 2005.)

have not diminished the extent of evaluation but allow flexibility in study design based on what is already known of the compound under investigation. The harmonized guideline suggests a three-study design that is likely to provide all of the developmental toxicity data necessary to support product registration, assuming no untoward toxicity. Our view of this approach is generally represented in Figure 8.7. Should toxicity be demonstrated, further mechanistic studies would be conducted to clarify effects and determine whether the responsible mechanisms would be applicable to humans. The results from previous subchronic and chronic studies (e.g., evidence suggesting an effect on spermatogenesis upon histopathologic examination of the testes) are critical in the design of an appropriate reproduction package.

Women of child-bearing potential are generally first recruited into phase II clinical trials, and several countries strongly suggest that efficacy be demonstrated in male patients prior to recruitment of women of child-bearing potential into trials, regardless of the outcomes of the nonclinical reproductive studies. However, it should be noted that the FDA has encouraged the inclusion of women of child-bearing potential in early clinical trials, especially in the case of drugs intended to treat life-threatening conditions or in the study of disease states that more commonly affect women. Typically, prior to the inclusion of women of child-bearing potential into clinical trials, studies are conducted to evaluate effects on organogenesis (segment II) in two species (usually the rabbit and the rodent species selected for the subchronic and chronic studies). Female fertility assessments are also usually undertaken prior to longer-term treatment or addition of significant numbers of patients. Despite the availability of these data, clinical protocols frequently require that women of child-bearing potential use reliable contraceptive intervention. Evaluation of toxicity to male fertility is now incorporated into the ICH guidance [140] and is supported by histological evaluation of the testes at the conclusion of the subchronic and chronic nonclinical studies, and specific animal studies to examine drug effects on male fertility are generally not conducted until phase III. Unless there are concerns regarding a specific chemical class or mechanism of action, the more sophisticated analyses of peri- and postnatal development and behavior (segment III studies) are often conducted in conjunction with the phase III clinical trials. Frequently, as is the case with other toxicity evaluations, special studies may be conducted to determine the mechanisms of observed reproductive effects in an effort to assess whether these findings are meaningful to humans and whether use of the compound should be restricted depending on the reproductive status of the patient (i.e., the drug should not be administered to pregnant women). Finally, the inclusion of female patients in clinical trials may be allowed following a more limited assessment of reproductive or developmental parameters if the compound under development is shown to be efficacious or is thought to provide distinct advantages over available therapies in the treatment of life-threatening disease. The ICH guidance refers to the availability of other test systems such as mammalian cell systems, tissues, organs, or organism cultures to help understand mechanism of effects [140]. As with all in vitro or ex vivo approaches, these studies provide useful information in a very specific and limited environment; they lack the complexity of the developmental process and the dynamic interaction between maternal and developing organism [140].

Carcinogenicity Studies

Among the final toxicity studies to be conducted to support the registration of chronic-use therapeutics are the carcinogenicity bioassays [136,137]. These lifetime (2-year) studies are usually conducted in two rodent species (normally, rat and mouse). Selection of the top dose for the carcinogenicity bioassays has been an intensely debated topic of discussion in the international harmonization process. The international consensus has based the selection of the top dose for carcinogenicity bioassays on any one of the following [143,234]:

- MTD
  - AUC rodent-to-human ratio of 25-fold, which applies when no genotoxicity is identified
- Saturation of absorption
- Dose-limiting pharmacodynamics (i.e., hypotension, hypoglycemia, decreased blood clotting time)
  - Use of a limit dose (1000 mg/kg), which applies when the maximum recommended human dose is ≤500 mg/day and the AUC (rodent–human) is ≥10

The middle and low doses should also be selected to provide additional information to use in the risk assessment. The consideration of dose linearity, saturation of metabolic pathways, MOS, pharmacodynamics, specific animal physiology, threshold effects, and progression of toxic effects should be included in the selection of the middle and low doses for carcinogenicity evaluations.

The use of an MTD as the top dose in a carcinogenicity study has been a subject of much discussion and debate, as has the choice of an appropriate high dose in any nonclinical toxicology study [24]. The current ICH guidance [143] provides some general guidelines for dose selection in rodent bioassay studies, adequate MOS over human exposure; produces no significant physiological dysfunction or effects on survival, guided by animal and human data; and permits data interpretation in the context of clinical use. The MTD has been classically defined as the dose that causes no more than a 10% decrease in body weight and does not produce mortality, clinical signs of toxicity, or pathologic lesions that would be predicted to shorten the natural life span of an experimental animal for any reason other than the induction of neoplasms [143,209]. Because the MTD is often based on a body weight effect, Hoffman et al. [132] have proposed an improved evaluation of growth data in rodent toxicology studies using a powerful statistical analysis method that reduces the number of false positives and provides a comprehensive summary of compound-related effects. The MTD is suggested to produce a level of toxicity indicative of sufficient chemical challenge to define chronic toxic manifestations [128]. Many regulatory bodies default to the use of the MTD as the maximum dose in the rodent bioassays. A major concern with the MTD approach is that metabolic saturation, as discussed previously, may occur at high doses, leading to abnormal metabolism [178] or, in the case of inhaled therapeutics, abnormal clearance [177]. Chemicals administered at the MTD in animal bioassays tend to induce mitogenesis as a result of cell death due to frank intoxication, with the target tissues differing among species and sexes [113]. This stimulation of cell proliferation, a natural recovery process in response to severe toxicologic insult that does not normally occur at reasonable multiples of human exposure levels, can account for the carcinogenic response of nongenotoxic compounds [49]. Thus, the fact that a chemical is a carcinogen at MTD levels in rodents may provide little meaningful information relative to low-dose risk assessment in humans [2]. MTDs for chronic toxicity studies are usually estimated based on the results of subchronic toxicity studies; however, because compound distribution and disposition may be affected by the dose or duration of treatment, this may be a very crude estimate [178,182].

The choice of an MTD is a critical aspect of chronic toxicity evaluations [216]. Cell proliferation indices in subchronic toxicology studies may provide a useful estimation of an appropriate MTD by determining the highest dose that does not result in the phenomenon [26]. The use of kinetic parameters (C_{max}, AUC related to dose) would better predict the dose at which saturation (nonlinearity) might occur and therefore provide a better estimate of the MTD in a particular species. Also, changes in the urinary metabolite profile, in relation to dose, may be a good way of indicating metabolic overload and could aid in more accurately selecting upper dose levels in toxicology studies [242]. A systemic exposure-based alternative to the MTD for carcinogenicity studies has been presented by Contrera et al. [52]. This alternative was discussed as being suitable for nongenotoxic compounds with low rodent toxicity and similar metabolism in rodents and humans. The relevance to human risk assessment of a carcinogenicity finding for nongenotoxic compounds was addressed by Silva Lima and van der Lann [205]. They discussed the main causes of carcinogenicity from nongenotoxic compounds: chronic cell injury, immunosuppression, increased secretion of trophic hormones, receptor activation, and other mechanisms such as CYP450 induction. An overall approach to addressing the human relevance of the mode of animal carcinogenicity (i.e., genotoxic or nongenotoxic) has been addressed by Cohen et al. [48].

The utility of using two rodent species has also been an active area for discussion. The ICH has indicated that the rat would be preferable to the mouse for conducting carcinogenicity studies [136]. The rat seems to have been given a preference because background mechanistic data are usually available for rats (not mice), studies of metabolic disposition are more often carried out in rats than in mice, and mouse carcinogenicity studies are dominated by liver tumors of questionable relevance to humans. A review of the European regulatory database has concluded that studies in the mouse add little to the ability to detect carcinogenic risk from pharmaceuticals [53,227,228]; however, the review found that carcinogenicity studies in two rodent species are necessary to identify trans-species tumorigens. NCEs active across species are considered to pose a relatively greater risk to humans than NCEs positive in only one specie. The conduct of a study using an alternative in vivo carcinogenicity model along with a standard bioassay in one species was considered to be an acceptable alternative for assessing carcinogenic potential [43].

The use of alternative models for carcinogenicity assessment meets the desire of the FDA to have an assessment in two species and offers the advantages of using fewer animals, being of shorter duration and being capable of enhancing the predictability of the rodent bioassay [50,137]. A number of transgenic animal models are currently being evaluated as alternatives to the 2-year bioassay [8,42,111]; however, it is early yet to evaluate the validity of alternative models of carcinogenic potential to the risk assessment process, and it would be advisable to discuss the selection of alternative models with the appropriate regulatory agency prior to study initiation, as the use of alternative models is not routinely accepted by the FDA.

Within the FDA, it is highly recommended that protocols, dose justification documents, and supporting data be submitted to the Carcinogenicity Assessment Committee (CAC) for evaluation prior to initiation of carcinogenicity studies. The CAC provides consultation on study designs, ensures

consistency and quality in the analysis and interpretation of animal carcinogenicity studies across industry and within the agency, and monitors scientific developments to ensure that scientific standards of design and interpretation are upheld.

Since 1982, the number of NBEs presented for registration has risen; even so, these NBEs represent a relatively small number of molecules. Due, in part, to this lack of practical experience, the safety programs for the NBEs have been designed on a case-by-case basis. The ICH has provided two guidelines that address carcinogenicity studies with products of biotechnology [136,147]. It is generally acknowledged that carcinogenicity studies are not appropriate for biotechnology products given essentially as replacement therapy at physiological levels, especially when clinical experience exists (e.g., insulins, calcitonin, and pituitary-derived growth hormone) [121,136,147]. Product-specific assessment of carcinogenic potential may be necessary depending on clinical dosing regimen, patient population, and biological activity of the product. For products that have the potential to induce cell proliferation (e.g., growth factors), an in vitro evaluation of receptor expression in cells relevant to the patient population may be conducted. If these data indicate a need for further evaluation of carcinogenic potential, 2-year studies in a single rodent species should be considered.

Long-term carcinogenicity evaluations with endogenous peptides and proteins or their analogs are generally indicated when [136]

- Significant differences in biological effects to the natural substances are found
- Modifications lead to significant changes in structure compared to the natural substance
- Therapeutic exposure levels exceed those that normally occur in the systemic circulation or in tissues

A specific example of studies recommended for the analog of a naturally occurring decapeptide, GnRH, is presented under Biological Entities [192].

Regulatory guidelines as to when and why carcinogenicity studies should be conducted with naturally occurring substances and their analogs exist, as do opinions from industry, academia, and regulatory agencies regarding the propriety of conducting these studies [121,147]. For each NBE under development, the existing opinions and guidance must be considered, a reasonable plan to establish safety must be developed, and a discussion with the appropriate regulatory agency should be held to test the plan. Safety evaluation of NBEs is still very much a case-by-case consideration. The pharmaceutical industry must be careful not to overinterpret the position that carcinogenicity studies are not usually appropriate for biotechnology products.

CHEMICAL ENTITIES

The extent and types of safety testing of synthetic organic pharmaceutical agents in animal models depend on a variety of factors, including the potential duration of treatment of patients (e.g., short term, antibiotics; chronic, antihypertensives), route of administration, pharmacologic mechanism of action, proposed patient population, and clinical experience with other agents in that therapeutic class. Furthermore, the design of animal toxicity studies that occur later in development must carefully consider the results of previous tests in animals and humans relative to bioavailability, unanticipated toxic responses, and relevance of species selected.

Generally, toxicity testing in animals can be considered in four phases: testing to support candidate selection, testing to support FHD (single- and multiple-dose; phase I), testing to support longer-term and broader clinical efficacy studies (weeks to months; phase II), and testing to support final registration and, if appropriate, chronic treatment (phase III) (Figure 8.8). Although the great majority of testing is performed prior to registration, special studies may be requested by regulatory agencies during the review and approval processes. Following widespread clinical use of a new agent, further testing may be appropriate to examine potential mechanisms of action for unanticipated side effects that become evident in the increasing patient population or subpopulations. These may occur due to genetic differences, environmental factors, age, patient history, existence of other diseases or pathologies, and drug interactions. Other tests may be considered if new formulations of the drug are developed, if the drug is suggested for new indications, or if it will be used in patient populations that were not anticipated during initial development (e.g., pediatrics).

The primary purpose of initial nonclinical testing to support FHD is to determine the toxicity and pharmacokinetics (and oral bioavailability, if appropriate) of the drug candidate in humans following single or multiple doses. Usually, the drug is administered to humans at doses below the anticipated efficacious dose, and doses are escalated until a satisfactory multiple over the anticipated efficacious dose is achieved or toxicity becomes evident. Unless the drug candidate has known serious toxicity, as is the case with many oncolytics, it is usually first tested in a limited male, nonpatient population that is under constant observation. As indicated earlier, the FDA supports the early inclusion of women in clinical trials for new therapies, especially those to be used in the treatment of life-threatening diseases [87,89]. Because of this interest, studies of developmental toxicity, which usually occur later in drug development, may be moved to a much earlier point in the drug development process [184].

When designing animal studies to support FHD, a major consideration in dose selection must be the anticipated MOS between animals and humans. Ideally, doses in animal studies should provide exposure to the compound well in excess of what is anticipated at the highest doses to be tested in humans. As discussed previously, a comparison of these doses on a milligram-per-kilogram basis is no longer considered to provide adequate information in this respect due to potential species differences in absorption and rates and routes of metabolism; thus, a good estimate of the pharmacokinetic behavior of the agent in animals is an important

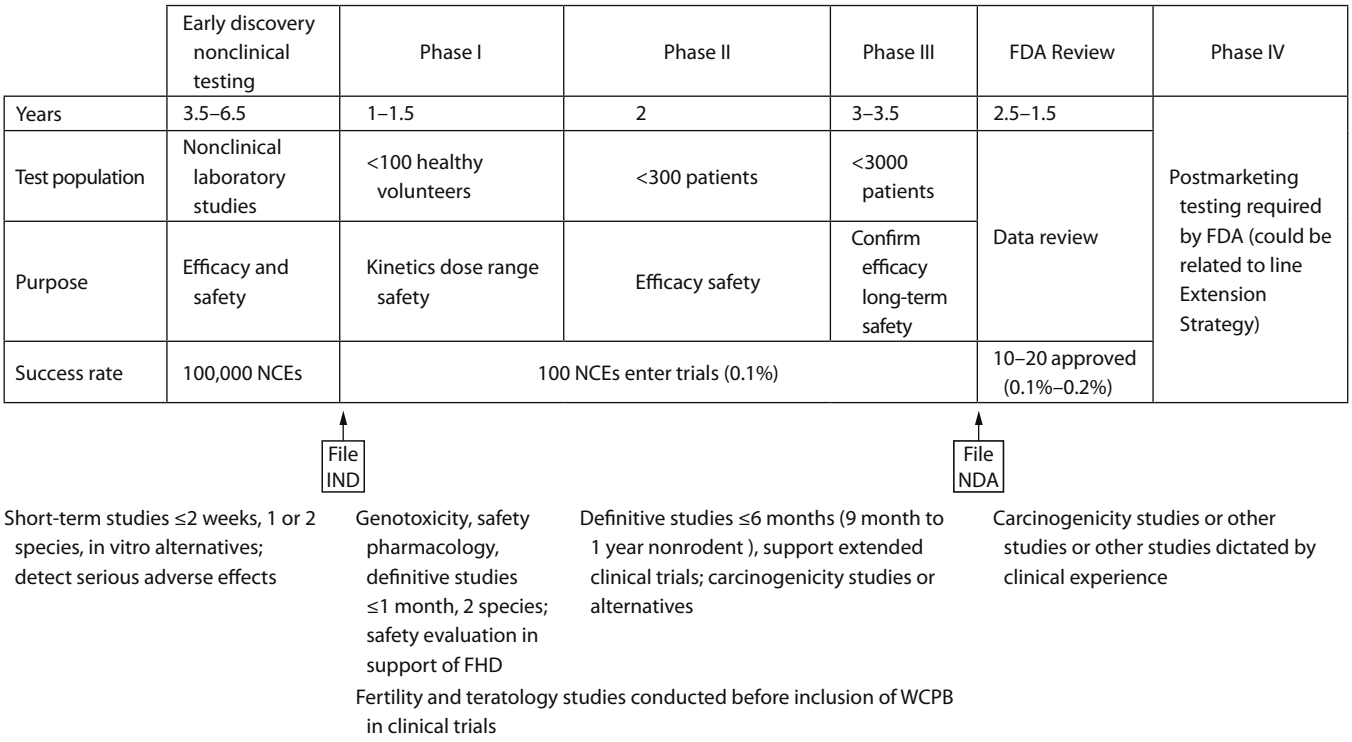

FIGURE 8.8 Schematic of the drug development and approval process in the United States. Similar processes are employed for worldwide pharmaceutical testing. (From Gordon, C.V. and Wierenga, D.E., *The Drug Development and Approval Process*, Pharmaceutical Manufacturers Association, Washington, DC, 1991.)

TABLE 8.5
International Guidelines for the Duration of Animal Toxicology Studies Necessary to Support Clinical Trials of Various Durations

	Toxicology Duration to Support Clinical Trials		Toxicology Duration to Support Marketing	
Clinical Trial Duration	**Rodents**	**Nonrodents**	**Rodents**	**Nonrodents**
Up to 2 weeks	2 weeks	2 weeks	1 month	1 month
Between 2 weeks and 6 months	Same as clinical trial	Same as clinical trial	—	—
>2 weeks to 1 month	—	—	3 months	3 months
>1 to 3 months	—	—	6 months	6 months
>3 months	—	—	6 months	9 months
>6 months	6 months	9 months	—	—

Source: Data from ICH, Guidance on non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals, ICH Topic M3(R2), Step 5, ICH Harmonized Tripartite Guideline, *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*, Geneva, Switzerland, 2009. See ICH website (http://www.ich.org) for further details and exceptions.

goal of nonclinical testing. No firm guideline exists regarding what should be considered an adequate MOS; however, a smaller margin between the potentially efficacious dose and a toxic dose is tolerated for those compounds under development for life-threatening diseases, particularly if they are expected to offer a distinct therapeutic advantage over other agents currently marketed in the class.

Based on the activities of the ICH, the designs and goals of clinical trials are similar throughout the world. Table 8.5 shows the recommendations for the duration of animal tests relative to proposed human exposure to NCEs. It is possible to discuss the nonclinical and clinical programs with regulatory bodies and, depending on the characteristics and proposed use of the chemical, modify these recommendations.

The duration of animal tests necessary to support a specific duration of clinical trials for NBEs is much more flexible than the comparable guidance for NCEs [147]. Short-term clinical trials for life-threatening conditions may be supported by

2-week nonclinical toxicology studies. Subchronic clinical trials can be supported with toxicology studies of 1–3-month duration. Clinical trials to support chronic therapy can be supported with 6-month toxicology studies.

Regulators throughout the world have recognized that resources could be used more efficiently and efficacious and safe drugs could be made available more rapidly, if guidelines for nonclinical testing and registration were comparable across countries. The ICH has developed a comprehensive set of safety guidelines to harmonize the regulatory requirements of the EU, United States, and Japan. The ICH Expert Working Groups (EWGs) have considered appropriate guidelines for all of the various types of toxicity tests, including acute and subchronic testing, chronic and carcinogenicity testing, reproduction and developmental toxicity studies, and mutagenicity testing. The required duration of animal studies to support human exposure has also been addressed. A good deal of collaboration has occurred between regulatory agencies and pharmaceutical companies in the development of the safety guidelines.

Dorato and Buckley [77] have provided a more complete discussion of toxicology testing and the role of the ICH process in drug development. Table 8.6 shows the ICH guidelines that address the various nonclinical studies required to support clinical trials and registrations in the three major regions (EU, United States, and Japan) and to qualify impurities. A proposal on nonclinical studies to support pharmaceutical excipient development has been made by Baldrick [15]. Selected Internet websites providing information on the design of toxicology studies to support clinical trials are shown in Table 8.7.

As mentioned previously, the speed with which safe and effective new drugs enter clinical trials and eventually the market is a focal topic for regulatory agencies and the pharmaceutical industry. In the past, the FDA [88] and the European Committee for Human Medicinal Products (CHMP) [44] have published guidelines that provide an exception to the minimum 2-week rodent and nonrodent study requirements of the ICH and allow for single-dose toxicity studies in support of targeted clinical trials. The FDA guidance [88] was intended to facilitate choosing compounds to enter phase I human studies. The CHMP [46] guidance was intended to facilitate early characterization of pharmacokinetic properties or receptor selectivity profiles using, for example, positron emission tomography (PET). The clinical trials could test several closely related pharmaceuticals with the intent of choosing the preferred candidate or formulation for further development. The CHMP guidance recommended a maximum dose of 100 μg. Following these attempts, the FDA, in keeping with their Critical Path Initiative [92], published a guidance on exploratory investigational new drug (eIND) studies [93]. The FDA guidance clarifies the nonclinical and clinical approaches to the conduct of exploratory studies that occur very early in phase I, involve very limited human exposure, and have no therapeutic intent (e.g., screening). The dosing duration in the clinical study is expected to be ≤7 days. The flexibility available for drug development and examples of the use of the eIND approach are presented. This is a proposal of an approach that could facilitate the development of safe and effective drugs.

Specific Agents

As mentioned previously, two NCEs (omeprazole and zidovudine) were selected for discussion due to their different target patient populations, which drove the design of customized toxicology testing strategies. While these still represent good examples of the drug development process from a toxicology perspective, updated regulatory guidances and recommendations govern current approaches. For example, the establishment of new limit doses (1000 mg/kg), use of transgenic mouse models in place of the traditional 2-year mouse carcinogenicity study, and the acceptability of a 9-month chronic nonrodent study are just a few that should be considered. As with any drug development program, strong partnership with the regulatory agencies is essential in providing the most appropriate safety package to support the registration of an NCE. The discussion that follows for omeprazole and zidovudine is limited primarily to the data that supported their initial approvals (and, in the case of zidovudine, include postapproval commitments) to demonstrate how disease state and early toxicology findings can influence subsequent development strategy.

Omeprazole (Prilosec®)

Omeprazole is a substituted benzimidazole that is a potent inhibitor of H^+/K^+ ATPase (proton pump) at the secretory surface of the gastric parietal cell, thereby inhibiting gastric acid secretion [20,158,196,231]. Omeprazole is indicated for the short-term (4–12-week) treatment of active duodenal and gastric ulcer, gastroesophageal reflux disease (GERD), severe erosive esophagitis, and maintenance of healing of erosive esophagitis, as well as the long-term treatment of pathological hypersecretory conditions such as the Zollinger–Ellison syndrome. It is also approved for use with clarithromycin and amoxicillin for the treatment of patients with *Helicobacter pylori* infection and duodenal ulcer disease. Omeprazole OTC, an over-the-counter product, is approved only for frequent heartburn occurring 2 or more days a week. The recommended dosage for short-term indications is 20–40 mg daily (approximately 0.4–0.8 mg/kg in a 50 kg individual). For long-term indications, the recommended initial dose is 60 mg daily; however, doses up to 120 mg three times daily have been administered [187,188]. Table 8.8 lists the toxicology studies that were submitted to the FDA [86] to support the U.S. registration of omeprazole. The content of the toxicology package suggests that the intravenous route may have also been a considered route for therapy.

The results of acute, subchronic, and chronic studies suggested that the toxicology profile of omeprazole was generally unremarkable [36,79,86,125]. The acute toxicity of the compound in rats and mice was low, as demonstrated by the oral LD_{50} values (the dose that kills 50% of the animals tested), generally in excess of 4 g/kg. Multiple-dose studies in rats

TABLE 8.6
ICH Guidelines for the Conduct of Nonclinical Studies

Topic	Number[a]	Title and Contents
Toxicity testing	S4	*Duration of Chronic Toxicity Testing in animals, Rodent and Non Rodent Toxicity Testing (Step 5)* Repeated-dose toxicity testing and reduction in duration of longest-term dose toxicity study in rodents from 12 to 6 months; reduction of duration of repeated-dose toxicity studies in nonrodents from 12 to 9 months
Carcinogenicity studies	S1A	*Need for Carcinogenicity Studies of Pharmaceuticals (Step 5)* Definition of circumstances requiring carcinogenicity studies, taking into account known risks, indications, and duration of exposure
	S1B	*Testing for Carcinogenicity in Pharmaceuticals (Step 5)* Need for studies in two species; additional in vivo tests for carcinogenicity
	S1C(R2)	*Dose Selection for Carcinogenicity Studies in Pharmaceuticals (Step 5)* Criteria for selection of high dose
Genotoxicity studies	S2(R1)	*Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use (Step 5)* Specific guidance for in vitro and in vivo tests plus glossary of terms; identification of a standard set of assays; extent of confirmatory experimentation
Reproductive toxicology	S5(R2)	*Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility (Step 5)* Specific guidance for testing reproductive toxicity
Toxicokinetics and pharmacokinetics	S3A	*Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies (Step 5)* Integration of kinetic information into toxicity testing
	S3B	*Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies (Step 5)* Need for tissue distribution studies when appropriate data cannot be derived from other sources
Biotechnology products	S6(R1)	*Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (Step 5)* Nonclinical safety studies, use of animal models of disease, and other alternative methods; need for genotoxicity and carcinogenicity studies; impact of antibody formation
Joint safety/efficacy (multidisciplinary)	M3(R2)	*Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals (Step 5)* Principles for the development of nonclinical testing strategies (address full range of studies to support clinical trials for NCEs)
	M7	*Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk (Step 2)* Final concept paper
Pharmacology studies	S7A	*Safety Pharmacology Studies for Human Pharmaceuticals (Step 5)*
	S7B	*The Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals (Step 5)*
Immunotoxicology studies	S8	*Immunotoxicology Studies for Human Pharmaceuticals (Step 5)*
Anticancer pharmaceuticals	S9	*Nonclinical Evaluation for Anticancer Pharmaceuticals (Step 5)*
Photosafety	S10	*Photosafety Evaluation of Pharmaceuticals (Step 2)* Final concept paper
Impurities in new drug substances	Q3A(R2)	*Impurities in New Drug Substances (Step 5)*
Impurities in new drug products	Q3B(R2)	*Impurities in New Drug Products (Step 5)*
Specifications	Q6A	*Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (Step 5)*
	Q6B	*Test Procedures and Acceptance Criteria for Biotechnological/Biological Products (Step 5)*

[a] The most recent information on ICH guidelines can be found on the ICH website (http://www.ich.org).

were conducted at doses up to 414 mg/kg/day for 3 months and up to 138 mg/kg/day for 6 months. No consistent effects on body weight or feed consumption were reported in those studies. Treatment-related findings that occurred at high doses in these studies included decreases in several erythrocytic parameters and decreases in plasma glucose and triiodothyronine. The latter finding was ascribed to a reduction in the peripheral conversion of thyroxine to triiodothyronine. Increased liver and kidney weights were observed in both studies, as well as in the 24-month rat oncogenicity study. Elevated kidney weights were correlated with an apparent exacerbation of the progress of chronic nephropathy that normally occurs in aging Sprague Dawley rats.

Dogs were treated with omeprazole for 3 months at doses up to 138 mg/kg/day or 12 months at doses up to 28 mg/kg/day. Clinical chemistry findings were generally

TABLE 8.7
Internet Websites Providing Information on the Design and Expectations of Nonclinical Toxicology Studies

http://www.fda.gov/Drugs/GuidanceCompliance RegulatoryInformation/Guidances	Access to guidance documents representing the agency's current thinking on a particular subject relating to the drug development process; includes access to adopted and draft ICH guidelines
www.ema.europa.eu/ema	Enter "Safety Working Party" to access documents covering safety evaluation in EU
www.Ich.org	The process and the adopted and draft safety guidelines of the ICH
www.ema.europa.eu/ema	The EMA

TABLE 8.8
Summary of Toxicology Studies Conducted to Support the Registration of Omeprazole in the United States

Acute Toxicology
- Oral study in mice
- Intravenous study in mice
- Oral study in rats
- Intravenous study in rats
- Oral study in dogs

Subchronic Toxicology
- 2-week intravenous study in rats
- 1-month intravenous study in rats
- 1-month intravenous study in dogs
- 3-month oral study in rats
- 3-month oral study in mice
- 3-month oral study in dogs
- 3-month oral study in dogs with 3-month recovery

Chronic Toxicology
- 6-month oral study in rats
- 3-month and 6-month oral studies in rats with 2-week to 6-month recovery
- 2-year study in female rats to examine gastrin-dependent variables
- 1-year oral study in dogs with 4-month recovery
- 5-year oral study in dogs (ongoing at time of application)

Genetic Toxicology
- Ames *Salmonella* test with/without metabolic activation
- Mouse lymphoma forward mutation assay
- Mouse micronucleus test
- Mouse chromosome aberrations
- Rat liver DNA damage assay

Reproductive and Developmental Toxicology
- Segment I oral fertility in rats
- Segment II oral teratology in rats
- Segment II oral teratology in rabbits
- Segment III oral perinatal and postnatal in rats
- Segment III extended oral perinatal and postnatal in rats

Carcinogenicity Studies
- 78-week oral study in mice
- 104-week oral study in rats
- 104-week oral study in female rats

unremarkable, although, as observed in the rat, some decreases in hematology parameters and plasma triiodothyronine were noted. The most significant nonclinical finding in both rats and dogs was a reversible gastric mucosal cell hyperplasia with increases in mucosal thickness and folding. In the 6-month study in rats, omeprazole induced a dose-related eosinophilia of the zymogen granules of the pepsinogen-secreting chief cells, with slight atrophy of these cells occurring at the high dose. Slight chief cell atrophy was also observed in dogs given the high dose of omeprazole for 3 or 12 months. To characterize these gastric changes more rigorously, a rather extensive reversibility study was conducted in rats in which animals were treated with either 0 or 138 mg/kg/day omeprazole for 14 days or 1, 3, or 6 months. Other groups of animals were treated with that dose of omeprazole for 3 or 6 months, followed by recovery periods of 14 days or 1, 3, or 6 months. This study demonstrated the time dependency and complete reversibility of the gastric lesions in rats. A 3-month recovery period following 3 months of treatment in dogs showed that the slight chief cell atrophy observed at 3 months was reversible. Also, a 4-month recovery period following 12 months of treatment in dogs demonstrated the reversibility of mucosal hyperplasia and chief cell atrophy, although increased mucosal folding was still evident.

No teratologic findings were observed in the rat at omeprazole doses up to 138 mg/kg/day administered on days 6–15 of pregnancy. The two highest doses tested in rabbits (approximately 70 and 140 mg/kg), administered during days 6–18 of pregnancy, resulted in maternal toxicity as evidenced by anorexia and reduced water intake. Signs were sufficiently severe that treatment of animals at the high dose was discontinued on day 14. Fetal mortality was increased in conjunction with maternal toxicity, but fetal development was unaffected by maternal omeprazole treatment. The major finding of the fertility and peri- and postnatal studies was a decrease in the weight gain of pups of maternal animals given the high dose of 138 mg/kg/day during late pregnancy and lactation. This correlated with a decrease in maternal body weight and feed consumption during late lactation. Whether the decrease in pup weight gain was the result of the decrease in maternal feed consumption or whether it may have been a direct effect on offspring via the breast milk transfer of the compound is not known.

In the mouse oncogenicity study, animals were treated with up to 138 mg/kg/day omeprazole for 18 months. A decrease in survival was noted at the high dose, but no neoplasia was observed in any organ. Different results, however, were obtained in the rat oncogenicity study, in which animals were treated with 13.8, 43, or 138 mg/kg/day omeprazole for 24 months. Enterochromaffin-like (ECL) cell hyperplasia, progressing to ECL cell carcinoids, occurred in dose-related fashion in these animals, with males being affected at doses of 43 and 138 mg/kg/day and females being affected at all

dose levels. These positive findings resulted in the temporary suspension of the clinical trial program. The carcinoids were characterized as *end-of-life* tumors, as the first was discovered at 82 weeks of treatment in an animal that had died prematurely. Carcinoid tumors were not identified as the cause of death in any animals, and no metastases were found. A 2-year study was repeated in females in an attempt to define a dose at which ECL cell carcinoids did not occur; however, carcinoid formation again occurred in a dose-related fashion, including at the lowest dose tested (1.7 mg/kg/day).

A major question that must be addressed following positive results in a carcinogenicity bioassay is whether tumorigenesis was the direct result of chemical insult or could be related to the pharmacologic mechanism of action of the compound. Furthermore, whether the model is appropriate for extrapolation of these findings to humans requires evaluation. For example, at this late stage in the development of a compound, sufficient pharmacokinetic data should be available in both the animal species tested and humans to determine whether a finding might be restricted to a species that metabolizes the compound quite differently from humans. If this is the case, further mechanistic studies can be designed to support or refute the applicability of the findings.

A number of in vivo and in vitro mutagenicity studies were conducted with omeprazole (Table 8.8). An initial mouse micronucleus test with omeprazole administered to animals at a high dose of 5000 mg/kg produced equivocal results, with slight increases in the mean numbers of micronucleated cells compared to controls (approximately twofold, compared to 30-fold following a 0.4 mg/kg dose of the positive control, mitomycin C). It was noted that the dose of 5000 mg/kg was not well tolerated. A second mouse micronucleus test conducted with a maximum dose of approximately 800 mg/kg did not show evidence of mutagenic potential. All other mutagenicity tests conducted produced negative results, suggesting that the tumorigenesis observed in the 2-year rat studies was not the result of a genotoxic action of omeprazole or its metabolites.

Mechanistic studies in dogs and rats, combined with correlative data from clinical trials, ultimately provided the information to support the safe use and registration of omeprazole. At the doses selected for the toxicity studies, the sustained decrease in luminal pH of the stomach resulting from the inhibition of gastric acid secretion by omeprazole caused a substantial increase in the release of gastrin into the blood. In fed rats, gastrin levels in plasma normally range from 150 to 200 pg/mL. Omeprazole administered at doses of 13.8–138 mg/kg/day increased plasma gastrin concentrations to 1000–3000 pg/mL. Gastrin has a trophic effect on the gastric mucosa and results in the hyperplasia of several cell types, including ECL cells, and consequent mucosal thickening. These data suggest that omeprazole does not inherently cause ECL cell hyperplasia and resulting carcinoid formation. Indeed, in antrectomized dogs (the major source of gastrin is surgically removed), high doses of omeprazole for 1 year resulted in neither hypergastrinemia nor mucosal hyperplasia [79]. Similarly, antrectomy in rats prevents the hypergastrinemia and ECL cell hyperplasia associated with omeprazole treatment [158].

The course of development of omeprazole demonstrates the importance of conducting mechanistic studies to elucidate the significance of findings in animal safety studies and whether the effects can meaningfully be extrapolated to humans. Clearly, a close collaboration between the toxicologist and clinician during advancing human trials is critical to resolve questions related to human safety. The extensive clinical experience with omeprazole has confirmed its safe and effective use for treatment of the described indications [20,188]. In a group of patients who required continuous treatment with 40 mg of omeprazole for up to 4 years, no evidence was found for dysplastic or neoplastic changes [58]. Over 12,000 endoscopic biopsies further supported the clinical safety of omeprazole relative to its potential for causing hyperplastic changes.

It should be noted that development of omeprazole probably took years longer than anticipated due to the need to conduct the supplementary mechanistic and carcinogenicity nonclinical studies, as well as additional clinical investigations. Clearly, in these situations, the sponsor needs to evaluate whether the additional time and expense required for approval are supported by the market need. In this case, in 2001, 12 years after approval and prior to being approved for over-the-counter distribution, Prilosec was the world's second biggest selling drug.

Zidovudine (Retrovir®/AZT)

Zidovudine (azidothymidine [AZT]) inhibits viral RNA-dependent DNA polymerase (reverse transcriptase) and, thus, viral replication. Furthermore, as a thymidine analog, zidovudine becomes incorporated into growing strands of DNA by viral reverse transcriptase and inhibits the further addition of nucleotides. It is intended for use in the management of adult patients with human immunodeficiency virus (HIV) infection when antiretroviral therapy is warranted [189]. It is also indicated for the prevention of maternal–fetal HIV transmission during gestation and labor and in the neonate after birth. The recommended dose for adults is 600 mg/day in divided doses in combination with other antiretroviral agents. Zidovudine is also available for intravenous infusion in patients with advanced disease and for use in women during labor and delivery. In spite of the intended long-term use of the compound, it was approved with a minimal toxicology package due to the serious nature of the disease and lack of alternative efficacious therapies.

The studies listed in the upper portion of Table 8.9 were either submitted as part of the original NDA in December of 1986 or as amendments to the application shortly thereafter [85]. At the time of the initial pharmacology/toxicology review, a variety of chronic toxicity studies were still underway or planned. The FDA commentary indicated that "Nonclinical toxicity data submitted in support of the application include results of studies in rats, dogs, and cynomolgus monkeys. FDA guidelines would have prescribed more extensive non-clinical testing than that reported thus far; however, the urgency for developing an anti-AIDS drug has been so great that clinical testing has preceded the usual/customary nonclinical testing. For example, while data from a 6-month *clinical* study are available, results for the

TABLE 8.9
Summary of Toxicology Studies Submitted for Initial FDA Review to Support the Registration of Zidovudine in the United States

Acute Toxicology
- Intravenous study in mice
- Intravenous study in rats

Subchronic Toxicology
- 2-week intravenous study in dogs with 2-week recovery
- 2-week oral study in rats
- 2-week oral study in dogs
- 2-week oral study in monkeys
- 1-month intravenous study in rats with 2-week recovery
- 3-month oral study in rats with 2-week recovery
- 3-month oral study in monkeys with 6-week recovery
- 6-month oral study in rats with 2-month recovery

Reproductive and Developmental Toxicology
- Segment II oral teratology in rats
- Segment II oral teratology in rabbits

Genetic Toxicology
- Mouse lymphoma assay
- Ames *Salmonella* test with/without metabolic activation
- Cell transformation assay
- In vivo cytogenetic study in rats
- In vitro cytogenetic study in human lymphocytes

Summary of Toxicology Studies Planned or in Progress at the Time of Initial FDA Review of Zidovudine
- 6-month oral study in monkeys
- Segment I oral reproduction/fertility study in rats
- Segment III oral peri- and postnatal study in rats
- Segment II oral teratology study in rabbits
- 1-year oral study in rats
- 1-year oral study in monkeys
- Oral carcinogenicity study in rats
- Oral carcinogenicity study in mice

supporting 6-month *nonclinical* toxicity studies have not yet been submitted" [emphasis added]. An approvable letter issued by the FDA in March of 1987, less than 4 months following submission of the NDA, stipulated the timing for the conduct of these outstanding studies. Comprehensive reviews of the acute, subchronic, chronic, genetic, carcinogenic, reproductive, and developmental toxicity studies have been published [12,13,118].

Zidovudine demonstrated relatively low acute toxicity with intravenous median lethal doses (MLDs) of greater than 750 mg/kg in rats and mice. The most consistent findings in the subchronic and chronic studies with rats, dogs, and monkeys were effects on hematologic parameters. In rats given two divided doses of zidovudine at approximately 50, 150, or 500 mg/kg/day orally for 3 or 6 months, reversible decreases were observed in red blood cell counts and hemoglobin concentration, primarily in the mid- and high-dose groups. The severity of these effects appeared to progress slightly between 3 and 6 months of treatment. No remarkable histopathology was noted in these studies. A subsequent 1-year study, submitted well following the initial approval of zidovudine, revealed a toxicity profile similar to that observed in the 3- and 6-month studies. The severity of anemia did not progress between 6 and 12 months of exposure, and effects were again reversible following discontinuation of treatment.

Dogs were more sensitive to zidovudine treatment. In a 2-week study in which animals were administered 125–500 mg/kg/day orally in divided doses, leukopenia, thrombocytopenia, and decreases in erythroid values were observed at all dose levels. Cytostatic effects were observed in the small intestine at the high dose and were also evidenced by slight-to-moderate non-dose-related lymphoid depletion and mild-to-marked dose-related bone marrow hypocellularity at all dose levels. No cytostatic effects were observed at similar or higher doses in either rats or monkeys. Studies revealed that zidovudine was metabolized almost identically in monkeys and humans, and as a result, the continued nonclinical development of the drug was conducted in the monkey rather than the dog. The species differences in metabolism were not of sufficient magnitude, however, to account for the much greater sensitivity of the dog to zidovudine, and the design of subsequent nonclinical and clinical studies continued to respect the significant findings in this species.

Monkeys responded to a 2-week treatment at divided doses of 125–500 mg/kg/day with a slight reduction in hemoglobin concentrations in one animal at the low dose and in both monkeys given the high dose. In a 3-month monkey study, at divided doses of 35–300 mg/kg/day, dose-related decreases in erythron parameters were noted as early as day 15 of treatment and progressed to live-phase termination. Platelet counts were also increased. Values returned to normal during the 6-week recovery phase of the study. Subsequent (postapproval) 6- and 12-month studies were conducted in the monkey. In addition to the findings in the 3-month study, bone marrow cytology revealed changes consistent with the hematology findings, and marginal decreases in white blood cell counts were observed at the 300 mg/kg/day dose. All findings were reversible.

Teratology studies were also carried out in rats and rabbits prior to approval of zidovudine. Divided doses up to 500 mg/kg/day resulted in no evidence for teratogenicity in either species, but non-treatment-related low fertility rates and mortalities in the rabbit study prompted the FDA to request that a second study be initiated prior to drug approval. Effects were limited to an increase in fetal resorptions and an associated decrease in fetal body weights at the maternally toxic high dose. The potential use of zidovudine in pregnant women to inhibit transplacental HIV transmission prompted additional in vitro and in vivo reproductive and developmental toxicity studies following the initial approval of the compound. These subsequent studies demonstrated that zidovudine is embryotoxic in rats at doses that are not overtly maternally toxic. The postnatal survival, growth, and development of offspring from zidovudine-treated rats were unaffected following several treatment regimens. In general, exposure levels

associated with the effects observed in the reproductive and developmental toxicity studies were significantly higher than those observed clinically.

No evidence for mutagenicity by zidovudine was observed in the Ames *Salmonella* study either with or without mammalian metabolic activation. The compound was weakly mutagenic in the mouse lymphoma assay without metabolic transformation at concentrations of 4000 and 5000 μg/mL; it was also weakly mutagenic with metabolic activation at concentrations greater than or equal to 1000 μg/mL. A positive response was obtained in the mammalian cell transformation assay at concentrations of 0.5 μg/mL or greater. In an in vitro cytogenetic assay in human lymphocytes, zidovudine caused structural chromosomal abnormalities at concentrations equal to or greater than 3 μg/mL; however, in an in vivo rat assay, no chromosomal abnormalities were noted following the intravenous administration of doses up to 300 mg/kg (plasma levels over 400 μg/mL). Subsequent in vivo micronucleus studies in mice and rats revealed dose-related increases in micronucleated erythrocytes, reflecting chromosome breakage or mitotic spindle damage.

Carcinogenicity studies in mice were initiated using single-daily doses of 30, 60, or 120 mg/kg. These doses were reduced to 20, 30, or 40 mg/kg at 3 months of treatment due to treatment-related anemia. Rats were dosed with 80, 220, or 600 mg/kg/day, with the high dose being reduced to 450 then 300 mg/kg. As expected, hematologic changes were observed, but no deaths or morbidities occurred that were considered treatment related in either study. In the mouse study, one benign vaginal neoplasm occurred at 30 mg/kg, and five malignant and two benign neoplasms occurred at 40 mg/kg. Two vaginal neoplasms occurred at the high dose of the rat study. In both cases, the tumors were late occurring and nonmetastasizing. An eloquent argument was put forth suggesting that these vaginal tumors resulted from high local exposure to zidovudine due to the retrograde flow of urine containing high levels of the excreted compound into the vagina. An additional lifetime mouse study to support this hypothesis was conducted in which animals were administered zidovudine intravaginally. Thirteen vaginal squamous cell carcinomas occurred in animals receiving the highest concentration in that study, supporting the contention that systemic exposure to the drug was unlikely to be responsible for the neoplasia observed in the oral studies.

A variety of adverse reactions have been documented in patients receiving zidovudine. Due to the wide range of symptoms associated with the opportunistic infections seen in patients with autoimmune deficiency syndrome (AIDS), it is difficult to assess which adverse reactions are clearly the result of zidovudine therapy. The animal safety studies, however, were highly predictive of the major hematologic toxicities of zidovudine described in humans: granulocytopenia and severe anemia. Similar to the earlier discussion regarding the development of omeprazole, additional mechanistic studies were conducted with zidovudine to explain toxicity findings, even though the drug was intended for the treatment of a potentially fatal disease.

Although the toxicology support package for zidovudine ultimately responded to existing guidelines for registration of a chronic-use pharmaceutical in the United States, its development history demonstrates that the approval system allows considerable flexibility in cases where the market for a life-threatening disease is clearly not satisfied (i.e., antivirals, antifungals, oncolytics). This type of development strategy can only occur with close collaboration between the submitter and regulatory agency and after careful consideration of the risk/benefit assessments.

BIOLOGICAL ENTITIES

Specific Agents

The development of highly purified species-specific protein pharmaceutical agents, made possible through advances in rDNA technology, presents a significant challenge to toxicologists. The major question raised is "What nonclinical toxicology evaluations should be conducted to ensure safety in human clinical trials?" The major issue is the testing of these specific proteins in nonhomologous animal species, where the possibility of immunogenicity, not applicable to the clinical trial, exists [219]. Decisions on appropriate nonclinical study designs for NBEs are generally made on a case-by-case basis. The general consensus that nonclinical toxicity evaluations with species-specific proteins reveal little more than enhanced pharmacodynamic activity rather than predicting the potential for adverse effects needs constant reevaluation. Furthermore, the toxicity observed in animal studies may be the result of an immunologic response to the foreign protein. Toxicology studies with NBEs should demonstrate that the product has no adverse effects other than those specifically related to pharmacodynamics and that safety for the expected clinical dose range, rather than exaggerated toxicity (MTD), should be demonstrated [19,186,219]. The approach to nonclinical safety evaluation of biopharmaceuticals has been extensively reviewed [38].

Regulatory agencies have placed great emphasis on chemical characterization of the NBE as a means of establishing that it is identical with the naturally occurring protein (manufacturing contaminant issues aside). Establishing this identity has allowed for appropriate modification of toxicology requirements and abbreviation of the toxicology support package; however, NBEs are being developed that contain amino acid sequences that have been purposefully manipulated to differ from the naturally occurring protein to result, for example, in a prolonged duration of action over the naturally occurring agent. These molecules may require a more comprehensive toxicology package, such as those established for NCEs (see preceding text).

Safety testing of NBEs can be presented in three categories (Table 8.10). The reasonably clear-cut time sequence of nonclinical and clinical studies established with NCEs is not often feasible with NBEs. The interactions between toxicologists and clinicians are important in addressing suspected adverse reactions in the clinical trials [19]. Nonclinical

TABLE 8.10
Safety Testing of Biotechnology Products

Category	Requirements
1	Identity, purity, pharmacology, safety pharmacology
2	Category 1, plus detailed pharmacologic activity (human, animal), relationship of plasma concentration and antibody titer (human, animal, in vitro), tolerance, and selected toxicologic testing
3	Categories 1 and 2, plus studies guided by indication and studies guided by duration of treatment

Source: Bass, R. and Scheibner, E., *Arch. Toxicol*., 11(Suppl.), 182, 1987.

toxicology evaluations of NBEs should be designed according to the risks anticipated from the type of product, the contaminant profile, and the intended clinical use [19]. Major questions and differences of opinion will continue to exist relative to the evolution of nonclinical toxicology testing strategies of NBEs. The major questions will arise concerning appropriate species [74,219], the need to conduct genetic toxicology studies [14,209], the conduct of reproductive toxicology assessments [219], and the need for classical carcinogenicity studies. As examples of NBEs, we have chosen to discuss toxicology support for the registration of the GnRH analogs, IFN, and human insulin. The development of IFN has provided a great deal of guidance for nonclinical toxicity testing of NBEs. The pharmacologic effects of insulin are well known from extensive clinical experience. This experience has aided the relatively rapid approval of rDNA insulin products and has allowed the chemical characterization of test material to play a major role in supporting a more limited toxicology profile. Human insulin, therefore, provides an example of an NBE that was approved rapidly, in approximately 5 months [158] (insulin was approved through FDA CDER division, not CBER).

The ICH S6 guidance [147] addresses the international consensus on safety testing for biotechnology-derived pharmaceuticals. It is expected that the product used in the outlined pharmacology and toxicology evaluations is comparable to the product proposed for the initial clinical studies. The evaluations are also expected to be conducted in relevant animal models, for example, models in which the test material is pharmacologically active. The options around relevant test species [41] and the use of either one or two species are discussed in the published guidance [147]. The guidance confirms the generally held opinion that conventional toxicology approaches are generally not appropriate for biopharmaceuticals; options and alternatives around carcinogenicity, mutagenicity, reproductive toxicity, safety pharmacology, etc., are discussed in the guidance [147]. In developing plans for evaluation of biopharmaceuticals, it must be remembered that not all studies used to define safety will be able to comply with GLP guidance. The application of GRPs, in those cases, with attention to documentation and reproducibility/reconstruction of a study is necessary.

The use of homologous proteins is a potential option when no relevant species is identified. This approach has the ability to identify hazards but is not generally useful for quantitative risk assessment and is generally of little value in assessing the potential for carcinogenicity, should that be part of the development plan [147].

Gonadotropin-Releasing Hormone Analogs

GnRH analogs are either agonists or antagonists of the receptor for the naturally occurring hypothalamic decapeptide. The chemical modifications either increase the biological activity and duration of action or affect the solubility, potency, and kinetics of the molecule. GnRH analogs were first introduced for the treatment of cancer (e.g., prostatic carcinoma), and their toxicologic assessment was less complete than usually recommended for new drugs. Since their introduction, the use of GnRH analogs has expanded into treatment of non-life-threatening conditions, and they now are expected to have to undergo the same rigorous toxicology evaluation as other new drugs [192]. In the case of GnRH analogs, the FDA has allowed the multiple-dose toxicity studies to be conducted at a multiple of human exposure (30- to 50-fold) rather than at doses that define the toxic limits of the compound. Due to the chronic nature of therapy and the chemical dissimilarity with native GnRH, the FDA has recommended that both rat and mouse carcinogenicity studies be conducted. As is the case with multiple-dose toxicity studies, the FDA has allowed that the MTD need not be used but some multiple of the human clinical exposure must be used to set the top dose in the carcinogenicity studies (e.g., 15- to 50-fold). The full toxicity profile recommended for GnRH analogs includes single-dose acute toxicity (rodent and nonrodent), repeated-dose toxicity studies through 6 months in rodents and 9 months in nonrodents, genetic toxicology, developmental toxicology, and carcinogenicity [192].

Interferon

IFNs are classified as IFN-α (leukocyte), IFN-β (fibroblast), or IFN-γ (immune). IFN-α consists of a family of at least 14 highly homologous species. The amino acid sequence homology of the IFN-α subtypes has been reported to be 52%–75% [210,211,224]. The biologic activities of IFNs include antiviral, anticellular, and immunomodulatory activities [224]. The properties of IFNs and their potential uses have been reviewed by Bocci and Trotta [21,223].

The adverse clinical experiences reported with the use of IFNs include fever, chill/rigor, headache, tremor, nausea, vomiting, myalgia, anxiety, fatigue, malaise, anorexia, confusion, local inflammation, cardiovascular toxicity, hepatotoxicity, and abnormal electroencephalograms (EEGs) [96,202,203,210,230]. The most commonly reported adverse effects are fever, fatigue, and leukopenia [210]. The effects that cause the most distress in clinical subjects are those related to CNS depression [230]. The toxicity seen with very pure and single-clone IFN preparations is almost identical to that reported for the less pure, more heterogeneous preparations of IFNs. The responses reported, particularly the influenza-like

syndrome, therefore, are likely intrinsically related to IFNs and not to a contaminant or impurity [210,230].

The species specificity of highly purified human IFNs implies that classical animal (nonhomologous) efficacy and toxicity models are not applicable in evaluation of these materials. Nonclinical safety testing of IFNs has not identified an appropriate animal model [100,127,193,199,210,253], supporting the recommendation that the routine safety tests applied to NCEs should not be applied haphazardly [205] to NBEs. Yet, given the traditional significance and predictive nature of nonclinical toxicology evaluations and acknowledging the lack of generally accepted and validated nonclinical animal models for the testing of these entities, drug regulatory agencies have published safety testing guidelines that place NBEs on a level with conventional drugs relative to the comprehensive requirements for animal safety studies [103,150]. A representative example of these guidelines and requirements is given in Table 8.11.

IFN-α2a (Roferon®-A) is a commercially available NBE identical to 1 of the 15 subtypes of human leukocyte IFN [224,225]. At the time that clinical trials were initiated with this drug, considerable clinical data were available from studies with other leukocyte IFNs to indicate the types of adverse reactions, described earlier, that might be expected [210,229]. The species specificity of recombinant IFN-α2a has led to production of neutralizing antibodies in rodent and nonrodent species [224]. This has impaired the ability of toxicology studies to detect the expected adverse clinical signs in common toxicology species.

Acute, single-dose toxicology studies (Table 8.12) were conducted in a variety of species in an attempt to disclose any unexpected acute toxicity related to the clinical dosage form (excipients, active ingredients). No mortalities were noted in the species tested. The LD_{50} of IFN-α2a was determined to be >22.8 × 106 units/kg i.v. These studies were conducted at multiples of a single clinical dose (3×10^6 units/kg), ranging from 10- to 167-fold. Multiple-dose toxicology studies were conducted over a range of 5–26 weeks at 3- to 78-fold the weekly clinical dose (9×10^6 units/kg) (Table 8.13). A low frequency of treatment-related adverse findings was reported: slight weight loss in rats; a slight, reversible increase in platelets and total leukocytes in mice; a slight decrease in hemoglobin and hematocrit in squirrel monkeys; dose-dependent anorexia and weight loss in *Macaca mulatta*; and transient anorexia in *Macaca fascicularis*. In studies longer than 2 weeks, neutralizing antibodies developed in rabbits, guinea pigs, and *M. fascicularis* [216]. These results were expected and may have affected the signs of toxicity. Reproductive studies carried out in *M. mulatta* indicated that a dose-dependent increase in abortion was related to the administration of IFN-α2a.

TABLE 8.11
Recommendations for Interferon Testing by the French Ministry of Social Affairs

Toxicologic Test	Recommendation
Acute	Two species, both sexes in one species; two routes; 2-week observation
Subchronic	Two species, rodent and primate; 3 months of daily injections
Reproduction	Segments I, II, and III
Mutagenicity	In vivo and in vitro clastogeneses
Carcinogenicity	Not required
Pyrogenicity	Rabbit
Safety pharmacology	In vivo cardiopulmonary, isolated organs; neurobehavioral studies
Cell culture	Cytostatic and cytotoxic effects

Source: Wolf, F.J., *J. Environ. Pathol. Toxicol.*, 3, 113, 1980.

TABLE 8.12
Acute Toxicology Studies Conducted with Interferon-2α

Species	Route[a]	Dose (Units × 10^6/kg)	Clinical Multiple[b]
Mouse	i.v.	30,250	83×
	i.m.	30,500	167×
	s.c.	30	10×
Rat	i.v. and i.m.	30,100	≤33×
	s.c.	30	10×
Rabbit	i.v. and i.m.	100	33×
	i.m. and s.c.	30	10×

[a] i.v., intravenous; i.m., intramuscular; s.c., subcutaneous.

[b] Recommended clinical dose = 3×10^6 units/kg i.m. or s.c., three times weekly.

Insulin

The nonclinical toxicity of BHI was evaluated in an unconventional way. The use of graded increments of dose representing multiples of the projected clinical exposure was not feasible due to the pharmacologic effect of hypoglycemia caused by the insulin molecule. Because the pharmacologic effects of insulin were well known, a primary goal of the

TABLE 8.13
Multiple-Dose Toxicology Studies Conducted with Interferon-2α

Species	Route[a]	Duration (Weeks)	Dose (Units × 10^6/kg)	Clinical Multiple[b]
Mouse	i.m.	5	0,1.4,2.8,5.7	≤4×
Rat	i.m. and i.v.	5	0,1,10,100	≤78×
Rat	i.m.	26	0,7.5,15,30	≤23×
Saimiri sciureus	i.m.	2	0,2.5	2×
M. mulatta	i.m.	4	0,2.5,10,25	≤19×
M. fascicularis	i.m.	13	0,2,10	≤3×

[a] i.v., intravenous; i.m., intramuscular.

[b] Recommended clinical dose = 3×10^6 units/kg three times weekly (9 × 106 units/kg/week).

TABLE 8.14
Acute Toxicology Studies Conducted with Biosynthetic Human Insulin

Species	Route[a]	Dose (Units/kg)	Clinical Multiple[b]
Mouse	s.c.	10	40×
Rat	s.c.	10	40×
Dog	s.c.	2	8×
Monkey	i.v.	0.1	—

[a] s.c., subcutaneous; i.v., intravenous.
[b] Anticipated clinical dose = 0.24 units/kg/day, s.c.

toxicology evaluations was to determine whether BHI contained potentially toxic contaminants or impurities (e.g., *Escherichia coli* proteins, endotoxins) that are introduced as a result of the synthetic process. Toxicology studies on BHI were conducted simultaneously with purified porcine pancreatic insulin (PPI) as a positive control, at doses previously established to produce hypoglycemia but not mortality. The doses selected for toxicology studies were varied according to species sensitivity, route of administration, and duration of treatment. In acute, single-dose toxicity studies (Table 8.14), the minimal lethal dose of BHI to rats and mice was >10 units/kg s.c. Dogs given single doses of 2 units/kg BHI s.c. showed the expected hypoglycemia, but no toxicity. No toxic effects were seen in either rats or dogs given BHI s.c. or i.v. for 1 month (Table 8.15). Chronic toxicity, reproductive toxicity, and carcinogenicity studies were not conducted due to the extensive clinical experience with animal-derived insulin and the extensive chemical analysis of the NBE, establishing its identical nature with natural human insulin. BHI was negative in a genetic toxicology screen composed of bacterial mutation, DNA repair, and sister chromatid exchange evaluations. BHI was also not pyrogenic. Overall, BHI did not induce any effects different from those induced by PPI, and all effects seen were extensions of known insulin pharmacology. Investigations demonstrating the virtual absence of endogenous *E. coli* proteins and the absence of antigenic response in rats and guinea pigs sensitized with *E. coli* polypeptides further addressed the safety of the rDNA-derived human insulin product.

TABLE 8.15
Multiple-Dose Toxicology Studies Conducted with Biosynthetic Human Insulin

Species	Route[a]	Duration (Weeks)	Dose (Units/kg)	Clinical Multiple[b]
Rat	s.c.	4	2.4	10×
Dog	s.c.	4	2.0	8×
Dog	i.v.	4	0.1	—

[a] s.c., subcutaneous; i.v., intravenous.
[b] Anticipated clinical dose = 0.24 units/kg/day, s.c.

SPECIAL ISSUES

No-Observed-Adverse-Effect Level

The NOAEL is an important concept in the evaluation of potentially toxic agents [30,162]. The use of the NOAEL in the development of pharmaceuticals has been reviewed by Dorato and Engelhardt [78]. The NOAEL for pharmaceuticals may be defined as the "highest dose/exposure that does not cause important increases in the frequency or severity of adverse effects between exposed and control groups based on careful biological and statistical analysis. While minimum toxic effects or pharmacodynamic responses may be observed at this dose, they are not considered to be adverse to human health or as precursors to serious adverse events with continued duration of exposure" [78]. This approach fits very well with the ICH position that the effect to be determined is the toxicologically relevant effect; that is, the effect that may endanger human health [131].

In addition to the more traditional approach to NOAEL determination, alternatives have been discussed. The benchmark dose approach claims to be a more powerful statistical tool than traditional NOAEL approaches [98]. The term *hormesis* may be considered to describe a response that is stimulatory at low doses and inhibitory at high doses; however, no universally accepted definition of hormesis relative to its use in safety assessment exists. Hormesis theory has been proposed as a method to improve toxicology risk assessment [28,29,31,32]. As a perspective, Axelrod et al. [11] have presented an argument that the existing toxicology data do not support a universal extension of the hormesis concept to regulatory policy. The last 40 years of drug safety evaluations support the more traditional NOAEL methodology as an appropriate approach to risk/benefit for pharmaceuticals. Toxicologists should be open, however, to alternative approaches and challenges to established practices.

Immunotoxicology

Immunotoxicology can be defined as the discipline concerned with the study of adverse effects on the immune system as a result of exposure to xenobiotics [68]. The development of immunotoxicology, since the 1970s, has been reviewed by Koller [155]. It is not the purpose of this section to review in detail the specific evaluations conducted to define immunotoxicity [67,68,221,242] but to discuss the use of these evaluations in a hazard assessment tier approach. Adverse responses of the immune system are known to occur secondary to malnutrition, radiation exposure, neonatal thymectomy, and exposure to certain drugs and chemicals [71,228]. Historically, few chemicals have been shown to be immunosuppressive in toxicology evaluations, probably because the lymphoid organs and the immune system, in general, have been poorly examined. It would be desirable, therefore, to establish an effective tier approach to detect immunotoxicity in standard toxicology studies and also to evaluate the functional nature of the changes observed as a result of drug exposure. It is presumed that a functional change detected in the immune system is predictive of adverse health effects [242]. It must be remembered that a critical function

must be depressed beyond a defined, minimal point (reserve capacity) to indicate a health risk [169]. The tier approach is encouraged because it more carefully directs the use of resources, and a single immune function assay may not comprehensively characterize the myriad of potential toxic effects on the immune system [68]. Specific immune function tests for increasing the capability of toxicology studies to reveal effects on lymphoid tissue and to evaluate more fully the risk of chemical exposure by determining the functional significance of the responses observed have been reviewed [67]. It is known that acute and chronic effects of drug exposure on the immune system can result in three principal undesirable effects: immunosuppression or enhancement, autoimmunity, and allergic reactions [169]. Standard toxicology studies can be used to detect potential immunosuppression through the evaluation of hematologic changes, immune system organ weights and histology, changes in serum globulins, increased incidence of infections, and increased occurrence of tumors in the absence of other explanations [141]. This fits with the outcome of an early consensus meeting held by the National Institute of Environmental Health Sciences (1979) that resulted in the development of a list of relevant immunologic parameters for evaluating chemically induced immunotoxicity. This immunology screening panel has been reviewed [67] and includes pathotoxicology, hematology, host resistance, radiometric delayed hypersensitivity, lymphoproliferation, humoral immunity, and evaluation of bone marrow progenitor cells.

One of the first guidelines for immunotoxicology testing was that developed by the EU in the late 1970s. The focus of these guidelines was to evaluate the potential risk of chemical exposure by evaluating the functional significance of any histopathologic or hematologic effects seen on lymphoid organs in routine toxicity studies [182]. The intention was to pursue the significance of these effects with specific function tests as necessary. It is known that immunotoxicity following drug exposure may take the form of changes in lymphoid tissue organ weights or histology or changes in bone marrow or peripheral leukocytes [68]. Norbury [182], however, pointed out that the evaluation of drug effects on the immune system is related to immune responsiveness and is not simply a single-point examination of lymphoid tissue using histopathology and hematology. Histopathologic changes are generally not believed to be sensitive indicators of drug-induced immunotoxicity, are seen only at fairly high dose levels, and do not necessarily equate with functional immune alterations [68,182,242]. The route and time of exposure relative to the maturational development of the immune system are important considerations in designing an immunotoxicity protocol [39,69].

The application of nonspecific immunotherapy for bacterial and viral diseases has led to an increased level of importance in the determination of immunotoxic effects. The standard toxicology studies that form the basis of toxicologic evaluations should be complemented with specific evaluations useful in determining functional effects on the immune system, especially if the agent in question is a known immune modulator [141]. Immune system function results from a balance of the activities of various cellular components and their

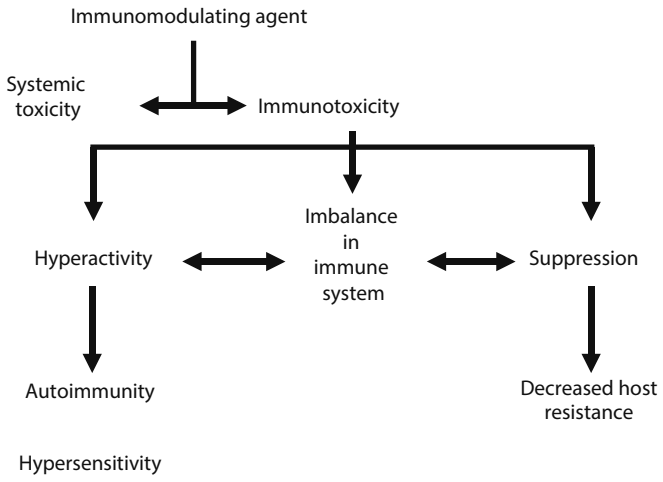

FIGURE 8.9 Potential toxic responses of immunomodulating agents. (Adapted from Falchetti, R. et al., in *Current Problems in Drug Toxicology*, Zbinden, G. et al., eds., John Libbey Euro-text, London, U.K., pp. 248–263, 1983.) Toxicity to the immune system encompasses a variety of adverse effects, including suppression or enhancement of immune response. (From ICH, Immunotoxicology studies for human pharmaceuticals, Topic S8, Step 5, ICH Harmonized Tripartite Guideline, *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*, Geneva, Switzerland, 2005.)

soluble factors [195], and an alteration in any factor could result in an imbalance of the entire system [82]. The effects of immunomodulating agents, therefore, could result in either enhancement (e.g., hypersensitivity, autoimmunity) or suppression (e.g., decreased host resistance) (Figure 8.9).

Several tier approaches to immunotoxicity testing have been proposed [66,169]; for example, the National Toxicology Program (NTP) has proposed an immunotoxicology testing strategy that includes a limited number of functional and host resistance assays [66]. The two-tier approach consists of a screen (tier 1), which represents a limited effort that includes the assessment of cell-mediated immunity, humoral immunity, and immunopathology. Tier 1 provides little information on the specificity of an observed immune defect or its relevance to the host; however, it can detect an immune alteration resulting from drug exposure (163). Tier 2 represents an in-depth evaluation, initiated only if functional changes are seen in tier 1 at otherwise nontoxic doses [169]. The in-depth immune function and host resistance evaluations provide information on the mechanisms of the immunotoxicity and aid risk assessment. Luster et al. [169] have reported that no compound evaluated to date has been found to produce an effect in tier 2 without demonstrating some effect in tier 1. The NTP procedure for detection of immune alterations following chemical or drug exposure in rodents is shown in Table 8.16. The concept of performing functional tests is critical to defining potential mechanisms of the toxic response and their applicability to humans. An international collaborative effort has focused on the evaluation of limited pathology or enhanced pathology evaluations to better understand potential immunotoxicity [208,220]. The enhanced pathology

TABLE 8.16
NTP Immunotoxicology Procedure

Immunopathology	Hematology (complete and differential blood count)	Immunopathology	Quantitation of splenic B and T cells
	Organ weights (spleen, thymus, kidney, liver)	Humoral-mediated immunity	IgG response to sheep RBCs
	Body weight	Cell-mediated immunity	Delayed hypersensitivity
	Cellularity (spleen)	Nonspecific immunity	Macrophage function
	Histology (spleen, thymus, lymph node)	Host resistance	Syngenic tumor cells (tumor incidence)
Humoral-mediated immunity	Plaque-forming cells		*Listeria monocytogenes* (mortality)
Cell-mediated immunity	Lymphocyte response to mitogens		Influenza (mortality)
Nonspecific immunity	Natural killer cell activity		*Plasmodium yoelii* (parasitemia)

approach (e.g., weight determination, examination of additional lymphoid organs, grading of changes in the principal compartments of lymphoid tissue) was determined to provide an advantage in revealing effects on the immune system.

The direction for pharmaceutical development is to include tests of potential immune system involvement in the traditional toxicologic evaluations for subchronic and chronic toxicity. Due to the sensitivity of the immune system to toxicants that could adversely affect the critical balance of the various immune factors and the adverse health effects that could ensue, it is extremely important to define any potential interaction of a new drug and immune system function [242].

As can be anticipated from the previous discussion, immunogenicity is a major scientific issue relative to the development of biotechnology products. Concern has been raised over comparison of the recombinant protein and the naturally occurring protein, as animal models are thought to be inadequate to assess the chemically subtle, but potentially immunologically significant, differences in the human response to these molecules. It has been assumed that a recombinant protein, designed for human use, would produce a number of adverse effects (e.g., the production of neutralizing antibodies) in experimental animals. It has now become clear, through chronic exposures in nonclinical studies, that some low molecular weight human proteins are not immunogenic in animals or are only weakly so. They have also been observed not to produce neutralizing antibodies. In the case where antibodies to human proteins have been detected in nonclinical studies, they do not necessarily cause expected immunopathology or neutralization activity. The rhesus monkey has been shown to predict the relative immunogenicity of several recombinant proteins in humans [254] and may serve as a good model.

A further question is, "Should all rDNA products be routinely screened in animals prior to their introduction into humans?" The major reason for conducting immunotoxicity evaluations in experimental animals is to detect those compounds that could induce anaphylaxis or anaphylactoid reactions in humans (Wierda, D. (2006): Personal communication. Eli Lilly and Company, Greenfield, IN.). New molecules, previously minimally tested in animals, such as enkephalins, would have a greater potential risk than well-known molecules, such as insulin. An approach to testing recombinant proteins as well as NCEs for immunogenicity or antigenicity has been suggested based on the extent of the clinical database and the existing regulatory requirements (Figure 8.10). Although studies in animals seem

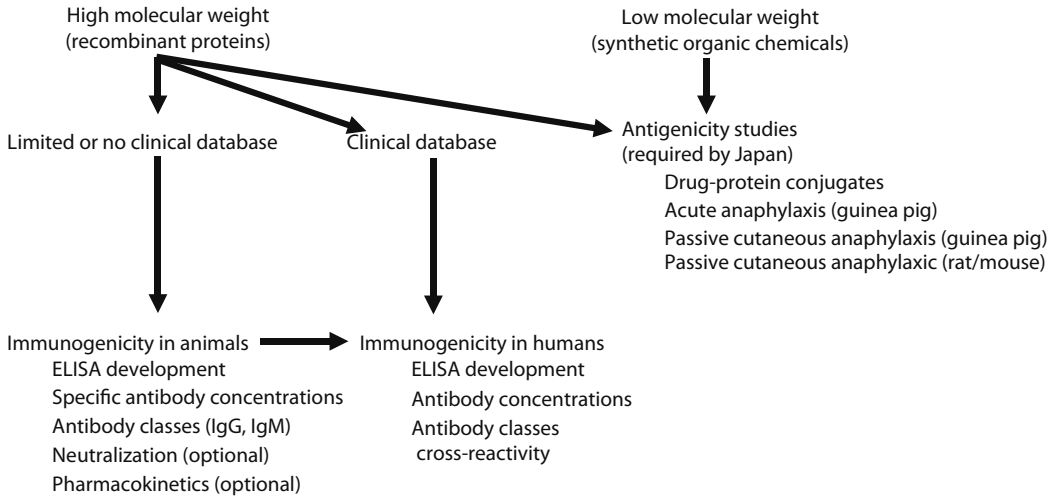

FIGURE 8.10 Proposed approach for addressing immunogenicity and antigenicity issues with established and novel biotechnology products and NCEs. (From Wierda, D., Personal communication, Eli Lilly and Company, Greenfield, IN, 1992.)

well justified for poorly characterized chemicals, it remains an open question whether or not regulatory agencies will accept an existing extensive clinical database as justification for not performing immunogenicity evaluations. Again, the chemical characterization of the recombinant product relative to the natural material will have some bearing on this debate.

The importance of defining the potential interaction between a new drug and the immune system is emphasized in the new regulatory approaches addressing immunotoxicology. The FDA has issued a guidance for industry [91] that identifies five major areas of immunotoxicology:

- Immunosuppression (decreased immune function)
- Immunogenicity (immune reaction to drugs)
- Hypersensitivity (immunological sensitization)
- Autoimmunity (immune reaction to self-antigens)
- Adverse immunostimulation (antigen-nonspecific uncontrolled activation of the immune system)

European Medicines Agency (EMA) has also published a position on immunotoxicology [81]. The major difference between the guidance documents is the mandatory requirement for functional testing in the EMA guidance [65]. The FDA guidance [91] supports a weight-of-evidence approach involving standard tests for each potential new drug [129]. Despite the new guideline approaches, histopathologic evaluation of lymphoid organs and tissues has been important in identifying potential immunotoxicology [157].

The ICH has agreed to harmonize the immunotoxicology guidance among the United States, EU, and Japan (Table 8.6). They have published a guidance on a weight-of-evidence decision-making approach to address nonclinical testing for immunosuppression induced by low molecular weight drugs; the guidance does not apply to biotechnology-derived pharmaceuticals [141]. For the purpose of the guideline, immunotoxicity is defined as unintended immunosuppression or enhancement. Although the guidance does not specify how each immunotoxicity study is performed, it does address the general approach to immunotoxicity testing for new and marketed drugs and provides a flow diagram for the application of recommended immunotoxicity evaluations. The guidance does make a strong connection with the use of the standard toxicity studies and to the use of in vivo pharmacology studies to provide information leading to a weight-of-evidence decision.

Genetic Toxicology

Genotoxicity has been defined as the ability of either a chemical or physical agent to damage DNA, resulting in a mutation [45]. An important element of toxicology is the early identification of potentially hazardous substances [200]. Because the actions of toxicants are ultimately exerted at the cellular level, isolated cell systems represent an important model for identifying toxic effects. In vitro assays allow a greater control over xenobiotic metabolism (e.g., addition of enzymes or inhibitors) and facilitate mechanistic studies that could not be performed in vivo [152]. In vitro tests generally provide a reasonable approximation of the potential for an agent to have an effect on genetic material; in vivo procedures provide a better test for the potential for genetic alterations to occur in the intact organism [154]. The short-term in vitro tests for genotoxicity, potentially predictive of in vivo carcinogenicity, are among the most important techniques available for the rapid determination of potential severe undesirable effects of compounds selected for development. They are also useful in the prioritization of compounds to be studied in the more extended and expensive in vivo toxicology studies. A number of assays are available to evaluate the potential for genotoxicity. The majority opinion is in favor of a battery approach to identify potential genotoxic activity because different assays assess different types of genetic damage and that no one assay is capable of detecting all genotoxic mechanisms relevant in tumorigenesis [152,201]. The ICH has published an updated guideline on how to conduct genotoxicity tests that includes the recommended standard genotoxicity testing battery for evaluations of pharmaceuticals [146]. The ICH test battery includes

- Gene mutation in bacteria to detect relevant genetic changes and the majority of genotoxic rodent carcinogens
- In vitro mammalian cell chromosomal aberration, the in vitro micronucleus assay, or in vitro mouse lymphoma thymidine kinase (TK) assay to detect chromosomal damage
- In vivo assessment of chromosomal effects that allows evaluation of additional relevant factors (e.g., absorption, distribution, metabolism, excretion), through the analysis of micronuclei in erythrocytes or chromosome aberrations in metaphase cells of the bone marrow

This battery may be expanded or modified when appropriate, such as when compounds with structural alerts are negative in the three standard tests or replacing an in vitro mammalian cell assay with the well conducted in vivo assays [146].

Excellent reviews of methods to study the genotoxic potential and issues concerning nongenotoxic but carcinogenic chemicals are available [23,25,149,235,237]. In vivo exposure assays have the advantage of an intact metabolic system to effectively assay those compounds that must be activated (metabolized to a reactive entity) to achieve an effect. The in vitro assays may be conducted with or without the addition of a postmitochondrial supernatant fraction from livers of rats treated with polychlorinated biphenyls to maximally induce drug-metabolizing enzyme activities and thus enhance the detection of indirect-acting agents.

In vitro genotoxicity assays are often used in the early drug discovery process aimed at selecting drug candidates for further development. Often when positive genotoxicity results

emerge, in silico and in vitro screens are commonly used much earlier in the discovery process to guide the chemistry to molecules with no genotoxic risk. The definitive in vitro genotoxicity evaluations of mutation and chromosomal damage are generally submitted prior to the FHD. The complete battery of recommended tests should be submitted prior to phase II clinical development.

Conducting genotoxicity screens on NBEs has been an area of much discussion. The ICH has recognized that the standard genotoxicity testing battery is not applicable to NBEs and generally should not be conducted [147].

Safety Pharmacology

Safety pharmacology involves establishing the pharmacologic profile of new drug candidates by evaluating the pharmacodynamics related to the therapeutic indication and by evaluating the pharmacodynamics on other organ systems not related to the therapeutic indication [56]. These studies are usually conducted at doses well below those used to establish the toxicology profile. It is not necessary or even desirable to produce frank toxicity to establish a valid pharmacologic profile. By definition [139], pharmacology studies are divided into three categories: primary pharmacodynamics, secondary pharmacodynamics, and safety. The traditional approach to safety pharmacology is focused on the third category. However, integration of these techniques with the basic pharmacologic assessment of the primary pharmacodynamics (i.e., cardiovascular pharmacology) is essential in making good decisions about drug development. The inclusion of safety pharmacology approaches in general toxicology studies should be considered whenever possible; the impact of potentially large doses must be considered in the ability to detect the desired responses. The dose–response design of toxicology studies should provide a dose where the evaluation of safety pharmacology parameters is appropriate.

Pharmacologic profiling was initially focused on guiding the synthetic chemist in the discovery of new pharmacologically active chemicals, rather than on the detection of adverse drug effects in humans [247]. Historically, in reviewing the common adverse drug findings in humans, Zbinden [247] pointed out that some responses are easily detected in both humans and experimental animals (e.g., sedation, anorexia, body weight changes, tremor, tachycardia), and some responses are only detectable in humans (e.g., tinnitus, vertigo, nausea, headache). In any event, when one considers the nature of the functional disturbances encountered in both nonclinical and clinical testing, it becomes evident that pharmacologic profiling is critical to the safety evaluation of potential therapeutic agents [238,239]. Every chemical that enters the body has the potential for creating effects that may or may not be related to its pharmacologic activity. Antihistamines, for example, produce sedation, which is related to their desired pharmacologic effects, and anticholinergic responses, which are not [238]. Pharmacologic profiling can help identify the potential incidence of effects unrelated to the known pharmacologic activity. Pharmacologic profiling also provides crucial information for the selection of NCEs during the early discovery process, for the design of toxicology studies, and for the approach to safety monitoring appropriate for clinical trials [167,191]. Traditionally, it is the objective of safety pharmacology to identify risks associated with the pharmacologic characteristics of a particular agent (some would call this toxicology), essential at therapeutically relevant doses and potentially over time, if indicated. There is an advantage in the active integration of basic pharmacology and safety pharmacology approaches; they need to be conducted independently, but with active communication around the outcomes. In the basic pharmacology assessments, imaging technologies are used to provide longitudinal assessments of compound effects in many therapeutic areas. In cardiovascular pharmacology, the use of echocardiography (ultrasound) provides assessment of drug-induced changes in cardiac function and is used in both safety and efficacy studies to great advantage [126].

Again, from an historical perspective, the Japanese Ministry of Health Labor and Welfare (MHLW) had published guidelines for general pharmacology studies (the original reference for safety pharmacology studies) [5]. These studies are designed to characterize effects and potency and to determine mechanism. The guidelines include studies to determine effects on general activity and behavior, the CNS, the autonomic nervous system and smooth muscle, the respiratory and cardiovascular systems, the gastrointestinal tract, and renal excretion.

The ICH guidelines now include select pharmacology guidance (Table 8.6). ICH Topic S7A [139] addresses the definition, objectives, and scope of safety pharmacology studies, as well as studies required prior to phase I clinical trials and prior to marketing approval. The safety pharmacology core battery has been built based on previously existing regional draft guidances [44] and now includes the following vital organ systems:

- CNS
- Cardiovascular system
- Respiratory system

Follow-up studies are recommended to provide a greater depth of understanding of the effects observed in the core battery or from clinical trials on pharmacovigilance: renal/urinary system, autonomic nervous system, gastrointestinal system, dependency potential, skeletal muscle, and immune and endocrine functions; in vitro and in vivo approaches can be used in concert [139,142].

The EU Committee for Proprietary Medicinal Products (CPMP) has published a *Points to Consider* document on QT interval prolongation [55]. The impact of QT prolongation and related occurrence of torsades de pointes with the use of quinolones has been reviewed by Frothingham [105]. The ICH Topic S7B [142] now provides recommendations for nonclinical studies to address the potential for QT interval prolongation. It also provides guidance on integrated risk assessments. The prolongation of the QT interval is one of

the few single physiological responses that could end the development of a potential new pharmaceutical [117].

Measure of Exposure

The relationship of administered dose to toxicologic response is not always a simple correlation. Traditionally, the administered dose (mg/kg) has been the most commonly used expression to compare toxicologic responses between species. The value of the administered dose term as the most appropriate comparator with toxicologic response, however, has been questioned in scientific and regulatory circles. It is becoming increasingly well recognized that both beneficial and toxic effects of therapeutic agents are dependent on the quantity of material reaching the target site [175,222,252]. This has led to in vivo exposure assessments, usually done by measurement of plasma or blood levels of the administered agent, being the most widely used and generally accepted approach to evaluate exposure. These measures also form the basis for any subsequent determinations of exposure multiples or safety margins.

Knowledge of the level of exposure is critical for understanding not only safety but also efficacy. Fully elucidating this knowledge base provides the most robust estimates of safety multiples. In its simplest representation, the safety multiple can be calculated by dividing the highest exposure observed without the presence of an adverse effect by the exposure needed to elicit efficacy. In practice, much more complexity is encountered in making this estimate. Variables such as exposure comparisons across species, the absence of exposure determinations at the target site of activity or toxicity, and nonlinearity of the dose/exposure–response complicate the assessment.

Measures of exposure are also useful in establishing nonlinearity in kinetics, which is important in explaining toxic responses seen in particular species [175]. It seems more rational to establish an upper dose in toxicology studies based on linearity of kinetics rather than at the MTD, as it is often the case that the MTD falls in the range of nonlinear kinetics, saturating normal metabolic processes. Thus, an animal treated at the MTD may be exposed to much higher levels of parent drug or toxic metabolites than would be observed at meaningful multiples of the clinical dose.

The exposure differences often observed across species can sometimes be explained by known differences in rates of absorption and/or metabolic clearance across species. Figure 8.11 illustrates the general relationship of clearance to body weight for dog, rat, and monkey. From this generic depiction, it is apparent how a lower administered dose on a milligram-per-kilogram basis produces a higher exposure in dog compared to rat. This relationship can be extrapolated further to humans, who, in general, would have even slower rates of clearance [179]. The relationship of administered dose to delivered dose remains a central issue in the interpretation of toxicology data. The measurement of plasma concentrations of parent compound and metabolites represents a partial resolution to this problem. There are limitations, however,

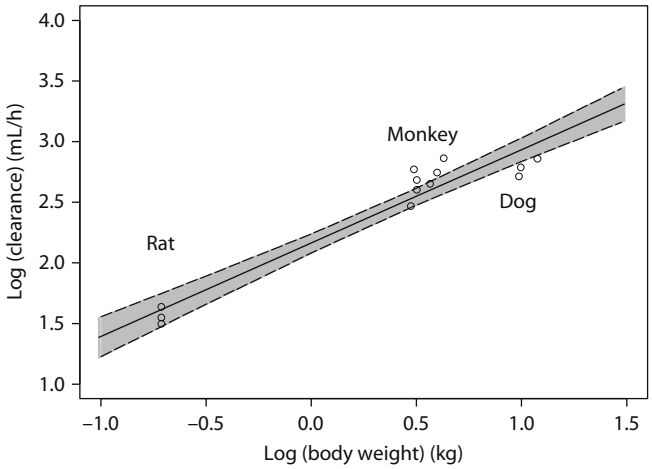

FIGURE 8.11 The general relationship of clearance to body weight for dog, rat, and monkey.

in using plasma concentration as a relevant measure of exposure for those compounds that are tissue sequestered [173]. It has been noted that, although many therapeutic agents achieve tissue levels proportional to plasma concentration, some continue to accumulate in tissue with continued dosing.

Levels of exposure of test compounds or their metabolites at sites of action can be assessed by measuring tissue kinetics. Lovastatin is an example of a compound that exerts its primary pharmacology and toxicity at the same site. In this case, the pharmacokinetics of both lovastatin and its active metabolite has been well characterized [123]. An increasingly more common method is the utilization of quantitative whole-body autoradiography. This technique uses radioactive drug (^{14}C or ^{3}H) to assess concentrations in tissues across the entire body (Figure 8.12). Concentration time profiles can be generated that subsequently can be used to derive various pharmacokinetic parameters. A shortcoming of this technique is its inability to distinguish parent compound from metabolite [42]. Perhaps the most critical aspect of the role of the toxicologist is to complete the multifaceted exposure comparisons, couple this knowledge with that of the observed effects of the test compound, and render an informed opinion of the overall safety profile of the test compound under study.

Clinical Trials in Pediatric Populations

The FDA has found that most products indicated for treatment of diseases that occur in both adults and children have little clinical trial support for pediatric use. As a result, a regulation requiring pediatric studies for certain NCEs and NBEs had been proposed and updated [90,95]. The CPMP had also concluded that specific age-dependent differences in pharmacokinetics, pharmacodynamics, growth process and development, and specific pathology require that therapeutic agents be tested in the target age group [54]. The European Medicines Agency (EMA) has published a guidance covering the conduct of juvenile animal studies in support of pediatric indications [80]. The ICH has recommended that pediatric

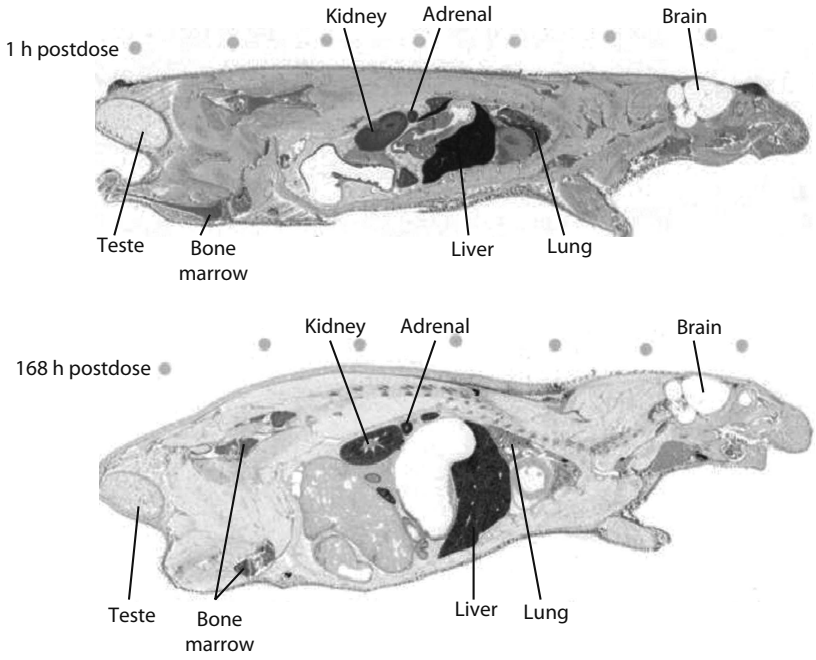

FIGURE 8.12 Phosphor images of whole-body sections from male Fischer 344 rats after receiving a single dose of [14C]-test compound.

clinical trials be supported by repeated-dose toxicity studies of an appropriate duration, all developmental toxicity studies, and the full battery of genotoxicity tests. These studies should be concluded before the pediatric clinical trials begin [145]. Due to the potentially long duration of treatment, carcinogenicity studies must be considered prior to the initiation of long-term pediatric clinical trials. The performance of nonclinical studies in juvenile animals may also be necessary if previous toxicology evaluations and human safety data are not sufficient or raise a cause for concern. In the relatively new guidance on nonclinical evaluation of anticancer drugs [144], it has been stated that the general approach to investigating anticancer drugs in pediatric patients is first to define a relatively safe dose in adult populations. This is the general approach that has existed for including pediatric populations for many years. For oncology indications specifically, juvenile animal toxicology studies are not usually conducted to support the inclusion of pediatric populations. Morford et al. [174] have reviewed the strategies, challenges, and current practice of providing nonclinical safety evaluations for inclusion of pediatric populations in the development of biopharmaceuticals. Pediatric safety assessments supported by nonclinical safety evaluations are now required for every NDA and Biologics License Application in the United States and for Marketing Authorization Applications in Europe [174].

Nonclinical Evaluation of Anticancer Drugs

As discussed earlier, the development of drugs for life-threatening diseases such as cancer and AIDS requires a modification of the approach established for the standard safety assessment of NCEs or NBEs. The treatment of cancer usually includes the use of potent agents designed to halt cell replication. The therapeutic index for these agents is often small. Due to the life-threatening nature of the disease, a greater tolerance of drug toxicity and a shorter nonclinical testing strategy are generally accepted for therapeutic agents in this class, as serious drug effects are often less threatening than the targeted disease; however, due to the greater intrinsic toxicity of the agents in this class, the early clinical trials are often conducted in patients rather than normal volunteers, as with other therapeutic agents.

The history of anticancer drug development has been extensively reviewed [70,119,198,222]. The basic approach for the development of anticancer drugs includes

- Establishment of a safe clinical trial entry dose
- Determination of potential dose-limiting target-organ toxicity
- Evaluation of reversibility of effects
- Determination of MTD in animals
- Determination of dose schedule toxicity

The use of nonclinical studies has been useful in accurately predicting MTDs in humans and safe starting doses for clinical trials [119]. The CPMP has provided a specific note for guidance on the nonclinical evaluation of anticancer agents [57]. Safety pharmacology is generally required prior to phase I studies, as are the determination of C_{max} (maximum plasma concentration of the drug) and AUC at the animal MTD. Other kinetic parameters are expected to be determined prior to phase II/III testing. Determination of the single-dose MTD in rodents and the approximate MTD in nonrodents, using a relevant route of exposure, is also expected prior to phase I. Repeated-dose toxicity studies in two rodent species

are expected prior to phase I clinical studies. Longer-term repeated-dose studies, in a rodent and nonrodent species, that are equal in duration to the clinical trial but less than 6 months are expected prior to phase II/III. Genotoxicity testing is not necessary prior to phase I/II, but the genotoxicity battery is expected to be conducted prior to phase II/III. Because cytotoxic anticancer agents are known to have an adverse effect on reproduction, developmental toxicity studies are not required but are encouraged. The development of anticancer drugs and other therapies for life-threatening diseases has unique characteristics based on the nature of the disease and the inherent toxicity of the therapeutic agents.

ICH guidance S9 [144] now addresses an international consensus on the nonclinical evaluation of pharmaceuticals for the treatment, but not for the prevention, of advanced cancer. The guidance [144] applies to both NCEs and NBEs. As stated earlier in the historical review, the development of therapy for advanced cancer requires a good deal of flexibility in the application of established nonclinical approaches. The nonclinical evaluation calls for the development of a nonclinical proof of principle as established by in vivo pharmacology studies of efficacy and in pharmacologic evaluations of effects on the heart, lungs, and CNSs. In the general toxicology approaches, the determination of the NOEL or NOAEL is not essential. The toxicology studies are designed to support the schedule of administration in the clinical trials and to help with an assessment of reversibility of (or recovery from) adverse events. Reproductive toxicology and genotoxicity evaluations are generally required for support of marketing. The study of carcinogenicity is not required. It is recommended that the reader review ICH S9 [144] for information on kinetics, combined therapies, pediatric populations, etc.

Alternative Methods for Carcinogenicity Determination

The testing for carcinogenic potential has relied primarily on the rodent bioassay [207]. Recently, through the ICH process, the rat has been identified as either the most acceptable or most relevant model for the 2-year bioassay. In addition to the rat bioassay, an alternative short-term method of carcinogenicity evaluation is recommended [144]. These approaches may include studies in transgenic mice (e.g., heterozygous $p53^{+/-}$ gene-deficient mouse or Tg.AC mouse) or the use of a neonatal rodent tumorigenicity model.

To evaluate and verify the available alternative models, the International Life Sciences Institute (ILSI) has initiated a collaborative effort among industry, academic, and government laboratories to study chosen chemicals in the alternative models and evaluate the results in light of the known bioassay data. Current information is insufficient to guide us in the choice of suitable alternative models for carcinogenicity evaluation. It is highly recommended that any deviation from the standard two species (mouse and rat) carcinogenicity bioassay be discussed with the FDA prior to implementation.

When fully validated, the proposed transgenic animal model may be used as follows [34]:

- To confirm results in equivocal 2-year rodent bioassays
- To set priorities for 2-year carcinogenicity bioassays
- As an alternative to the mouse 2-year bioassay, in conjunction with the rat 2-year bioassay
- To assess carcinogenic potential of new genotoxic contaminants or degradants in a drug product after 2-year bioassays are completed

In addition, the use of transgenic animals may support weight-of-evidence decisions; they are relatively short term and are generally less expensive than the 2-year rodent bioassay.

Several transgenic mouse models are available to complement the rat 2-year bioassay. The Tg.AC transgenic mouse, the heterozygous $p53^{+/-}$ gene-deficient mouse, and the $Tg/rasH_2$ mouse are discussed as examples. It must be remembered that these models are not fully validated.

The Tg.AC mouse model [210] examines chemically initiated skin as a target for tumorigenesis. The Tg.AC line may be able to differentiate carcinogens from noncarcinogens but may not be able to differentiate genotoxic carcinogens from those that cause only tumor promotion activity. This model may only be useful in combination with other transgenic animal models [202]. The heterozygous $p53^{+/-}$ gene-deficient mouse model is based on rendering mice heterozygous for the p53 tumor suppressor gene [111]. These animals are at elevated risk for tumor development. The model has been proposed to best approximate humans at risk for heritable forms of cancer. This model may be able to detect genotoxic carcinogens in a 6-month period. The $Tg/rasH_2$ transgenic mouse contains multiple copies of the human c-Ha-*ras* gene. It has been found to respond well to both genotoxic and nongenotoxic carcinogens [170].

The neonatal mouse assay has been available longer than the transgenic animal models. The detailed protocol for this 1-year study has been previously reviewed [101]. Neonates are treated with the test compound on days 8 and 15 of age and then observed to 1 year of age. At that time, the animals are evaluated for tumor production. This assay is sensitive to direct-acting carcinogens, primarily those that work through formation of covalently bound DNA adducts.

The assessment of carcinogenicity in the drug development process is at a crossroads. The rodent bioassay has been used for over 25 years and has provided useful data, although it is not a perfect system and has received much criticism. The investigation of alternatives to the standard bioassay in two rodent species has been encouraged by the ICH. Conducting a 2-year study in the rat and an alternative study in the mouse may provide an acceptable transition. The available alternative models generally accepted as useful in carcinogenicity testing include the $Tg/rasH_2$ transgenic mouse and the $p53^{+/-}$ knockout mouse. The $Tg/rasH_2$ model is preferred for testing nongenotoxic compounds, and the $p53^{+/-}$ knockout model is preferred for testing genotoxic compounds. The general

utility of available alternative models of carcinogenicity testing has been reviewed by MacDonald et al. [170]. Alternative assay results should not be considered on their own but should be included in a weight-of-evidence approach for risk assessment [170]. A historical perspective on the industry's experience with alternative carcinogenicity models has been provided by Ashton et al. [9]. Much work remains to be done before the alternative models are validated and fully useful as potential replacements in carcinogenicity risk assessments. The alternative assays are relative newcomers full of promise but short on experience. Undue enthusiasm about their ability to dramatically improve our carcinogenicity risk assessment process is not warranted. An alternative approach to carcinogenic risk evaluation, focusing on chemical exposure rather than the 2-year rodent bioassay, has been proposed by Cohen [47].

CONCLUSION

The future engagement of toxicologists in drug development decisions appears to be very promising. Physicians and patients continue to expect more efficacious and safer medications, more quickly and less expensively. Basic research efforts in biochemistry, physiology, and pharmacology have allowed the more precise characterization of receptors and the normal and perturbed sequelae of receptor binding, thus continuing to stimulate the development of more specific, potent modulators of cellular functions. The etiologies of human diseases are becoming better understood, thanks to the technologic ability to elucidate their characteristics at the molecular level. This has resulted in the potential to therapeutically modify the disease process at its origin: the human genome. These molecular approaches have already resulted in the development of agents that are highly species specific, and the use of these techniques to elucidate normal and pathological cellular function will only continue to escalate. The development of NBEs has already challenged the established norms of safety assessment. Consider the example of an agent that has shown selectivity for modifying the activity of a human-specific enzyme critical to a pathological process. Although traditional animal studies are likely to be predictive of toxicity that is unrelated to the pharmacology of the compound, they will not be useful for the prediction of adverse findings relative to the action of the drug at the enzyme, which will occur only in humans. Thus, not only may data generated from classical animal studies be inadequate to predict toxic responses in humans, but also the information may be irrelevant or misleading. A major concern is whether modification of this enzyme activity in the only responsive species (humans) might result in unanticipated, severe toxicity. How can this best be predicted prior to the initiation of clinical trials?

It is unlikely that the classical/traditional tools/approaches of toxicology will be sufficient to ensure the human safety of the new highly specific therapeutics forthcoming from sophisticated technologies. It would, however, be unwise to think that the strong foundation in classical toxicologic assessment and risk/benefit analysis has no place in the future of drug development; it is all about balance and the ability to apply new approaches based on a strong understanding of established procedures.

The future direction of discovery research suggests that industrial and regulatory toxicologists will need to collaborate more closely in the design of the toxicology studies to support registration, and ultimately these may have to be considered on a case-by-case basis. Indeed, the current guidelines resulting from global harmonization efforts repeatedly emphasize the need for defending the scientific rationale supporting the design of proposed toxicologic assessments. Although these guidelines are viewed as much more flexible than the country-specific regulations of the past, they also place a greater burden on toxicologists relative to defending the relevance of their studies. The era of *checking the "tox." box* has, thankfully, come to a close.

Another challenge on the horizon concerns the need to improve the efficiency of the drug development process without compromising its quality. Currently, the drug development and approval processes are taking longer and costing more than ever before. More stringent regulatory requirements have resulted in the conduct of more studies (in both animals and humans) that take more time and cost more money. These costs are passed on to the patient, who ultimately must also compensate for the resultant decreased market life, due to patent length restrictions, of the registered product. Furthermore, the use of large numbers of experimental animals remains a concern from both the ethical and financial points of view. One approach to solving these dilemmas is to ensure that the toxicology studies conducted meet the needs of the regulatory agency, the physician, and ultimately the patient. As suggested earlier, this can be most efficiently accomplished by early and routine interactions between the industry and these customers, especially in cases where the agent under development represents a unique therapeutic approach.

The application of omics technologies, such as genomics (toxico- or pharmaco-genomics), proteomics, and metabonomics, is expected to enhance the toxicology toolbox for evaluation of drug safety. Proteomics, the analysis of protein expression patterns, and metabonomics, the evaluation of metabolite profiles, are relative newcomers to drug safety evaluation. Toxicogenomics (or pharmacogenomics) has, perhaps, the greatest potential to affect nonclinical safety assessment [194]. Toxicogenomics focuses on the study of differential gene expression (DGE) as an adaptation to chemical or environmental stress. A basic assumption is that DGE underlies all drug-induced toxic events, with the possible exception of very rapid cell death [129,157]. A goal of toxicogenomics is to identify gene expression patterns that predict potential toxicity [83,195]. The identification of a causal relationship between DGE and delayed manifestations of frank toxicity can facilitate early drug development. It is anticipated that toxicogenomics will be increasingly integrated into all phases of drug development, particularly in mechanistic and predictive toxicology and biomarker identification [122].

The FDA has published a guidance document on submission of pharmacogenetic data [94]. Within the guidance, the FDA acknowledges that the toxicology database required to support clinical trials and marketing of drugs is well established. Any proposal for substitution of new animal genomics safety testing will involve the international scientific and drug development committees [94].

Finally, the major role of the toxicologist as a mechanistic scientist will continue to be enhanced. For the reasons discussed previously, the design of toxicology studies and the interpretation of toxicology data will become increasingly more sophisticated, requiring a *broad* knowledge base in a variety of other scientific disciplines and a strong foundation in classical toxicology approaches [204]. Elucidation of the mechanisms responsible for observed toxicities would improve the ability to achieve the traditional, ultimate purpose of the discipline of toxicology: appropriate extrapolation of these data to humans [243]. Achievement of this goal will surely become more challenging, but also more exciting, as the future approaches to drug development unfold.

QUESTIONS

8.1 What are the differences between NCEs and NBEs?
8.2 What are the safety testing suggestions for category 2 biotechnology products?
8.3 The U.S. FDA has issued guidance that identifies five major areas of immunotoxicology. Please name.
8.4 According to ICH S7A, the core battery of safety pharmacology includes what three vital organ systems?
8.5 What is the basic approach to the development of anticancer drugs?
8.6 Name one alternative method/test that has been proposed for carcinogenicity determination for a new drug.

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9 Statistics and Experimental Design for Toxicologists

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CONTENTS

INTRODUCTION

This chapter has been written (and updated six times) for both practicing and student toxicologists and pathologists as a practical guide to the common statistical problems encountered in toxicology and toxicological pathology and the methodologies that are available to solve them. The chapter has been enriched by the inclusion of discussions of why a particular procedure or interpretation is recommended, by the clear enumeration of the assumptions that are necessary for a procedure to be valid, and by the discussion of problems drawn from the actual practice of toxicology and toxicological pathology.

Since 1960, the field of toxicology has become increasingly complex and controversial in both its theory and practice. Much of this change is due to the evolution of the field and science and technology in general. As in all other sciences, toxicology started as a descriptive science. Living organisms, be they human or otherwise, were dosed with or exposed to chemicals or physical agents, and the adverse effects that followed were observed. But as a sufficient body of descriptive data was accumulated, it became possible to infer and study underlying mechanisms of action—to determine in a broader sense why adverse effects occurred. Toxicology has thus transitioned to the mechanistic stage, where active contributions to the field encompass both descriptive and mechanistic studies.

Studies continue to be designed and executed to generate increased amounts of data. Genomics and proteomics have even accentuated this process. The resulting problems of data analysis have then become more complex, and toxicology has drawn more deeply from the well of available statistical techniques. Statistics has also been very active and growing during the last 40 years, to some extent, at least, because of the parallel growth of toxicology. These simultaneous changes have led to an increasing complexity of data and, unfortunately, to the introduction of numerous confounding factors that severely limit the utility of the resulting data in all too many cases.

A major difficulty is the very real necessity to understand the biological realities and implications of a problem as well as to understand the peculiarities of toxicological data before procedures are selected and employed for analysis. These characteristics include the following:

1. It is necessary to work with a relatively small sample set of data collected from the members of a population (laboratory animals, cultured cells, and bacterial cultures) that is not actually our population of interest (i.e., humans or a target animal population).
2. Frequently, data obtained from a sample are censored on a basis other than by the investigator's design. By censoring, of course, we mean that not all data points were collected as might be desired. This censoring could be the result of either a biological factor (the test animal being dead or too debilitated to manipulate) or a logistic factor (equipment being inoperative or a tissue being missed in necropsy).
3. The conditions under which our experiments are conducted are extremely varied. In pharmacology (the closest cousin to at least classical toxicology), the possible conditions of interaction of a chemical or physical agent with a person are limited to a small range of doses via a single route over a short course of treatment to a defined patient population. In toxicology, however, all these variables (dose, route, time span, and subject population) are determined by the investigator.
4. The time frames available to solve our problems are limited by practical, regulatory, and economic factors, which conspire to compress time. This frequently means that there is not time to repeat a critical study if the first attempt fails, so a true iterative approach is not possible.

The training of most toxicologists in statistics remains limited to a single introductory course that concentrates on some theoretical basics. As a result, the armamentarium of statistical techniques of most toxicologists is limited, and the tools that are usually present (*t*-tests, chi-square, analysis of variance [ANOVA], and linear regression) are neither fully developed nor well understood. It is hoped that this chapter will help change this situation.

As a point of departure toward this objective, it is essential that any analysis of study results be interpreted by a professional who firmly understands three concepts: (1) the difference between biological significance and statistical significance, (2) the nature and value of different types of data, and (3) causality. For the first concept, we should consider the four possible combinations of these two different types of significance, for which we find the relationship shown as follows:

		Statistical Significance	
		No	**Yes**
Biological	No	Case I	Case II (false positive)
Significance	Yes	Case III (false negative)	Case IV

TABLE 9.1
Approximate Total Sample Sizes for Comparisons Using the *t*-Test and Equal Group Sizes

	β = 0.1		β = 0.2	
Δ/σ	α = 0.05	α = 0.10	α = 0.05	α = 0.10
0.25	672	548	502	396
0.50	168	138	126	98
0.75	75	62	56	44
1.00	42	34	32	24
1.25	28	22	20	16
1.50	18	16	14	12

Note: Δ is the difference in the treatment group means, and σ is the standard deviation.

Cases I and IV give us no problems, for the answers are the same statistically and biologically, but cases II and III present problems. In case II (the *false positive*), we have a circumstance where there is a statistical significance in the measured difference between treated and control groups, but there is no true biological significance to the finding. This is not an uncommon happening, for example, in the case of clinical chemistry parameters. This is called a *type I* error by statisticians, and the probability of this happening is called the α (alpha) level. In case III (the *false negative*), we have no statistical significance, but the differences between groups are biologically or toxicologically significant. This is called a *type II* error by statisticians, and the probability of such an error happening by random chance is called the β (beta) level. An example of this second situation is when we see a few of a very rare tumor type in treated animals. In both of these latter cases, numerical analysis, no matter how well done, is no substitute for professional judgment. Along with this, however, one must have a feeling for the different types of data and for the value or relative merit of each. Note that the two error types interact, and in determining sample size, we need to specify both α and β levels. Table 9.1 demonstrates this interaction in the case of the *t*-test.

The reasons why biological and statistical significance are not identical are multiple, but a central one is certainly causality. Through our consideration of statistics, we should keep in mind that just because a treatment and a change in an observed organism are seemingly or actually associated with each other, this does not *prove* that the former caused the latter. Although this fact is now widely appreciated for correlation (e.g., the fact that the number of storks' nests found each year in England is correlated with the number of human births that year does not mean that storks bring babies), it is just as true in the general case of significance. Timely establishment and proof that treatment causes an effect require an understanding of the underlying mechanism and proof of its validity. At the same time, it is important that we realize that not finding a good correlation or suitable significance associated with a treatment and an effect likewise does not prove that the two are not associated—that a treatment does not cause an effect. At best, it gives us a certain level of confidence that, under the conditions of the current test, these items are not associated.

These points will be discussed in greater detail in the "Assumptions and Formulations" sections for each method, along with other common pitfalls and shortcomings associated with the method. To help in better understanding the discussion to come, terms frequently used throughout this chapter should first be considered. These are presented in Table 9.2.

TABLE 9.2
Some Frequently Used Terms and Their General Meanings

Term	Meaning
95% Confidence interval	A range of values (above, below, or above and below) the sample (mean, median, mode, etc.) that has a 95% chance of containing the true value of the population (mean, median, mode); also called the fiducial limit equivalent to $p < 0.05$
Bias	Systemic error as opposed to a sampling error; for example, selection bias may occur when each member of the population does not have an equal chance of being selected for the sample
Degrees of freedom	The number of independent deviations; usually abbreviated df
Independent variables	Also known as predictors or explanatory variables
p-Value	Another name for significance level; usually 0.05
Power	The effect of the experimental conditions on the dependent variable relative to sampling fluctuation. When the effect is maximized, the experiment is more powerful. Power can also be defined as the probability that there will not be a type II error (1-beta); conventionally, power should be at least 0.07
Random	Each individual member of the population having the same chance of being selected for the sample
Robust	Having inferences or conclusions little affected by departure from assumptions
Sensitivity	The number of subjects experiencing each experimental condition divided by the variance of scores in the sample
Significance level	The probability that a difference has been erroneously declared to be significant, typically 0.05 and 0.01, corresponding to 5% and 1% chance of error
Type I error (false positives)	Concluding that there is an effect when there really is not an effect; its probability is the alpha level
Type II error (false negatives)	Concluding that there is no effect when there really is an effect; its probability is the beta level

Each measurement we make—each individual piece of experimental information we gather—is called a *datum*; however, we gather and analyze multiple pieces at one time, the resulting collection being called *data*. Data are collected on the basis of their association with a treatment (intended or otherwise) as an effect (a property) that is measured in the experimental subjects of a study, such as body weights. These identifiers (i.e., treatment and effect) are termed *variables*. Our treatment variables (those that the researcher or nature control and which can be directly controlled) are termed *independent*, and our effect variables (such as weight, life span, and number of neoplasms) are termed *dependent* variables; their outcome is believed to be dependent on the treatment being studied.

All the possible measures of a given set of variables in all the possible subjects that exist are termed the *population* for those variables. Such a population of variables cannot be truly measured; for example, one would have to obtain, treat, and measure the weights of all the Fischer-344 rats that were, are, or ever will be. Instead, we deal with a representative group: a *sample*. If our sample of data is appropriately collected and of sufficient size, it serves to provide good estimates of the characteristics of the parent population from which it was drawn.

Bias and Change

Any toxicological study aims to determine whether a treatment elicits a response. An observed difference in response between treated and control groups need not necessarily be a result of treatment. There are, in principle, two other possible explanations: *bias*, or systematic differences other than treatment between the groups, and *chance*, or random differences. A major objective of both experimental design and analysis is to try to avoid bias. Wherever possible, treated and control groups to be compared should be alike with respect to all other factors. Where differences remain, these should be corrected for in the statistical analysis. Chance cannot be wholly excluded, as identically treated animals will not respond identically. Although even the most extreme difference in theory might be due to chance, a proper statistical analysis will allow the experimenter to assess this possibility. The smaller the probability of a false positive, the more confident the experimenter can be that the effect is real. Good experimental design improves the chance of picking up a true effect with confidence by maximizing the ratio between *signal* and *noise*.

Hypothesis Testing and Probability (p) Values

A relationship of treatment to some toxicological endpoint is often stated to be *statistically significant* ($p < 0.05$). What does this really mean? A number of points have to be made. First, statistical significance need not necessarily imply biological importance, if the endpoint under study is not relevant to the animal's well-being. Second, the statement will usually be based only on the data from the study in question and will not take into account prior knowledge. In some situations, such as when one or two of a very rare tumor type are seen in treated animals, statistical significance may not be achieved, but the finding may be biologically extremely important, especially if a similar treatment was previously found to elicit a similar response. Third, the p value does not describe the probability that a true effect of treatment exists; rather, it describes the probability of the observed response, or one more extreme, occurring on the assumption that treatment actually had no effect whatsoever. A p value that is not significant is consistent with a treatment having a small effect not detected with sufficient certainty in this study. Fourth, there are two types of p values. A one-tailed (or one-sided) p value is the probability of getting by chance a treatment effect in a specified direction as great as or greater than that observed. A two-tailed p value is the probability of getting, by chance alone, a treatment difference in either direction that is as great as or greater than that observed. By convention, p values are assumed to be two-tailed unless the contrary is stated. Where one can rule out in advance the possibility of a treatment effect except in one direction (which is unusual), a one-tailed p value should be used. Often, however, two-tailed tests are to be preferred, and it is certainly not recommended to use one-tailed tests and *not* report large differences in the other direction. In any event, it is important to make it absolutely clear whether one- or two-tailed tests have been used.

It is a great mistake, when presenting results of statistical analyses, to mark, as do some laboratories, results simply as significant or not significant at one defined probability level (usually $p < 0.05$). This poor practice does not allow the reader any real chance to judge whether or not the effect is a true one. Some statisticians present the actual p value for every comparison made. Although this gives precise information, it can make it difficult to assimilate results from many variables. One practice we recommend is to mark p values routinely using plus signs to indicate positive differences (and minus signs to indicate negative differences) as follows: $+++p$, 0.001; $++0.001 \le p < 0.01$; $+0.01\ p < 0.05$ ($+0.05 \le p < 0.1$). This highlights significant results more clearly and also allows the reader to judge the whole range from *virtually certain treatment effect* to *some suspicion*. Note that, when using two-tailed tests, bracketed plus signs indicate findings that would be significant at the conventional $p < 0.05$ level using one-tailed tests but are not significant at this level using two-tailed tests. In interpreting p values, it is important to realize they are only an aid to judgment to be used in conjunction with other available information. One might validly consider a $p < 0.01$ increase as chance when it was unexpected, occurred only at a low dose level with no such effect seen at higher doses, and was evident in only one subset of the data. In contrast, a $p < 0.05$ increase might be convincing if it occurred in the top dose and was for an endpoint one might have expected to be increased from known properties of the chemical or closely related chemicals.

Multiple Comparisons

When a p value is stated to be <0.05, this implies that, for that particular test, the difference could have occurred by chance less than 1 time in 20. Toxicological studies frequently involve making treatment–control comparisons for large numbers of variables and, in some situations, also for various subsets of animals. Some statisticians worry that the larger the number of tests, the greater the chance of picking up statistically significant findings that do not represent true treatment effects. For this reason, an alternative *multiple comparisons* procedure has been proposed in which, if the treatment was totally without effect, then 19 times out of 20 *all* the tests should show nonsignificance when testing at the 95% confidence level. Automatic use of this approach cannot be recommended. Not only does it make it much more difficult to pick up any real effects, but also there is something inherently unsatisfactory about a situation where the relationship between a treatment and a particular response depends arbitrarily on which other responses happened to be investigated at the same time. It is accepted that in any study involving multiple endpoints will inevitably exist a gray area between those showing highly significant effects and those showing no significant effects, where there is a problem distinguishing chance and true effects. However, changing the methodology so the gray areas all come up as nonsignificant can hardly be the answer.

Estimating the Size of the Effect

It should be clearly understood that a p value does not give direct information about the size of any effect that has occurred. A compound may elicit an increase in response by a given amount, but whether a study finds this increase to be statistically significant will depend on the size of the study and the variability of the data. In a small study, a large and important effect may be missed, especially if the endpoint is imprecisely measured. In a large study, on the other hand, a small and unimportant effect may emerge as statistically significant.

Hypothesis testing tells us whether an observed increase can or cannot be reasonably attributed to chance but not how large it is. Although much statistical theory relates to hypothesis testing, current trends in medical statistics are toward confidence interval (CI) estimation, with differences between test and control groups expressed in the form of a best estimate, coupled with the 95% CI. Thus, if one states that treatment increases response by an estimated 10 units (95% CI, 3–17 units), this would imply a 95% chance that the indicated interval includes the true difference. If the lower 95% confidence limit exceeds zero, this implies that the increase is statistically significant at $p < 0.05$ using a two-tailed test. One can also calculate, for example, 99% or 99.9% confidence limits, corresponding to testing for significance at $p < 0.01$ or $p < 0.001$. In screening studies of standard design, the tendency has been to concentrate mainly on hypothesis testing; however, presentation of the results in the form of estimates with CIs can be a useful adjunct for some analyses and is very important in studies aimed specifically at quantifying the size of an effect.

Two terms refer to the quality and reproducibility of our measurements of variables. The first, *accuracy*, is an expression of the closeness of a measured or computed value to its actual or true value in nature. The second, *precision*, reflects the closeness or reproducibility of a series of repeated measurements of the same quantity. If we arrange all of our measurements of a particular variable in order as points on an axis marked as to the values of that variable and if our sample were large enough, the pattern of distribution of the data in the sample would begin to become apparent. This pattern is a representation of the frequency distribution of a given population of data—that is, of the incidence of different measurements, their central tendency, and dispersion.

The most common frequency distribution—and one we will talk about throughout this chapter—is the normal (or Gaussian) distribution. The normal distribution is such that two-thirds of all values are within one standard deviation (SD) of the mean (or average value for the entire population) and 95% are within 1.96 SDs of the mean. Symbols used are μ for the mean and σ for the SD.

In all areas of biological research, optimal design and appropriate interpretation of experiments require that the researcher understand both the biological and technological underpinnings of the system being studied and of the data being generated. From the point of view of the statistician, it is vitally important that the experimenter both know and be able to communicate the nature of the data and understand its limitations. One classification of data types is presented in Table 9.3.

The nature of the data collected is determined by three considerations: (1) the biological source of the data (the system being studied), (2) the instrumentation and techniques being used to make measurements, and (3) the design of the experiment. The researcher has some degree of control over each of these, least over the biological system (the researcher normally has a choice of only one of several models to study) and most over the design of the experiment or study. Such choices, in fact, dictate the type of data generated by a study.

TABLE 9.3
Types of Variables (Data) and Examples of Each Type

Classified by	Type	Example
Scale	Scalar	Body weight
Continuous	Ranked	Severity of a lesion
Discontinuous	Scalar	Weeks until the first observation of a tumor in a carcinogenicity study
	Ranked	Clinical observations in animals
	Attribute	Eye colors in fruit flies
	Quantal	Dead/alive or present/absent
Frequency distribution	Normal	Body weights
	Bimodal	Some clinical chemistry parameters
	Others	Measures of time to incapacitation

Statistical methods are based on specific assumptions. Parametric statistics (those that are most familiar to the majority of scientists) have more stringent underlying assumptions than do nonparametric statistics. Among the underlying assumptions for many parametric statistical methods (such as the ANOVA) is that the data are continuous. The nature of the data associated with a variable (as described earlier) imparts a value to that data, the value being the power of the statistical tests that can be employed.

Continuous variables are those that can at least theoretically assume any of an infinite number of values between any two fixed points (such as measurements of body weight between 2.0 and 3.0 kg). Discontinuous variables, meanwhile, are those that can have only certain fixed values, with no possible intermediate values (such as counts of five and six dead animals, respectively).

Limitations on our ability to measure constrain the extent to which the real-world situation approaches the theoretical, but many of the variables studied in toxicology are in fact continuous. Examples of these are lengths, weights, concentrations, temperatures, periods of time, and percentages. For these continuous variables, we may describe the character of a sample with measures of central tendency and dispersion that we are most familiar with: the mean, denoted by the symbol x and also called the arithmetic average, and the SD, which is denoted by the symbol σ and is calculated as being equal to

$$\sqrt{\frac{\sum x^2 - \left(\left(\sum x\right)^2 \Big/ N\right)}{N-1}}$$

where

x is the individual datum

N is the total number of data in the group

Contrasted with these continuous data, however, we have discontinuous (or discrete) data, which can assume only certain fixed numerical values. In these cases, our choice of statistical tools or tests is, as we will find later, more limited.

Probability

Probability is simply the frequency with which, in a sufficiently large sample, a particular event will occur or a particular value be found. Hypothesis testing, for example, is generally structured so the likelihood of a treatment group being the same as a control group (the so-called *null hypothesis*) can be assessed as being at less than a selected low level (very frequently 5%), which implies that we are $1.0 - \alpha$ (i.e., 1.0 – 0.05, or 95%) sure that the groups are *not* equivalent.

FUNCTIONS OF STATISTICS

Statistical methods may serve to perform any combination of three possible tasks. The one we are most familiar with is hypothesis testing—that is, determining if two (or more) groups of data differ from each other at a predetermined level of confidence. A second function is the construction and use of models that may be used to predict future outcomes of chemical–biological interactions. This is most commonly seen in linear regression or in the derivation of some form of correlation coefficient. Model fitting allows us to relate one variable (typically, a treatment or independent variable) to another. The third function, reduction of dimensionality, continues to be less commonly utilized than the first two. This final category includes methods for reducing the number of variables in a system while only minimally reducing the amount of information, thus making a problem easier to visualize and understand. Examples of such techniques are factor analysis and cluster analysis. A subset of this last function is the reduction of raw data to single expressions of central tendency and variability (such as the mean and SD). There is also a special subset of statistical techniques that is part of both the second and third functions of statistics. This is data transformation, which includes such things as the conversion of numbers to log or probit values.

Descriptive Statistics

Descriptive statistics are used to summarize the general nature of a dataset. As such, the parameters describing any single group of data have two components. One of these describes the location of the data, and the other gives a measure of the dispersion of the data in and about this location. Often overlooked is the fact that the choice of which parameters are used to give these pieces of information implies a particular type of distribution for the data.

Most commonly, location is described by giving the (arithmetic) mean and dispersion by giving the SD or the *standard error of the mean* (SEM). The calculation of the first two of these has already been described. If we again denote the total number of data in a group as N, then the SEM would be calculated as

$$\text{SEM} = \frac{\text{SD}}{\sqrt{N}}$$

The use of the mean with either the SD or SEM implies, however, that we have reason to believe that the sample of data being summarized is from a population that is at least approximately normally distributed. If this is not the case, then we should instead use a set of statistical descriptions that do not require a normal distribution. These are the *median* (for location) and the *semiquartile distance* (for a measure of dispersion). These somewhat less familiar parameters are characterized as follows.

When all the numbers in a group are arranged in a ranked order (i.e., from smallest to largest), the *median* is the middle value. If the group has an odd number of values, then the middle value is obvious; for example, in the case of 13 values, the seventh largest is the median. When the number of values in the sample is even, the median is calculated as the midpoint between the $(N/2)$th and the $([N/2] + 1)$th number;

for example, in the series of numbers 7, 12, 13, and 19, the median value would be the midpoint between 12 and 13, which is 12.5.

The SD and the SEM are related to each other but yet are quite different. The SEM is quite a bit smaller than the SD, making it very attractive to use in reporting data. This size difference is because the SEM actually is an estimate of the error (or variability) involved in measuring the means of samples and not an estimate of the error (or variability) involved in measuring the data from which means are calculated. This is implied by the *central limit theorem*, which tells us three major things:

- The distribution of sample means, which will be approximately normal regardless of the distribution of values in the original population from which the samples were drawn
- The mean value of the collection
- The SD of the collection of all possible means of samples of a given size, called the standard error of the mean, which depends on both the SD of the original population and the size of the sample

The SEM should be used only when the uncertainty of the estimate of the mean is of concern, which is almost never the case in toxicology; rather, we are concerned with an estimate of the variability of the population, for which the SD is appropriate.

When all the data in a group are ranked, a quartile of the data contains one ordered quarter of the values. Typically, we are most interested in the borders of the middle two quartiles, Q_1 and Q_3, which together represent the semiquartile distance and which contain the median as their center. Given that there are N values in an ordered group of data, the upper limit of the fourth quartile (Q) may be computed as being equal to the [($jN \div 1$)/fourth] value. Once we have used this formula to calculate the upper limits of Q_1 and Q_3, we can then compute the semiquartile distance (which is also called the *quartile deviation* and as such is abbreviated as QD) with the formula $QD = (Q_3 - Q_1)/2$. For example, for the 15-value dataset 1, 2, 3, 4, 4, 5, 5, 5, 6, 6, 6, 7, 7, 8, 9, we can calculate the upper limits of Q_1 and Q_3 as

$$Q_1 = \frac{1(15+1)}{4} = \frac{16}{4} = 4$$

$$Q_2 = \frac{3(15+1)}{4} = \frac{48}{4} = 12$$

The 4th and 12th values in this dataset are 4 and 7, respectively. The semiquartile distance can then be calculated as

$$QD = \frac{7-4}{2} = 1.5$$

There are times when it is desired to describe the relative variability of one or more sets of data. The most common way of doing this is to compute the *coefficient of variation* (CV), which is calculated simply as the ratio of the SD to the mean, or

$$CV = \frac{SD}{\bar{X}}$$

A CV of 0.2 or 20% thus means that the SD is 20% of the mean. In toxicology, the CV is frequently between 20% and 50% and may at times exceed 100%.

Sampling

Sampling—the selection of which individual data points will be collected, whether in the form of selecting which animals to collect blood from or to remove a portion of a diet mix from for analysis—is an essential step upon which all other efforts toward a good experiment or study are based. Three assumptions about sampling are common to most of the statistical analysis techniques that are used in toxicology: the sample is collected without bias, each member of a sample is collected independently of the others, and members of a sample are collected with replacements. Precluding bias, both intentional and unintentional, means that at the time of selection of a sample to measure, each portion of the population from which that selection is to be made has an equal chance of being selected. Independence means that the selection of any portion of the sample is not affected by and does not affect the selection or measurement of any other portion. Finally, sampling with replacement means that, in theory, after each portion is selected and measured, it is returned to the total sample pool and thus has the opportunity to be selected again. This is a corollary of the assumption of independence. Violation of this assumption (which is almost always the case in toxicology and all the life sciences) does not have serious consequences if the total pool from which samples are drawn is sufficiently large (say, 20 or greater) that the chance of reselecting that portion is small anyway.

The four major types of sampling methods are *random*, *stratified*, *systematic*, and *cluster*. Random is by far the most commonly employed method in toxicology. It stresses the fulfillment of the assumption of avoiding bias. When the entire pool of possibilities is mixed or randomized, then the members of the group are selected in the order in which they are drawn from the pool.

Stratified sampling is performed by first dividing the entire pool into subsets or strata, then doing randomized sampling from each strata. This method is employed when the total pool contains subsets that are distinctly different but in which each subset contains similar members. An example is a large batch of a powdered pesticide in which it is desired to determine the nature of the particle size distribution. Larger pieces or particles are on the top, progressively smaller particles have settled lower in the container, and at the very bottom, the material has been

packed and compressed into aggregates. To determine a timely representative answer, proportionally sized subsets from each layer or strata should be selected, mixed, and randomly sampled. This method is used more commonly in diet studies.

In systematic sampling, a sample is taken at set intervals (such as every fifth container of reagent or taking a sample of water from a fixed sample point in a flowing stream every hour). This is most commonly employed in quality assurance or (in the clinical chemistry lab) in quality control.

In cluster sampling, the pool is already divided into numerous separate groups (such as bottles of tablets), and we select small sets of groups (such as several bottles of tablets) then select a few members from each set. What one gets then is a cluster of measures. Again, this is a method most commonly used in quality control or in environmental studies when the effort and expense of physically collecting a small group of units is significant.

In classical toxicology studies, sampling arises in a practical sense in a limited number of situations. The most common of these are as follows:

- Selecting a subset of animals or test systems from a study to make some measurements (which either destroys or stresses the measured system, or is expensive) at an interval during a study; this may include such cases as doing interim necropsies in a chronic study or collecting and analyzing blood samples from some animals during a subchronic study.
- Analyzing inhalation chamber atmospheres to characterize aerosol distributions with a new generation system.
- Analyzing diet in which test material has been incorporated.
- Performing quality control on an analytical chemistry operation by having duplicate analyses performed on some materials.
- Selecting data to audit for quality assurance purposes.

We have now become accustomed to developing exhaustively detailed protocols for an experiment or study prior to its conduct. A priori selection of statistical methodology (as opposed to the post hoc approach) is as significant a portion of the process of protocol development and experimental design as any other and can measurably enhance the value of the experiment or study (see Table 9.4). Prior selection of statistical methodologies is essential for proper design of other portions of a protocol such as the number of animals per group or the sampling intervals for body weight. Implied in such a selection is the notion that the toxicologist has both an in-depth knowledge of the area of investigation and an understanding of the general principles of experimental design, for the analysis of any set of data is dictated to a large extent by the manner in which the data are obtained.

The four basic statistical principles of experimental design are *replication*, *randomization*, *concurrent* (*local*) *control*, and *balance*. In abbreviated form, these may be summarized as follows:

- *Replication*—Any treatment must be applied to more than one experimental unit (animal, plate of cells, litter of offspring, etc.). This provides more accuracy in the measurement of a response than can be obtained from a single observation, because underlying experimental errors tend to cancel each other out. It also supplies an estimate of the experimental error derived from the variability among each of the measurements taken (or replicates).

TABLE 9.4
Rules for Form Design and Preparation

1. Forms should be used when some form of repetitive data must be collected. They may be either paper or electronic.
2. If only a few (two or three) pieces of data are to be collected, they should be entered into a notebook and not onto a form. This assumes that the few pieces are not a daily event, with the aggregate total of weeks/months/years ending up as lots of data to be pooled for analysis.
3. Forms should be self-contained but should not try to repeat the content of the standard operating procedures or method descriptions.
4. Column headings on forms should always specify the units of measurement and other details of entries to be made. The form should be arranged so sequential entries proceed down a page, not across. Each column should be clearly labeled with a heading that identifies what is to be entered in the column. Any fixed part of entries (such as °C) should be in the column header.
5. Columns should be arranged from left to right so there is a logical sequential order to the contents of an entry as it is made. An example would be date/time/animal number/body weight/name of the recorder. The last item for each entry should be the name or unique initials of the individual who made the data entry.
6. Standard conditions that apply to all the data elements to be recorded on a form, or the columns of the form should be listed as footnotes at the bottom of the form.
7. Entries of data on the form should not use more digits than are appropriate for the precision of the data being recorded.
8. Each form should be clearly titled to indicate its purpose and use. If multiple types of forms are being used, each should have a unique title or number.
9. Before designing the form, carefully consider the purpose for which it is intended. What data will be collected, how often, with what instrument, and by whom? Each of these considerations should be reflected in some manner on the form.
10. Those things that are common or standard for all entries on the form should be stated as such once. These could include such things as instrument used, scale of measurement (°C, °F, or K), or the location where the recording is made.

In practice, this means that an experiment should have enough experimental units in each treatment group (i.e., a large enough *N*) so that reasonably sensitive statistical analysis of data can be performed. The estimation of sample size is addressed in detail later in this chapter.

- *Randomization*—This is practiced to ensure that every treatment has its fair share of extreme high and extreme low values. It also serves to allow the toxicologist to proceed as if the assumption of independence is valid; that is, there is no avoidable (known) systematic bias in how one obtains data.
- *Concurrent control*—Comparisons between treatments should be made to the maximum extent possible between experimental units from the same closely defined population; therefore, animals used as a control group should come from the same source, lot, age, etc., as test group animals. Except for the treatment being evaluated, test and control animals should be maintained and handled in exactly the same manner.
- *Balance*—If the effect of several different factors is being evaluated simultaneously, the experiment should be laid out in such a way that the contributions of the different factors can be separately distinguished and estimated. There are several ways of accomplishing this using one of several different forms of design, as will be discussed later.

The four basic experimental design types used in toxicology are the *randomized block*, *Latin square*, *factorial design*, and *nested design*. Other designs that are used are really combinations of these and are rarely employed in toxicology. Before examining these four basic types, however, we must first examine the basic concept of blocking.

Blocking is, simply put, the arrangement or sorting of the members of a population (such as all of an available group of test animals) into groups based on certain characteristics that may (but are not sure to) alter an experimental outcome. Such characteristics that may cause a treatment to give a differential effect include genetic background, age, sex, and overall activity levels, among others. The process of blocking then acts (or attempts to act) so each experimental group (or block) is assigned its fair share of the members of each of these subgroups.

We should now recall that randomization is aimed at spreading out the effect of undetectable or unsuspected characteristics in a population of animals or some portion of this population. The merging of the two concepts of randomization and blocking leads to the first basic experimental design, the randomized block. This type of design requires that each treatment group has at least one member of each recognized group (such as age), the exact members of each block being assigned in an unbiased (or random) fashion.

The second type of experimental design assumes that we can characterize treatments (whether intended or otherwise) as belonging clearly to separate sets. In the simplest case, these categories are arranged into two sets that may be thought of as rows (for, say, source litter of test animal, with the first litter as row 1, the next as row 2, etc.) and the secondary set of categories as columns (for, say, our ages of test animals, with 6–8 weeks as column 1, 8–10 weeks as column 2, and so on). Experimental units are then assigned so each major treatment (control, low dose, intermediate dose, etc.) appears once and only once in each row and each column. If we denote our test groups as A (control), B (low), C (intermediate), and D (high), such an assignment would appear as shown in Table 9.5.

TABLE 9.5
Sample Table

	Age (Weeks)			
Source Litter	**6–8**	**8–10**	**10–12**	**12–14**
1	A	B	C	D
2	B	C	D	A
3	C	D	A	B
4	D	A	B	C

The third type of experimental design is the factorial design, which has two or more clearly understood treatments, such as exposure level to test chemical, animal age, or temperature. The classical approach to this situation (and to that described under the Latin square) is to hold all but one of the treatments constant and at any one time to vary just that one factor. In the factorial design, however, all levels of a given factor are combined with all levels of every other factor in the experiment. When a change in one factor produces a different change in the response variable at one level of a factor than at other levels of this factor, there is an interaction between these two factors that can then be analyzed as an interaction effect.

The last of the major varieties of experimental design is the nested design, where the levels of one factor are nested within (or are subsamples of) another factor; that is, each subfactor is evaluated only within the limits of its single larger factor.

Another concept that is essential to the design of experiments in toxicology is *censoring*. Censoring is the exclusion of measurements from certain experimental units, or indeed of the experimental units themselves, from consideration in data analysis or inclusion in the experiment at all. Censoring may occur either prior to initiation of an experiment (where, in modern toxicology, this is almost always a planned procedure), during the course of an experiment (when they are almost universally unplanned, resulting from such as the death of animals on test), or after the conclusion of an experiment (when usually data are excluded because of being identified as some form of outlier).

In practice, a priori censoring in toxicology studies occurs in the assignment of experimental units (such as animals) to test groups. The most familiar example is in the common practice of assignment of test animals to acute, subacute, subchronic, and chronic studies, where the results of otherwise

random assignments are evaluated for body weights of the assigned members. If the mean weights are found not to be comparable by some preestablished criterion (such as a 90% probability of difference by ANOVA), then members are reassigned (censored) to achieve comparability in terms of starting body weights. Such a procedure of animal assignment to groups is known as *censored randomization*.

The first precise or calculable aspect of experimental design encountered is determining sufficient test and control group sizes to allow one to have an adequate level of confidence in the results of a study (i.e., in the ability of the study design with the statistical tests used to detect a true difference—or effect—when it is present). The statistical test contributes a level of power to such a detection. Remember that the power of a statistical test is the probability that a test results in rejection of a hypothesis (say, H_0) when some other hypothesis (say, H) is valid. This is considered the power of the test with respect to the (alternative) hypothesis H.

If there is a set of possible alternative hypotheses, the power, regarded as a function of H, is termed the *power function* of the test. When the alternatives are indexed by a single parameter θ, simple graphical presentation is possible. If the parameter is a vector θ, then one can visualize a *power surface*.

If the power function is denoted by β(θ) and H_0 specifies $\theta = \theta_0$, then the value of β(P)—the probability of rejecting H_0 when it is in fact valid—is the significance level. The power of a test is greatest when the probability of a type II error is the least. Specified powers can be calculated for tests in any specific or general situation.

Some general rules to keep in mind are as follows:

- The more stringent the significance level, the greater the necessary sample size. More subjects are needed for a 1%-level test than for a 5%-level test.
- Two-tailed tests require larger sample sizes than one-tailed tests. Assessing two directions at the same time requires a greater investment.
- The smaller the critical effect size, the larger the necessary sample size. Subtle effects require greater efforts.
- Any difference can be significant if the sample size is large enough.
- The larger the power required, the larger the necessary sample size. Greater protection from failure requires greater effort. The smaller the sample size, the smaller the power (i.e., the greater the chance of failure).
- The requirements and means of calculating the necessary sample size depend on the desired (or practical) comparative sizes of test and control groups.

This number (N) can be calculated, for example, for equal-sized test and control groups using the formula:

$$N = \frac{\left(t_1 + t_2\right)^2}{d^2} S$$

where

t_1 is the one-tailed t value with ($N - 1$) degrees of freedom corresponding to the desired level of confidence

t_2 is the one-tailed t value with ($N - 1$) degrees of freedom corresponding to the probability that the sample size will be adequate to achieve the desired precision

S is the sample SD, derived typically from historical data and calculated as

$$S = \sqrt{\frac{1}{N-1}\sum\left(V_1 - V_2\right)^2}$$

A number of aspects of experimental design are specific to the practice of toxicology. Before we look at a suggestion for step-by-step development of experimental designs, these aspects should first be considered as follows:

1. Frequently, the data gathered from specific measurements of animal characteristics are such that there is wide variability in the data. Often, such wide variability is not present in a control or low-dose group, but in an intermediate-dosage group, variance inflation may occur; that is, a large SD may be associated with the measurements from this intermediate group. In the face of such a set of data, the conclusion that there is no biological effect based on a finding of no statistically significant effect might well be erroneous.
2. In designing experiments, a toxicologist should keep in mind the potential effect of involuntary censoring on sample size. In other words, although a study might start with five dogs per group, this provides no margin should any die before the study is ended and blood samples are collected and analyzed. Just enough experimental units per group frequently leave too few at the end to allow meaningful statistical analysis, and allowances should be made accordingly in establishing group sizes.
3. It is certainly possible to pool the data from several identical toxicological studies. For example, if we performed an acute inhalation study where only three treatment group animals survived to the point at which a critical measure (such as analysis of blood samples) was performed, we would not have enough data to perform a meaningful statistical analysis. We could then repeat the protocol with new control and treatment group animals from the same source. At the end, after assuring ourselves that the two sets of data are comparable, we could combine (or pool) the data from survivors of the second study with those from the first. The costs of this approach, however, would then be both a greater degree of effort expended (than if we had performed a single study with larger groups) and increased variability in the pooled samples (decreasing the power of our statistical methods).

4. Another frequently overlooked design option in toxicology is the use of an unbalanced design—that is, of different group sizes for different levels of treatment. There is no requirement that each group in a study (control, low dose, intermediate dose, and high dose) has an equal number of experimental units assigned to it. Indeed, there are frequently good reasons to assign more experimental units to one group than to others, and all the major statistical methodologies have provisions to adjust for such inequalities, within certain limits. The two most common uses of the unbalanced design have larger groups assigned to either the highest dose, to compensate for losses due to possible deaths during the study, or the lowest dose, to give more sensitivity in detecting effects at levels close to an effect threshold or more confidence to the assertion that no effect exists.
5. We are frequently confronted with the situation where an undesired variable is influencing our experimental results in a nonrandom fashion. Such a variable is called a *confounding variable*; its presence makes the clear attribution and analysis of effects at best difficult and at worst impossible. Sometimes such confounding variables are the result of conscious design or management decisions, such as the use of different instruments, personnel, facilities, or procedures for different test groups within the same study. Occasionally, however, such confounding variables are the result of unintentional factors or actions, in which case the variable is referred to as a *lurking variable*. Examples of such variables are almost always the result of standard operating procedures being violated (e.g., water not being connected to a rack of animals over a weekend, a set of racks not being cleaned as frequently as others, or a contaminated batch of feed being used).
6. Finally, some thought must be given to the clear definition of what is meant by experimental unit and concurrent control. The experimental unit in toxicology encompasses a wide variety of possibilities. It may be cells, plates of microorganisms, individual animals, litters of animals, etc. The importance of clearly defining the experimental unit is that the number of such units per group is the *N* that is used in statistical calculations or analyses and critically affects such calculations. The experimental unit is the unit that receives treatments and yields a response that is measured and becomes a datum. What this means in practice is that, for example, in reproduction or teratology studies where we treat the parental generation females and then determine results by counting or evaluating offspring, the experimental unit is still the parent; therefore, the number of litters, not the number of offspring, is the *N* [8]. A true concurrent control is one that is identical in every manner with the treatment groups except for the treatment being evaluated. This means that all manipulations, including gavaging with equivalent volumes of vehicle or exposing to equivalent rates of air exchanges in an inhalation chamber, should be duplicated in control groups just as they occur in treatment groups.

The goal of the four principles of experimental design (*replication*, *randomization*, *concurrent control*, and *balance*) is statistical efficiency and the economizing of resources. The single most important initial step in achieving such an outcome is to clearly define the objective of the study and have a clear statement of what questions are being asked.

EXPERIMENTAL DESIGN

Toxicological experiments generally have a twofold purpose. The first question is whether or not an agent results in an effect on a biological system. The second question, never far behind, is how much of an effect is present. It has become increasingly desirable that the results and conclusions of studies aimed at assessing the effects of environmental agents be as clear and unequivocal as possible. It is essential that every experiment and study yield as much information as possible and that the results of each study have the greatest possible chance of answering the questions it was conducted to address. The statistical aspects of such efforts, so far as they are aimed at structuring experiments to maximize the possibilities of success, are called *experimental design*.

Ten facets of any study may affect its ability to detect an effect of a treatment. The first six concern minimizing the role of chance; the last four relate to avoidance of bias:

- *Choice of species and strain*—Ideally, the responses of interest should be rare in untreated control animals but should be reasonably readily evoked by appropriate treatments. Some species or specific strains, perhaps because of inappropriate diets [1], have high background tumor incidences that make increases both difficult to detect and difficult to interpret when detected.
- *Dose levels*—This is a very important and controversial area. In screening studies aimed at hazard identification, it is normal, to avoid requiring huge numbers of animals, to test at dose levels higher than those to which humans will be exposed but not so high that marked toxicity occurs. A range of doses is usually tested to guard against the possibility of misjudgment of an appropriate high dose and that the metabolic pathways at the high doses differ markedly from those at lower doses and, perhaps, to ensure that no large effects occur at dose levels in the range to be used by humans. In studies aimed more at risk estimation, more and lower doses may

be tested to obtain fuller information on the shape of the dose–response curve.

- *Number of animals*—This is obviously an important determinant of the precision of the findings. The calculation of the appropriate number depends on (1) the critical difference (i.e., the size of the effect it is desired to detect); (2) the false-positive rate (i.e., the probability of an effect being detected when none exists; equivalent to the α level or type I error); (3) the false-negative rate, (i.e., the probability of no effect being detected when one of exactly the critical size exists; equivalent to the β level or type II error); and (4) some measure of the variability in the material. Tables relating numbers of animals required to obtain values of critical size α and β are given in Lee [2], and software (e.g., nQUERY ADVISOR) is also available for this purpose. As a rule of thumb, to reduce the critical difference by a factor n for a given value of α and β, the number of animals required will have to be increased by a factor of n^2.
- *Duration of the experiment*—It is obviously important not to terminate the study too early for fatal conditions, which are normally strongly age related. Less obviously, going on for too long in a study can be a mistake, partly because the last few weeks or months may produce relatively few extra data at a disproportionate cost and partly because diseases of extreme old age may obscure the detection of tumors and other conditions of more interest. For nonfatal conditions, the ideal is to kill the animals when the average prevalence is around 50%.
- *Accuracy of determinations*—This is of obvious importance. Although good laboratory practices and advances in technology have improved the situation here, it is necessary for those taking part in the study to be diligent.
  - *Sampling*—Sampling is an essential step upon which any meaningful experimental result depends. Sampling may involve selection of the individual data points that will be collected, determining which animals tissue samples will be collected from or taking a sample of a diet mix for chemical analysis. Three assumptions about sampling are common to most of the statistical analysis techniques used in toxicology. The assumptions are that the sample is collected without bias, each member of a sample is collected independently of the others, and members of a sample are collected with replacements.
  - *Stratification*—To detect a treatment difference with accuracy, it is important that the groups being compared are as homogeneous as possible with respect to other known causes of the response. In particular, suppose that there is another known important cause of the response for which the animals vary, so the animals are a mixture of hyper- and hyporesponders from this cause. If the treated group has a higher proportion of hyperresponders, it will tend to have a higher response even if the treatment has no effect. Even if the proportion of hyperresponders is the same as in the controls, it will be more difficult to detect an effect of treatment because of the increased between-animal variability. Given that this other factor is known, it will be sensible to take it into account in both the design and the analysis of the study. In the design, it can be used as a blocking factor so animals at each level are allocated equally (or in the correct proportion) to control and treated groups. In the analysis, the factor should be treated as a stratifying variable, with separate treatment–control comparisons made at each level and the comparisons combined for an overall test of difference. This is discussed later, where we refer to the factorial design as one example of the more complex designs that can be used to investigate the separate effect of multiple treatments.
  - *Balance*—If the effect of several different factors is being evaluated simultaneously, then the experiment should be laid out in such a way that the contributions of the different factors can be separately distinguished and estimated. There are several ways to accomplish this using different forms of design, as will be discussed later. It is important to recognize that mathematical comparisons are best when group sizes are similar. It may be tempting to place more animals in the treated group to *see* the effect, but such an action weakens the statistical analysis of the experiment.
  - *Randomization*—Random allocation of animals to treatment groups is a prerequisite of good experimental design. If not carried out, one can never be sure whether treatment–control differences are due to treatment or due to confounding by other relevant factors. The ability to randomize easily is a major advantage animals experiments have other over methods such as epidemiology. Although randomization eliminates bias (as least in expectation), simple randomization of all animals may not be the optimal technique for producing a sensitive test. If there is another major source of variation (e.g., sex or batch of animals), it will be better to carry out stratified randomization (i.e., separate randomizations within each level of the stratifying variable). The need for randomization

applies not only to the allocation of animals to the treatment but also to anything that can materially affect the recorded response. The same random number that is used to apply animals to treatment groups can be used to determine cage position, order of weighting, order of bleeding for clinical chemistry, order of sacrifice at terminations, and so on.

- *Adequacy of control group*—Although, on occasion, historical control data can be useful, a properly designed study demands that a relevant concurrent control group be included with which results for the test group can be compared. The principle that like should be compared with like, apart from treatment, demands that control animals should be randomized from the same source as the treatment animals. Careful consideration should also be given to the appropriateness of the control group. Thus, in an experiment involving the treatment of a compound in a solvent, it would often be inappropriate to include only an untreated control group, as any differences observed could be attributed only to the treatment–solvent combination. To determine the specific effects of the compound, a comparison group given the solvent only, by the same route of administration, would be required. It is not always generally realized that the position of the animal in the room in which it is kept may affect the animal's response. An example is the strong relationship between the incidence of retinal atrophy in albino rats and closeness to the lighting source. Systematic differences in cage position should be avoided, preferably via randomization.

For the reader who would like to further explore experimental design, a number of more detailed texts are available that include more extensive treatments of the statistical aspects of experimental design [3–8].

GENERALIZED METHODOLOGY SELECTION

One approach for the selection of appropriate techniques to employ in a particular situation is to use a decision-tree method. Figure 9.1 is a decision tree that leads to the choice of one of three other trees to assist in technique selection, with each of the subsequent trees addressing one of the three functions of statistics that was defined earlier in this chapter. Figure 9.2 illustrates the selection of hypothesis-testing procedures, Figure 9.3 modeling procedures, and Figure 9.4 the reduction of dimensionality procedures. For the vast majority of situations, these trees will guide the user to the choice of the proper technique. The tests and terms used in these trees will be explained subsequently.

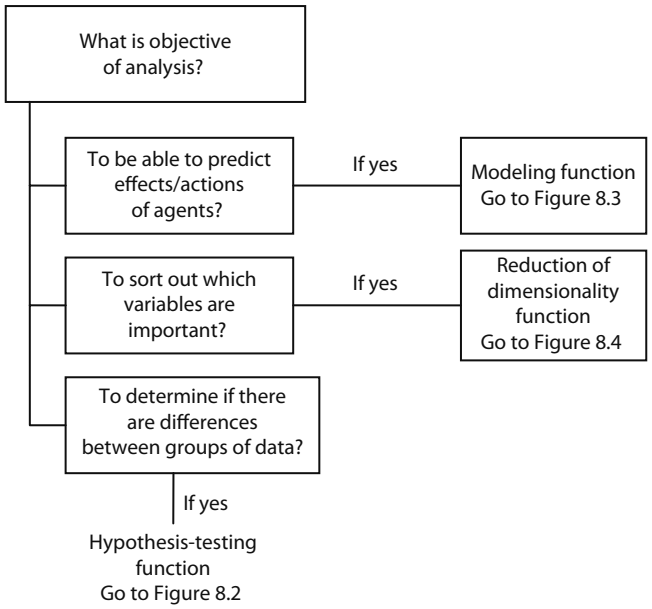


FIGURE 9.1 Overall decision tree for selecting statistical procedures.

GENERAL CONSIDERATIONS AND DATA CHARACTERIZATION FOR STATISTICAL ANALYSIS

Variables to Be Analyzed

Although some pathologists still regard their discipline as providing qualitative rather than quantitative data, it is abundantly clear that pathology, when applied to routine screening of animal toxicity and carcinogenicity studies, has to be quantitative to at least some degree so statistical statements can be made about possible treatment effects. Inevitably, there will be some descriptive text that will not be appropriate for statistical analysis. However, the main objective of the pathologist should be to provide information on the presence or absence (with severity grade or size where appropriate) of a list of conditions, consistently recorded from animal to animal by well-defined criteria, that can be validly used in a statistical assessment.

Given that statistical analysis is worth doing and data are available that would be analyzed, should one then analyze all the endpoints recorded? Some arguments have been put forward against analyzing all the endpoint studies, none of which really holds water. One argument is that some endpoints are not of interest. Perhaps the study is essentially a carcinogenicity study, so nonneoplastic endpoints are not considered to be background pathology and almost per se unrelated to treatment. In our view, this is illogical. If the pathologist has gone to the trouble of recording the data, then surely, in general, they ought to be analyzed; otherwise, why record them in the first place? After all, the costs of the statistical analyses are much less than those of doing the study and the pathology. While one might justify failure to analyze nonneoplastic data where tumor analysis

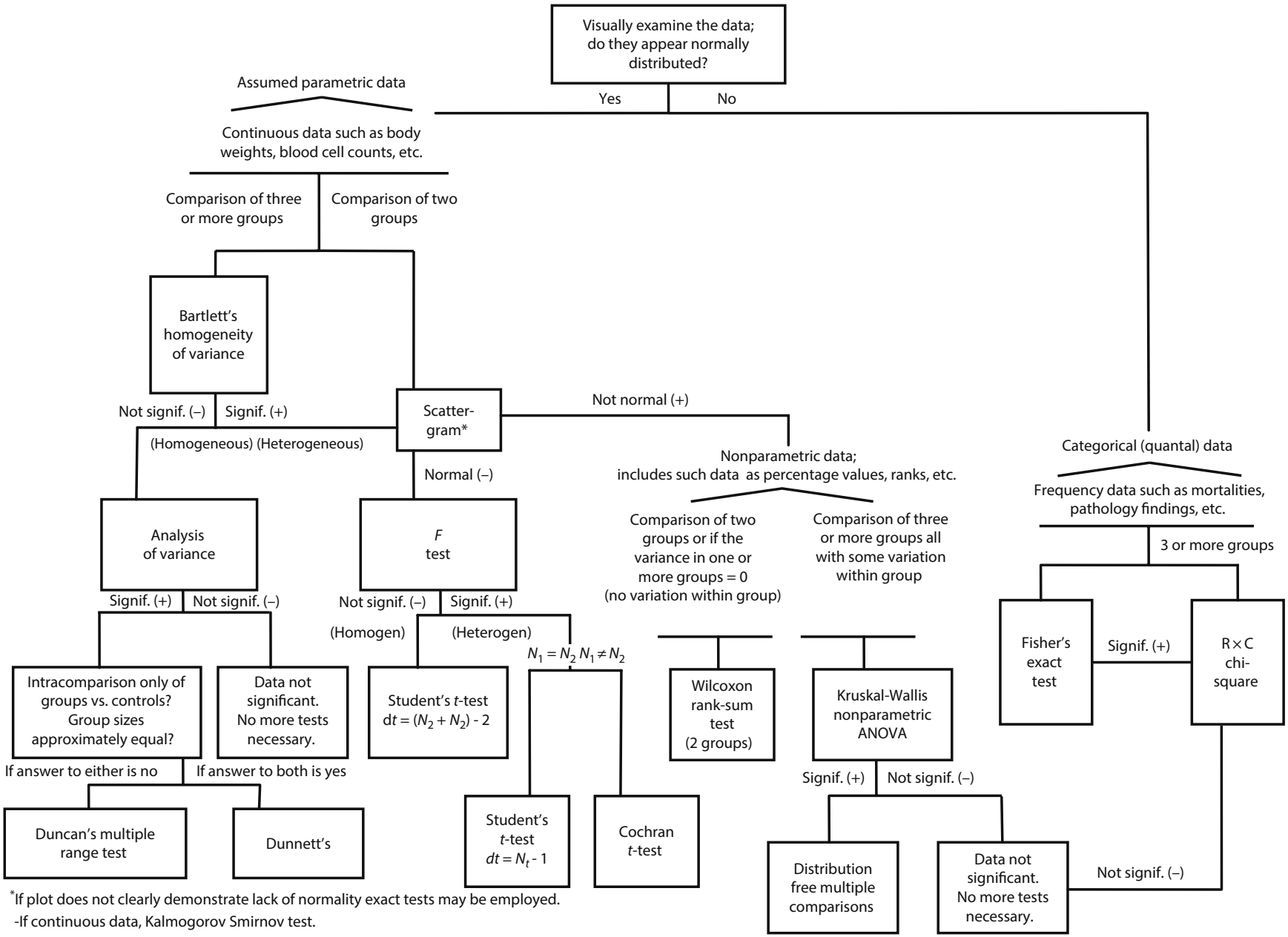


FIGURE 9.2 Decision tree for selecting hypothesis-testing procedures.

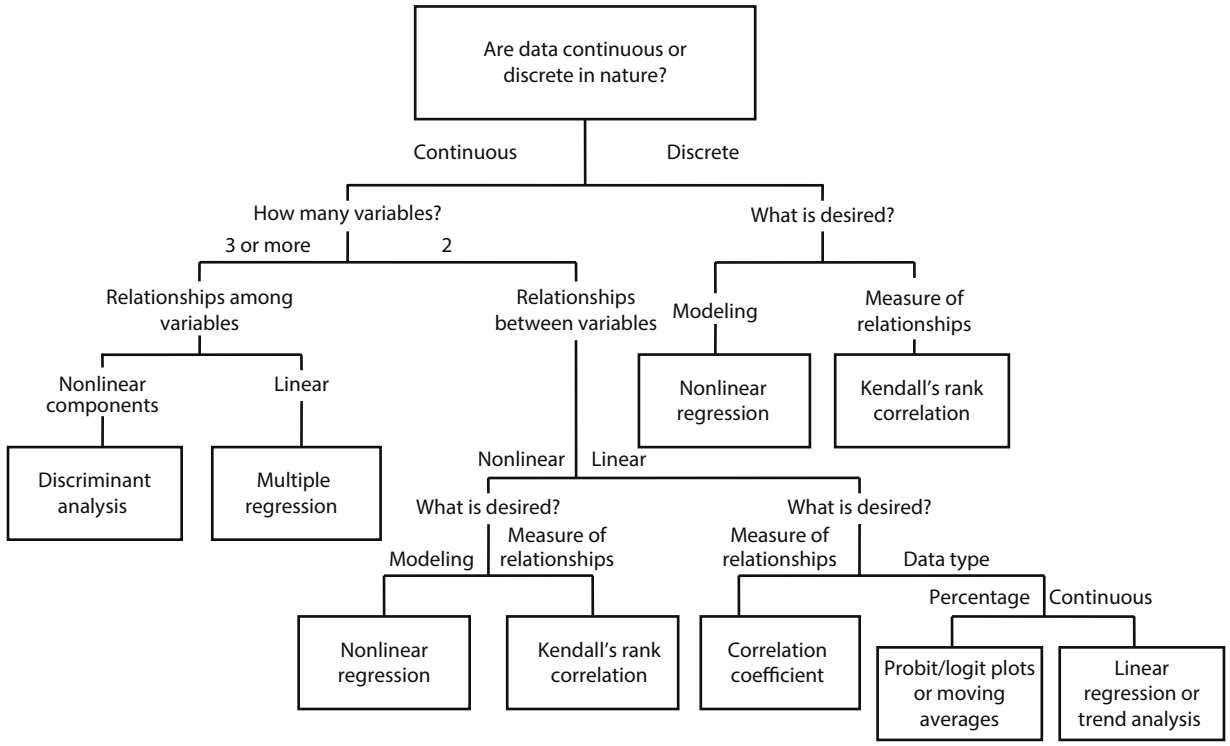


FIGURE 9.3 Decision tree for selecting modeling procedures.

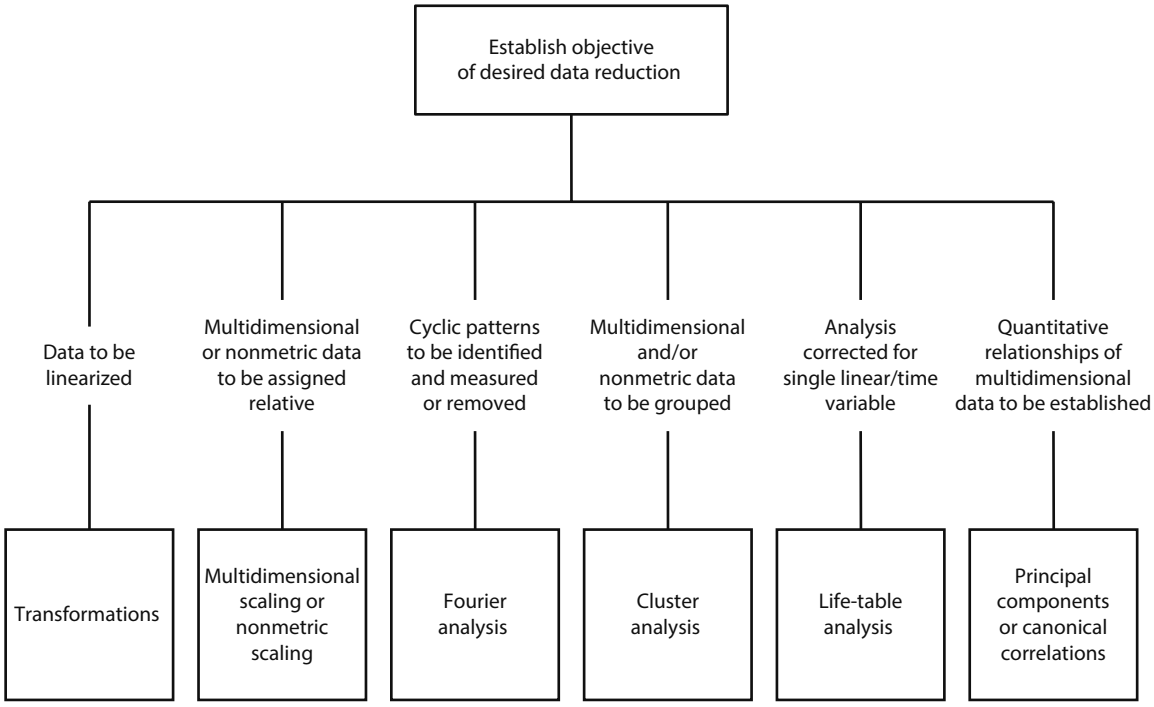


FIGURE 9.4 Decision tree for the selection of reduction of dimensionality procedures.

has already shown that the compound is clearly carcinogenic and no longer of market potential, the general rule ought to be to analyze everything that has been specifically investigated.

Another argument put forward against doing multiple analyses is that it may yield many chance significant p values that have to be explained away. This seems to us a poor reason for not exploring the data fully. A detailed look at the data can only aid interpretation, provided that one is not hide-bound by the false argument that statistical significance necessarily equates with biological importance and definitely indicates a true effect of treatment.

TABLE 9.6
Three Dimensions of Dose–Response

As Dose Increases
Incidence of responders in an exposed population increases.
Severity of response in affected individuals increases.
Time to occurrence of response or of progressive stage of response decreases.

Another reason not to analyze might be that visual inspection of summary tables reveals no suspicion of an effect for some endpoint. This seems to be, in this age of rapid and efficient computer programs, totally the wrong way to organize things. If the data are held on a computer, it is much better and quicker to do the actual analysis than to do the inevitably subjective, unreliable, and slow prescreening process. In any case, where substantial differences in survival between groups exist, it is very difficult to form a reliable view by inspection of non-age-adjusted frequencies on whether an effect might or might not have occurred.

A final, more valid, reason is that some endpoints occur only very rarely. One should, however, be clear what *very rarely* means. For a typical study with a control and three dose groups of equal size, one would get a significant trend statistics if all three cases occurred at the top dose level or in the control group (two-tailed $p \approx 0.03$), so a total of three cases will normally be enough for statistical analysis. Endpoints occurring once or twice only are not worth analyzing formally, although, if seen only in the top dose group, they may be worth noting in the report. This is especially true if they are lesions that are rarely reported (see Table 9.6).

Combination of Pathological Conditions

There are four main situations when one might consider combining pathological conditions in a statistical analysis. The first is when essentially the same pathological condition has been recorded under two or more different names or even under the same name in different places. Here, failure to combine these conditions in the analysis may severely limit the chances of detecting a true treatment effect. It should be noted, however, that grouping together conditions that are actually different may also result in the masking of a true treatment effect, particularly if the treatment has a very specific effect.

The second is when separately recorded pathological conditions form successive steps on the pathway of the same process. The most important example of this is the incidence of related types of malignant tumor, benign tumor, and focal hyperplasia. It will normally be appropriate to carry out analyses of (1) the incidence of malignant tumor, (2) the incidence of benign or malignant tumor, and, where appropriate, (3) the incidence of focal hyperplasia, benign tumor, or malignant tumor. It will not normally be appropriate to carry out analyses of benign tumor incidence only or of the incidence of hyperplasia only.

The third situation for combining is when the same pathological condition appears in different organs as a result of the same underlying process. Examples of this are the multicentric tumors (such as myeloid leukemia, reticulum cell sarcoma, and lymphosarcoma) or certain nonneoplastic conditions (such as arteritis/periarteritis and amyloid degeneration). Here, analysis will normally be carried out of only the incidence at any site, although in some situations, site-specific analyses might be worth carrying out.

The final situation where an analysis of combined pathological conditions is normal is for analyses of the overall incidence of malignant tumor at any site, of benign or malignant tumors at any site, or of multiple tumor incidence. Although analyses of tumor incidence at specific sites are normally more meaningful, because treatments often affect only a few specific sites, these additional analyses are usually required to guard against the possibility that treatment had some weak but general tumor-enhancing effect not otherwise evident.

In some situations, one might also envisage analyses of other combinations of specific tumors, such as tumors at related sites (e.g., endocrine organs if the compound had a hormonal effect) or of similar histological type.

Taking Severity into Account

The same line of argument that suggests that if the pathologist records data they should be analyzed also suggests that if the pathologist chooses to grade a condition for severity, then the grade should be taken into account in the analysis. There are two ways to carry out analysis when the grade has to be taken into account. In one, analyses are carried out not only of whether or not the animal has a condition but also of whether or not the condition is at least grade 2, at least grade 3, etc. In the other approach, nonparametric (rank) methods are used. The latter approach is more powerful, as it uses all the information in one analysis, although the output may not be so easily understood by those without some statistical training. Note that the analyses based on grade can be carried out only if grading has been consistently applied throughout. If a condition has been scored only as present/absent for some animals but has been graded for others, it is not possible to carry out graded analyses unless the pathologist is willing to go back and grade the specific animals showing the condition.

Using Simple Methods That Avoid Complex Assumptions

Different methods for statistical analysis can vary considerably in their complexity and in the number of assumptions they make. Although the use of statistical models has its place (more so for effect estimation than for hypothesis testing and more so in studies of complex design than in those of simple design), there are advantages in using, wherever possible, statistical methods that are simple, robust, and make

as few assumptions as possible. There are three reasons for this. First, such methods are more generally understandable to the toxicologist. Second, hardly ever enough data exist in practice to validate any given formal model fully. Third, even if a particular model is known to be appropriate, the loss of efficiency in using appropriate simpler methods is often only very small.

The methods we advocate for routine use for the analysis of tumor incidence tend, therefore, not to be based on the use of formal parametric statistical models. For example, when studying the relationship of treatment to incidence of a pathological condition and wishing to adjust for other factors (in particular, age at death) that might otherwise bias the comparison, methods involving stratification are recommended, rather than a multiple regression approach or time-to-tumor models. ANOVA methods can be useful in the case of continuously distributed data for estimating treatment effects; however, they involve underlying assumptions (normally distributed variables, variability equal in each group). If these assumptions are violated, nonparametric methods based on the rank of observations, rather than their actual value, may be preferable for hypothesis testing.

Using All of the Data

Often information is available about the relationship between treatment and a condition of interest for groups of animals differing systematically in respect to some other factor. Obvious examples are males and females, differing times of sacrifice and differing secondary treatments. Although it will be necessary, in general, to look at the relationship within levels of this other factor, it will also be advisable to try to come to some assessment of the relationship over all levels of the other factor and where a combined inference is not sensible, but in far more situations, this is not the case, and using all the data in one analysis allows a more powerful test of the relationship under study. Some scientists consider that conclusions for males and females should always be drawn separately, but there are strong statistical arguments for a joint analysis.

Combining, Pooling, and Stratification

Suppose, in a hypothetical study of a toxic agent that induces tumors that do not shorten the lives of tumor-bearing animals, the data regarding the number of animals with tumor out of the number examined are as shown in Table 9.7. It can be seen that if the time of death is ignored and the *pooled* data are studied, the incidence of tumors is the same in each group, resulting in the false conclusion that treatment had no effect. Looking within each time of death, however, an increased incidence in the exposed group can be seen. An appropriate statistical method would *combine* a measure of difference between the groups based on the early deaths and a measure of difference based on the late deaths and conclude correctly that incidence, after adjustment for time of death, is greater in the exposed groups.

TABLE 9.7
Dose–Response Effect on Time to Death

	Control	Exposed	Combined
Early deaths	1/20 (5%)	18/90 (20%)	19/110 (17%)
Late deaths	24/80 (30%)	7/10 (70%)	31/90 (34%)
Total	25/100 (25%)	25/100 (25%)	50/200 (25%)

In this example, time of death is the stratifying variable, with two strata—early deaths and late deaths. The essence of the methodology is to make comparisons only within strata (so one is always comparing like with like, except with respect to treatment) and then to combine the differences over strata. Stratification can be used to adjust for any variable or, indeed, combinations of variables.

Some studies are of factorial design, in which combinations of treatments are tested. The simplest such design is one in which four equal-sized groups of animals receive: (1) no treatment, (2) treatment A only, (3) treatment B only, and (4) treatments A and B. If one is prepared to assume that any effects of the two treatments are independent, one can use stratification to enable more powerful tests to be conducted of the possible individual treatment effects. Thus, to test for effects of treatment A, for example, one conducts comparisons in two strata, the first consisting of groups 1 and 2 not given treatment B and the second consisting of groups 3 and 4 given treatment B. Results combined from the two strata are based on twice as many animals and are therefore markedly more likely to detect possible effects of treatment A than is a simple comparison of groups 1 and 2. There is also the possibility of identifying interactions, such as synergism and antagonism, between the two treatments.

Multiple Control Groups

In some routine long-term screening studies, the study design involves 5 groups of (usually) 50 animals of each sex, 3 of which are treated with successive doses of a compound and 2 of which are untreated controls. Assuming that there is no systematic difference between the control groups (e.g., the second control group is in a different room or from a different batch of animals), it will be normal to carry out the main analyses with the control groups treated as a single group of 100 animals. It will usually be a sensible preliminary precaution to carry out additional analyses comparing incidences in the two control groups.

Trend Analysis, Low-Dose Extrapolation, and NOEL Estimation

Although comparisons of individual treated groups with the control group are important, a more powerful test of a possible effect of treatment will be to carry out a test for a dose-related trend. This is because most true effects of treatment tend to result in a response that increases (or decreases) with increasing dose and because trend tests take into account

all the data in a single analysis. In interpreting the results of trend tests, it should be noted that a significant trend does not necessarily imply an increased risk at lower doses, nor, conversely, does a lack of increase at lower doses necessarily indicate evidence of a threshold (i.e., a dose below which no increase occurs).

Note that the testing for trend is seen as a more sensitive way of picking up a possible treatment effect than simple pairwise comparisons of treated and control groups. Attempting to estimate the magnitude of effects at low doses, typically below the lowest positive dose tested in the study, is a much more complex procedure and is heavily dependent on the assumed functional form of the dose–response relationship.

Such low-dose extrapolation is typically conducted only for tumors believed to be caused by a genotoxic effect that some, but by no means all, scientists believe has no threshold. For other types of tumors and for many nonneoplastic endpoints, a threshold cannot be estimated directly from data at a limited number of dose levels and a no-observed-effect level can be estimated by finding the highest dose level at which there is no significant increase in effects.

Need for Age Adjustment

When marked differences in survival occur between treated groups, it is widely recognized that there is a need for an age adjustment (i.e., an adjustment for age at death or onset). This is illustrated in the earlier example, where, because of the greater number of deaths occurring early in the treated group, the true effect of treatment disappears if no adjustment is made. Thus, a major purpose of age adjustment is to avoid bias. It is not so generally recognized, however, that, even where there are no survival differences, age adjustment can increase the power to detect between-group differences. This is illustrated in Table 9.8. Here, treatment results in a somewhat earlier onset of a condition that occurs eventually in all animals. Failure to age-adjust will result in a comparison of 29/50 with 21/50, which is not statistically significant. Age adjustment will essentially ignore the early and late deaths, which contribute no comparative statistical information and will be based on the comparison of 9/10 with 1/10, which is statistically significant. Here, age adjustment sharpens the contrast, rather than avoiding bias, by avoiding the dilution of data capable of detecting treatment effects with data that are of little or no value for this purpose.

TABLE 9.8
Lethality Incidence

	Control	Exposed
Early deaths	0/20	0/20
Middle deaths	1/10	9/10
Late deaths	20/20	20/20
Total	21/50	29/50

Need to Take Context of Observation into Account

It is now widely recognized that age adjustment cannot properly be carried out unless the context of observation is taken into account. Three contexts are relevant, the first two relating to the situation where the condition is only observed at death (e.g., an internal tumor) and the third where it can be observed in life (e.g., a skin tumor). In the first context, the condition is assumed to have caused the death of the animal (i.e., to be *fatal*). In this case, the incidence rate for a time interval and a group is calculated by

$$\frac{\text{Number of animals dying in interval because of lesion}}{\text{Number of animals alive at start of interval}}$$

In the second context, the animal is assumed to have died of another cause (i.e., the condition is *incidental*). In this case, the rate is calculated by

$$\frac{\text{Number of animals dying in interval with lesion}}{\text{Total number of animals dying in interval}}$$

In the third context, where the condition is *visible*, the rate is calculated by

$$\frac{\text{Number of animals getting condition in interval}}{\text{Number of animals without condition at start of interval}}$$

A problem with the method of Peto et al. [9], which takes the context of observation into account, is that some pathologists are unwilling or feel unable to decide whether, in any given case, a condition is fatal or incidental. A number of points should be made here. First, where there are marked survival differences, it may not be possible to conclude reliably whether a treatment is beneficial or harmful unless such a decision is made. This is well illustrated by the example in Peto et al. [9], where assuming that all pituitary tumors were fatal resulted in the (false) conclusion that *N*-nitrosodimethylamine (NDMA) was carcinogenic and assuming that they were all incidental resulted in the (false) conclusion that NDMA was protective. Using, correctly, the pathologist's best opinion as to which were and which were not likely to be fatal resulted in an analysis that (correctly) concluded that NDMA had no effect. If the pathologist in this case had been unwilling to make a judgment as to fatality, believing it to be unreliable, no conclusion could have been reached. This state of affairs would, however, be a fact of life and *not* a position reached because an inappropriate statistical method was being used.

Although it will normally be a good routine for the pathologist to ascribe *factors contributory to death* for each animal that was not part of a scheduled sacrifice, it is in fact not strictly necessary to determine the context of observation for all conditions at the outset. An alternative strategy is to analyze under differing assumptions: (1) no cases fatal,

(2) all cases occurring in descendents fatal, and (3) all cases of same defined severity occurring in decedents fatal, with, under each assumption, other cases incidental.

If the conclusion turns out the same under each assumption or if the pathologist can say, on general grounds, that one assumption is likely to be a close approximation to the truth, it may not be necessary to know the context of observation for the condition in question for each individual animal. Using the alternative strategy might result in a saving of the pathologist's time by only having to make a judgment for a limited number of conditions where the conclusion seems to hang on the correct knowledge of the context of observation.

Finally, it should be noted that, although many nonneoplastic conditions observed at death are never causes of death, it is, in principle, as necessary to know the context of observation for nonneoplastic conditions as it is for tumors.

Experimental and Observational Units

In many situations, the animal is both the experimental unit and the observational unit, but this is not always so. To determine treatment effects by the methods of the next section, it is important that each experimental unit provides only one item of data for analysis, as the methods all assume that individual data items are statistically independent. In many feeding studies, where the cage is assigned to a treatment, it is the cage, rather than the animal, that is the experimental unit. In histopathology, observations for a tissue are often based on multiple sections per animal, so the section is the observational unit. Multiple observations per experimental unit should be combined in some suitable way into an overall average for that unit before analysis.

Missing Data

In many types of analysis, animals with missing data are simply removed from the analysis; however, in some situations, this can be an inappropriate thing to do. One situation is when carrying out an analysis of a condition that is assumed to have caused the death of the animal.

Although an animal dying at week 83 for which the section was unavailable for microscopic examination cannot contribute to the group comparison at week 83, one knows that it did not die because of any condition in previous weeks, so it should contribute to the denominator of the calculations in all previous weeks.

Another situation is when histopathological examination of a tissue is not carried out unless an abnormality is seen post mortem. In such an experiment, one might have the following data for that tissue:

- *Control group*—50 animals, 2 abnormal post mortem, 2 examined microscopically, 2 with tumor of specific type
- *Treated group*—50 animals, 15 abnormal post mortem, 15 examined microscopically, 14 with tumor of specific type

Ignoring animals with no microscopic sections, one would compare 2/2 = 100% with 14/15 = 93% and conclude that treatment nonsignificantly decreased incidence. This is likely to be a false conclusion, and it would be better here to compare the percentages of animals that had a post mortem abnormality that turned out to be a tumor—that is, 2/50 = 4% with 14/50 = 28%. Unless some aspects of treatment made tumors much easier to detect at post mortem, one could then conclude that treatment did have an effect on tumor incidence.

Particular care has to be taken in studies where the procedures for histopathological examination vary by group. In a number of studies conducted in recent years, the protocol has demanded full microscopic examination of a given tissue list in decedents in all groups and in terminally killed controls in high-dose animals. In other animals, terminally killed low- and mid-dose animals, microscopic examination of a tissue is conducted only if the tissue is found to be abnormal at post mortem. Such a protocol is designed to save money but leads to difficulty in comparing the treatment groups validly. Suppose, for example, that responses in terminally killed animals are 8/20 in the controls, 3/3 (with 17 unexamined) in the low-dose animals, and 5/6 (with 14 unexamined) in the mid-dose animals. Is one supposed to conclude that treatment at the low and mid doses increased response, based on a comparison of the proportions examined microscopically (40%, 100%, and 83%), or that it decreased response, based on the proportion of animals in the group (40%, 15%, and 25%)? It could well be that treatment had no effect but some small tumors were missed at post mortem. In this situation, a valid comparison can be achieved only by ignoring the low- and mid-dose groups when carrying out the comparison for the age stratum *terminal kill*. This, of course, seems wasteful of data, but these are data that cannot be usefully used due to the inappropriate protocol.

USE OF HISTORICAL CONTROL DATA

In some situations, particularly where incidences are low, the results from a single study may suggest an effect of treatment on tumor incidence but be unable to demonstrate it conclusively. The possibility of comparing results in the treated groups with those of control groups from other studies is then often raised; thus, a nonsignificant incidence of 2 cases out of 50 in a treated group may seem much more significant if no cases have been seen in, say, 1000 animals representing controls from 20 similar studies. Conversely, a significant incidence of 5 cases out of 50 in a treated group as compared with 0 out of 50 in the study controls may seem far less convincing if many other control groups had incidences around 5 out of 50. While not understating the importance of looking at historical control data, it must be emphasized that there are a number of reasons why variation between studies may be greater than variation within a study. Differences in diet, in duration of the study, in intercurrent mortality, and in who the study pathologist is may all contribute. Statistical techniques that ignore this and carry out simple statistical tests of

treatment incidence against a pooled control incidence may well give results that are seriously in error and are likely to overstate statistical significance considerably.

Methods for Data Examination and Preparation

The data from toxicology studies should always be examined before any formal analysis. Such examinations should be directed to determining if the data are suitable for analysis and, if so, what form the analysis should take (see Figure 9.2). If the data as collected are not suitable for analysis or if they are suitable only for low-powered analytical techniques, one may wish to use one of the many forms of data transformation to change the data characteristics so they are more amenable to analysis. For data examination, two major techniques are presented here: the *scattergram* and *Bartlett's test*. Likewise, for data preparation, two techniques are presented: *randomization* (including a test for randomness in a sample of data) and *transformation*. Exploratory data analysis (EDA) is presented and briefly reviewed later. This is a broad collection of techniques and approaches to probe data—that is, to both examine and perform some initial flexible analysis of the data.

Scattergram and Bartlett's Test

Two of the major points to be made throughout this chapter are (1) the use of the appropriate statistical tests and (2) the effects of small sample sizes (as is often the case in toxicology) on our selection of statistical techniques. Frequently, simple examination of the nature and distribution of data collected from a study can also suggest patterns and results that were unanticipated and for which the use of additional or alternative statistical methodology is warranted. It was these points that caused the author to consider a section on scattergrams and their use essential for toxicologists.

Bartlett's test may be used to determine if the values in groups of data are homogeneous. If they are, this (along with the knowledge that they are from a continuous distribution) demonstrates that parametric methods are applicable. But if the values in the (continuous data) groups fail Bartlett's test (i.e., are heterogeneous), we cannot be secure in our belief that parametric methods are appropriate until we gain some confidence that the values are normally distributed. With large groups of data, we can compute parameters of the population (kurtosis and skewness, in particular) and from these parameters determine if the population is normal (with a certain level of confidence). If our concern is especially marked, we can use a chi-square goodness-of-fit test for normality. But when each group of data consists of 25 or fewer values, these measures or tests (kurtosis, skewness, and chi-square goodness-of-fit) are not accurate indicators of normality. Instead, in these cases, we should prepare a scattergram of the data and then evaluate the scattergram to estimate if the data are normally distributed. This procedure consists of developing a histogram of the data and examining the histogram to gain a visual appreciation of the location and distribution of the data.

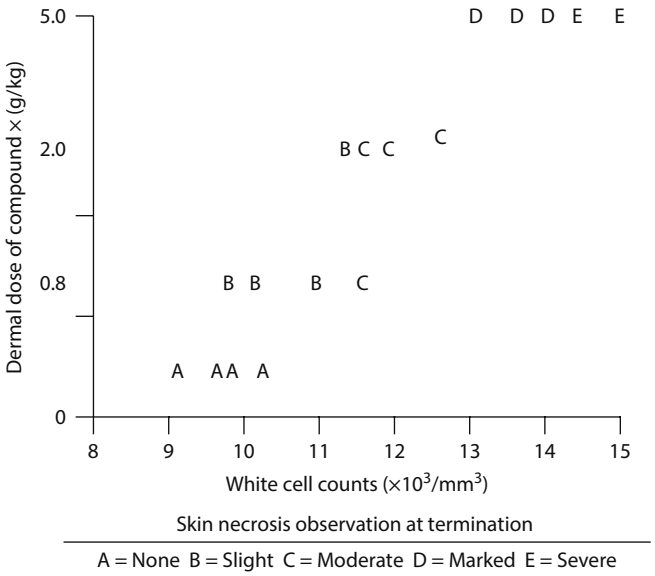


FIGURE 9.5 Exploratory data analysis.

The abscissa (or horizontal scale) should be in the same scale as the values and should be divided so the entire range of observed values is covered by the scale of the abscissa. Across such a scale, we then simply enter symbols for each of our values. Example 9.1 shows such a plot. Example 9.1 is a traditional and rather limited form of scatterplot, but such plots can reveal significant information about the amount and types of association between the two variables, the existence and nature of outliers, the clustering of data, and a number of other two-dimensional factors [10,11].

Current technology allows us to add significantly more graphical information to scatterplots by means of graphic symbols (letters, faces, or different shapes, such as squares and colors) for the plotted data points. One relatively simple example of this approach is shown in Figure 9.5, where the simple case of dose (in a dermal study), dermal irritation, and white blood cell (WBC) count is presented. This graph quite clearly suggests that as dose (variable x) is increased, dermal irritation (variable y) also increases; as irritation becomes more severe, WBC count (variable z), an indicator of immune system involvement suggesting infection or persistent inflammation, also increases. There is no direct association of variables x and z, however. Cleveland and McGill [12] presented an excellent, detailed overview of the expanded capabilities of the scatterplot, and the interested reader should refer to that article. Cleveland later expanded this to a book [13]. Tufte [14] has also expanded on this.

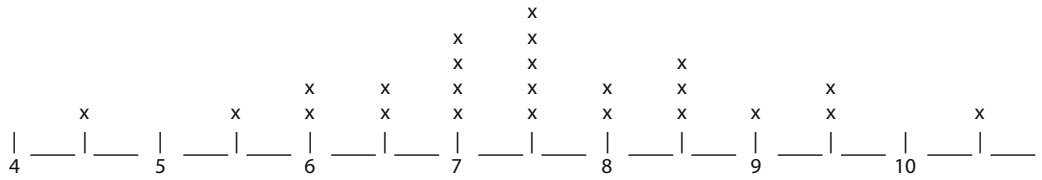
Suppose we have the following two datasets:

Group 1—4.5, 5.4, 5.9, 6.0, 6.4, 6.5, 6.9, 7.0, 7.1, 7.0, 7.4, 7.5, 7.5, 7.5, 7.6, 8.0, 8.1, 8.4, 8.5, 8.6, 9.0, 9.4, 9.5, and 10.4

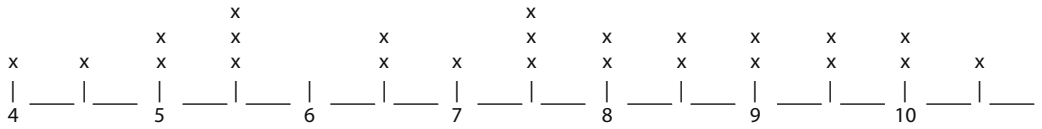
Group 2—4.0, 4.5, 5.0, 5.1, 5.4, 5.5, 5.6, 6.5, 6.5, 7.0, 7.4, 7.5, 7.5, 8.0, 8.1, 8.5, 8.5, 9.0, 9.1, 9.5, 9.5, 10.1, 10.0, and 10.4

Both of these groups contain 24 values and cover the same range. From them, we can prepare the following scattergrams:

Group 1



Group 2



Group 1 can be seen to approximate a normal distribution (bell-shaped curve); we can proceed to perform the appropriate parametric tests with such data. But group 2 clearly does not appear to be normally distributed; in this case, the appropriate nonparametric technique must be used.

Example 9.1

BARTLETT'S TEST FOR HOMOGENEITY OF VARIANCE

Bartlett's test [15] is used to compare the variances (values reflecting the degree of variability in datasets) among three or more groups of data, where the data in the groups are continuous sets (such as body weights, organ weights, red blood cell [RBC] counts, or diet consumption measurements). It is expected that such data will be suitable for parametric methods (normality of data is assumed), and Bartlett's test is frequently used as a test for the assumption of equivalent variances.

Bartlett's test is based on the calculation of the corrected χ^2 (chi-square) value by the formula:

$$\chi^2_{corr} = 2.3026 \frac{\sum \mathrm{df}\left(\log_{10}\left[\sum \mathrm{df}\left(S^2\right)/\sum \mathrm{df}\right]\right) - \sum\left[\mathrm{df}\left(\log_{10} S^2\right)\right]}{1+\frac{1}{3(K-1)}\left[\sum\frac{1}{\mathrm{df}} - \frac{1}{\sum \mathrm{df}}\right]}$$

where S^2 = variance, equal to

$$\frac{n\sum X^2 - \left(\sum X\right)^2}{(n/n-1)}$$

where

X is the individual datum within each group
n is the number of data within each group
df is the degrees of freedom for each group = (N – 1)
K is the number of groups being compared

The corrected χ^2 value yielded by the earlier calculations is compared to the values listed in the chi-square table according to the numbers of degrees of freedom [16]. If the calculated value is smaller than the table value at the selected p level (traditionally 0.05), the groups are accepted to be homogeneous, and the use of ANOVA is assumed proper. If the calculated χ^2 is greater than the table value, the groups are heterogeneous and other tests (as indicated by the decision tree in Figure 9.2) are necessary.

Assumptions and Limitations

- Bartlett's test does not test for normality but rather homogeneity of variance (also called equality of variances or homoscedasticity).
- Homoscedasticity is an important assumption for Student's *t*-test, ANOVA, and analysis of covariance (ANCOVA).
- The *F*-test is actually a test for the two-sample (i.e., control and one test group) case of homoscedasticity. Bartlett's test is designed for three or more samples.
- Bartlett's test is very sensitive to departures from normality. As a result, a finding of a significant chi-square value in Bartlett's test may indicate nonnormality rather than heteroscedasticity. Such a finding can be brought about by outliers, and the sensitivity to such erroneous findings is extreme with small sample sizes.

Statistical Goodness-of-Fit Tests

A goodness-of-fit test is a statistical procedure for comparing individual measurements to a specified type of statistical distribution; for example, a normal distribution is completely specified by its arithmetic mean and variance (the square of the SD). The null hypothesis that the data represent a sample from a single normal distribution can be tested by a statistical goodness-of-fit test. Various goodness-of-fit tests have been devised to determine if the data deviate significantly from a specified distribution. If a significant departure occurs, it

indicates that only the specified distribution can be rejected with some assurance. This does not necessarily mean that the true distribution contains two or more subpopulations. The true distribution may be a single distribution, based on a different mathematical relationship (e.g., lognormal). In the latter case, logarithms of the measurement would not be expected to exhibit by a goodness-of-fit test a statistically significant departure from a lognormal distribution.

Everitt and Hand [17] recommended the use of a sample of 200 or more to conduct a valid analysis of mixtures of populations. Even the maximum likelihood method, the best available method, should be used with extreme caution, or not at all, when separation between the means of the subpopulations is less than 3 SD and sample sizes are less than 300. None of the available methods conclusively establishes bimodality, which may, however, occur when separation between the two means (modes) exceeds 2 SD. Conversely, inflections in probits or separations in histograms less than 2 SD apart may arise from genetic differences in test subjects.

Mendell et al. [18] compared eight tests of normality to detect a mixture consisting of two normally distributed components with different means but equal variances. Fisher's skewness statistic was preferable when one component comprised less than 15% of the total distribution. When the two components comprised more nearly equal proportions (35%–65%) of the total distribution, the Engelman and Hartigan test [19] was preferable. For other mixing proportions, the maximum likelihood ratio test was best; thus, the maximum likelihood ratio test appears to perform very well, with only small loss from optimality, even when it is not the best procedure.

The method of *maximum likelihood* provides estimators that are usually quite satisfactory. They have the desirable properties of being consistent, asymptotically normal, and asymptotically efficient for large samples under quite general conditions. They are often biased, but the bias is frequently removable by a simple adjustment. Other methods of obtaining estimators are also available, but the maximum likelihood method is the most frequently used.

Maximum likelihood estimators also have another desirable property: *invariance*. Let us denote the maximum likelihood estimator of the parameter θ by $\hat{\sigma}$. Then, if $f(\theta)$ is a single-valued function of θ, the maximum likelihood estimator of $f(\theta)$ is $f(\hat{\sigma})$. Thus, for example,

$$\hat{\sigma} = \left(\hat{\sigma}^2\right)^{1/2}$$

The principle of maximum likelihood tells us that we should use that value as our estimate, which maximizes the likelihood of the observed event.

These maximum likelihood methods can be used to obtain *point estimates* of a parameter, but we must remember that a point estimator is a random variable distributed in some way around the true value of the parameter. The true parameter value may be higher or lower than our estimate. It is often useful therefore to obtain an interval within which we are reasonably confident the true value will lie, and the generally accepted method is to construct what are known as *confidence limits*.

The following procedure will yield upper and lower 95% confidence limits with the property that, when we say that these limits include the true value of the parameter, 95% of all such statements will be true and 5% will be incorrect:

1. Choose a (test) statistic involving the unknown parameter and no other unknown parameter.
2. Place the appropriate sample values in the statistic.
3. Obtain an equation for the unknown parameter by equating the test statistic to the upper 2.5% point of the relevant distribution.
4. The solution of the equation gives one limit.
5. Repeat the process with the lower 2.5% point to obtain the other limit.

One can also construct 95% CIs using unequal tails (e.g., using the upper 2% point and the lower 3% point). We usually want our CI to be as short as possible, however, and with a symmetric distribution such as the normal or *t*, this is achieved using equal tails. The same procedure very nearly minimizes the CI with other nonsymmetric distributions (e.g., chi-square) and has the advantage of avoiding rather tedious computation.

When the appropriate statistic involves the square of the unknown parameter, both limits are obtained by equating the statistic to the upper 5% point of the relevant distribution. The use of two tails in this situation would result in a pair of nonintersecting intervals. When two or more parameters are involved, it is possible to construct a region within which, we are reasonably confident, the true parameter values will lie. Such regions are referred to as *confidence regions*. The implied interval for p_1 does not form a 95% CI, however, nor is it true that an 85.7375% confidence region for p_1, p_2, and p_3 can be obtained by considering the intersection of the three separate 95% CIs, because the statistics used to obtain the individual CIs are not independent. This problem is obvious with a multiparameter distribution such as the multinomial, but it even occurs with the normal distribution because the statistic that we use to obtain a CI for the mean and the statistic that we use to obtain a CI for the variance are not independent. The problem is not likely to be of great concern unless a large number of parameters are involved.

Randomization

Randomization is the act of assigning a number of items (plates of bacteria or test animals, for example) to groups in such a manner that there is an equal chance for any one item to end up in any one group. This is a control against any possible bias in the assignment of subjects to test groups. A variation on this is censored randomization, which ensures that the groups are equivalent in some aspect after the assignment process is complete. The most common example of a

censored randomization is one in which it is ensured that the body weights of test animals in each group are not significantly different from those in the other groups. This is done by analyzing group weights both for homogeneity of variance and by ANOVA after animal assignment, then rerandomizing if there is a significant difference at some nominal level, such as $p \leq 0.10$. The process is repeated until there is no significant difference.

There are several methods for actually performing the randomization process. The three most commonly used are card assignment, use of a random number table, and use of a computerized algorithm. For the card-based method, individual identification numbers for items (plates or animals, for example) are placed on separate index cards. These cards are then shuffled and placed one at a time in succession into piles corresponding to the required test groups. The results are a random group assignment.

The random number table method requires only that one have unique numbers assigned to test subjects and access to a random number table. One simply sets up a table with a column for each group to which subjects are to be assigned. We start from the head of any one column of numbers in the random table (each time the table is used, a new starting point should be utilized). If our test subjects number less than 100, we utilize only the last 2 digits in each random number in the table. If they number more than 99 but less than 1000, we use only the last 3 digits. To generate group assignments, we read down a column, one number at a time. As we come across digits that correspond to a subject number, we assign that subject to a group (enter its identifying number in a column), proceeding to assign subjects to groups from left to right and filling one row at a time. After a number is assigned to an animal, any duplication of its unique number is ignored. We use as many successive columns of random numbers as we may need to complete the process.

The third (and now most common) method is to use a random number generator that is built into a calculator or computer program. Procedures for generating these are generally documented in user manuals.

One is also occasionally required to evaluate whether a series of numbers (such as an assignment of animals to test groups) is random. This requires the use of a randomization test, of which there are a large variety. The chi-square test can be used to evaluate the goodness-of-fit to a random assignment. If the result is not critical, a simple sign test will work. For the sign test, we first determine the middle value in the numbers being checked for randomness. We then go through a list of the numbers assigned to each group, scoring each as a "+" (greater than our middle number) or a "–" (less than our middle number). The number of pluses and minuses in each group should be approximately equal.

Transformations

If our initial inspection of a dataset reveals it to have an unusual or undesired set of characteristics (or to lack a desired set of characteristics), we have a choice of three courses of action. We may proceed to select a method or test appropriate to this new set of conditions or abandon the entire exercise or transform the variables under consideration in such a manner that the resulting transformed variates (X' and Y', e.g., as opposed to the original variates X and Y) meet the assumptions or have the characteristics that are desired.

The key to all this is that the scale of measurement of most (if not all) variables is arbitrary. Although we are most familiar with a linear scale of measurement, there is nothing that makes this the correct scale on its own, as opposed to a logarithmic scale (familiar logarithmic measurements are pH values or earthquake intensity [Richter scale]). Transforming a set of data (converting X to X') is really as simple as changing a scale of measurement.

There are at least four good reasons to transform data:

1. Normalize the data, making them suitable for analysis by our most common parametric techniques such as ANOVA. A simple test of whether a selected transformation will yield a distribution of data that satisfies the underlying assumptions for ANOVA is to plot the cumulative distribution of samples on probability paper (commercially available paper that has the probability function scale as one axis). One can then alter the scale of the second axis (i.e., the axis other than the one that is on a probability scale) from linear to any other (logarithmic, reciprocal, square root, etc.) and see if a previously curved line indicating a skewed distribution becomes linear to indicate normality. The slope of the transformed line gives us an estimate of the SD. And if the slopes of the lines of several samples or groups of data are similar, we accordingly know that the variances of the different groups are homogeneous.
2. Linearize the relationship between a paired set of data, such as dose and response. This is the most common use in toxicology for transformations and is demonstrated in the "Probit/Log Transforms and Regression" section.
3. Adjust data for the influence of another variable. This is an alternative in some situations to the more complicated process of ANCOVA. A ready example of this usage is the calculation of organ weight to body weight ratios in in vivo toxicity studies, with the resulting ratios serving as the raw data for an ANOVA performed to identify possible target organs.
4. Make the relationships between variables clearer by removing or adjusting for interactions with third, fourth, etc., uncontrolled variables that influence the pair of variables of interest.

Common transformations are presented in Table 9.9.

Exploratory Data Analysis

Over the past 20 years, an entirely new approach has been developed to get the most information out of the increasingly

TABLE 9.9
Common Data Transformations

Transformation	How Calculated[a]	Example of Use
Arithmetic	$x' = x/y$ or $x' = x + c$	Organ weight/body weight
Reciprocals	$x' = 1/x$	Linearizing data, particularly rate phenomena
Arcsine (also called angular)	$x' = \arcsine\sqrt{x}$	
Logarithmic	$x' = \log x$	pH Values
Probability (probit)	x' = probability x	Percentage responding[b]
Square roots	$x' = \sqrt{x}$	Surface area of animal from body weights
Box Cox	$x' = (x^v - 1)v$ for $v \neq 0$	A family of transforms for use when one has no prior knowledge of the appropriate transformation to use
	$x' = \ln x$ for $v = 0$	
Rank transformations	Depends on the nature of samples	As a bridge between parametric and nonparametric statistics

[a] x and y are original variables; x' and y' transformed values; c stands for a constant.
[b] Plotting a double reciprocal (i.e., $1/x$ vs. $1/y$) will linearize almost any dataset, so will plotting the log transforms of a set of variables.

larger and more complex datasets that scientists are faced with. This approach involves the use of a very diverse set of fairly simple techniques that comprise EDA. As expounded by Tukey [20], there are four major ingredients to EDA:

- *Displays*—These visually reveal the behavior of the data and suggest a framework for analysis. The scatterplot (presented earlier) is an example of this approach.
- *Residuals*—These are what remain of a set of data after a fitted model (such as a linear regression) or some similar level of analysis has been removed.
- *Reexpressions*—These involve questions of what scale would serve to best simplify and improve the analysis of the data. Simple transformations, such as those presented earlier in this chapter, are used to simplify data behavior (e.g., linearizing or normalizing) and clarify analysis.
- *Resistance*—This is a matter of decreasing the sensitivity of analysis and summary of data to misbehavior so the occurrence of a few outliers, for example, will not complicate or invalidate the methods used to analyze the data. For example, in summarizing the location of a set of data, the median (but not the arithmetic mean) is highly resistant.

These four ingredients are utilized in a process falling into two broad phases: an exploratory phase and a confirmatory phase. The exploratory phase isolates patterns in and features of the data and reveals them, allowing an inspection of the data before there is any firm choice of actual hypothesis-testing or modeling methods has been made.

Confirmatory analysis allows evaluation of the reproducibility of the patterns or effects. Its role not only is close to that of classical hypothesis testing but also often includes steps such as (1) incorporating information from an analysis of another closely related set of data and (2) validating a result by assembling and analyzing additional data. These techniques are in general beyond the scope of this text; however, Velleman and Hoaglin [21] and Hoaglin et al. [22] present a clear overview of the more important methods, along with codes for their execution on a microcomputer (they have also now been incorporated into Minitab). A short examination of a single case of the use of these methods, however, is in order.

Toxicology has long recognized that no population—animal or human—is completely uniform in its response to any particular toxicant. Rather, a population is composed of a (presumably normal) distribution of individuals, some resistant to intoxication (hyporesponders), the bulk responding close to a central value (such as an LD_{50}), and some being very sensitive to intoxication (hyperresponders). This population distribution can, in fact, result in additional statistical techniques. The sensitivity of techniques such as ANOVA is reduced markedly by the occurrence of outliers (extreme high or low values, including hyper- and hyporesponders), which, in fact, serve to markedly inflate the variance (SD) associated with a sample. Such variance inflation is particularly common in small groups that are exposed or dosed at just over or under a threshold level, causing a small number of individuals in the sample (who are more sensitive than the other members) to respond markedly. Such a situation is displayed in Figure 9.6, which plots the mean and SDs of methemoglobin levels in a series of groups of animals exposed to successively higher levels of a hemolytic agent.

Though the mean level of methemoglobin in group C is more than double that of the control group (A), no hypothesis test will show this difference to be significant because it has such a large SD associated with it. Yet this inflated variance exists because a single individual has such a marked response. The occurrence of the inflation is certainly an indicator that the data need to be examined closely. Indeed, all tabular data in toxicology should be visually inspected for both trend and variance inflation.

A concept related (but not identical) to resistance and EDA is that of *robustness*. Robustness generally implies insensitivity to departures from assumptions surrounding an

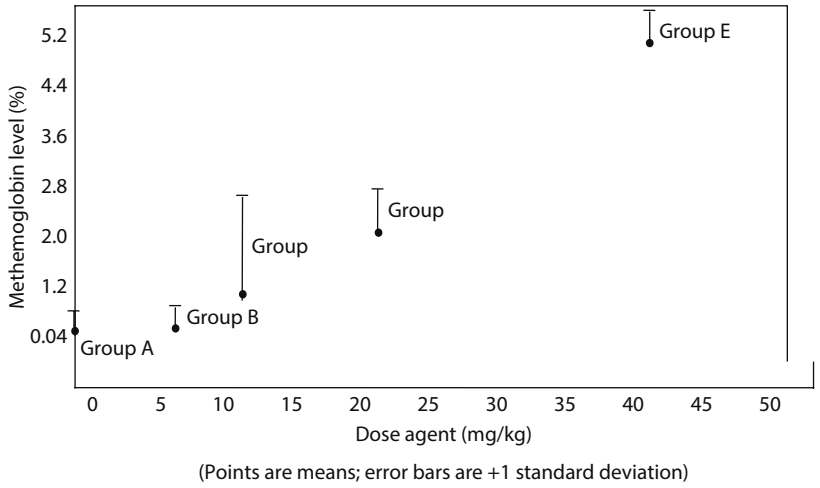


FIGURE 9.6 Variance inflation.

underlying model, such as normality. When summarizing the location of data, the median, though highly resistant, is not extremely robust, but the mean is both nonresistant and nonrobust.

HYPOTHESIS TESTING OF CATEGORICAL AND RANKED DATA

Categorical (or contingency table) presentations of data can contain any single type of data, but generally the contents are collected and arranged so they can be classified as belonging to treatment and control groups, with the members of each of these groups then classified as belonging to one of two or more response categories (such as tumor/no tumor or normal/hyperplastic/neoplastic). For these cases, two forms of analysis are presented: Fisher's exact test (for the 2 × 2 contingency table) and the R × C (R, row; C, column) chi-square test (for large tables). It should be noted, however, that versions of both of these tests permit the analysis of any size of contingency table.

The analysis of rank data—what is generally called *nonparametric statistical analysis*—is an exact parallel of the more traditional (and familiar) parametric methods. There are methods for the single-comparison case (just as Student's *t*-test is used) and for the multiple-comparison case (just as ANOVA is used) with appropriate post hoc tests for exact identification of the significance with a set of groups. Four tests are presented for evaluating statistical significance in rank data: the Wilcoxon rank-sum test, distribution-free multiple comparisons, Mann–Whitney U test, and the Kruskal–Wallis nonparametric ANOVA. For each of these tests, tables of distribution values for the evaluations of results can be found in any of a number of reference volumes [23]. It should be clearly understood that, for data that do not fulfill the necessary assumptions for parametric analysis, these nonparametric methods are either as powerful or, in fact, more powerful than the equivalent parametric test.

Fisher's Exact Test

Fisher's exact test should be used to compare two sets of discontinuous quantal (all or none) data. Small sets of such data can be checked by contingency data tables, such as those of Finney et al. [24]. Larger sets, however, require computation. These include frequency data such as incidences of mortality or certain histopathological findings. Thus, the data can be expressed as ratios. These data do not fit on a continuous scale of measurement but usually involve numbers of responses classified as either negative or positive—that is, a contingency table situation [15].

The analysis is started by setting up a 2 × 2 contingency table to summarize the numbers of positive and negative responses, as well as the totals of these, as follows:

	Positive	Negative	Total
Group I	A	B	$A + B$
Group II	C	D	$C + D$
Totals	$A + C$	$B + D$	$A + B + C + D = N_{total}$

Using the earlier set of symbols, the formula for P appears as follows*:

$$P = \frac{(A+B)!(C+D)!(A+C)!(B+D)!}{N!A!B!C!D!}$$

The exact test produces a probability (P) that is the sum of the earlier calculation repeated for each possible arrangement of the numbers in the earlier cells (i.e., A, B, C, and D) showing an association equal to or stronger than that between the two variables. The P resulting from these computations will be the exact one- or two-tailed probability depending on which of these two approaches is being employed. This value tells us if the groups differ significantly (with a probability less than 0.05, say) and the degree of significance.

* $A!$ is A factorial; for example, for 4!, this would be (4)(3)(2)(1) = 24.

Assumptions and Limitations

- Tables are available that provide individual exact probabilities for small sample size contingency tables (see Zar [25]).
- Fisher's exact test must be used in preference to the chi-square test for small cell sizes.
- The probability resulting from a two-tailed test is exactly double that of a one-tailed test from the same data.
- Ghent [26] has developed and proposed a good (although, if performed by hand, laborious) method extending the calculation of exact probabilities to 2 × 3, 3 × 3, and R × C contingency tables.
- Fisher's probabilities are not necessarily symmetric. Although some analysts will double the one-tailed *p*-value to obtain the two-tailed result, this method is usually overly conservative.

2 × 2 Chi-Square Test

Although Fisher's exact test is preferable for the analysis of most 2 × 2 contingency tables in toxicology, the chi-square test is still widely used and is preferable in a few unusual situations (particularly if cell sizes are large yet only limited computational support is available). The formula is simply

$$\chi^2 = \frac{(O_1 - E_1)^2}{E_1} = \frac{(O_2 - E_2)^2}{E_2}$$

$$= \sum \frac{(O_i - E_i)^2}{E_i}$$

where

*O*s are observed numbers (or counts)
*E*s are expected numbers

The common practice in toxicology is for the observed figures to be test or treatment group counts. The expected figure is calculated as

$$\mathrm{E} = \frac{(\text{column total})(\text{row total})}{\text{grand total}}$$

for each box or cell in a contingency table. Our degrees of freedom are (R − 1)(C − 1) = (2 − 1)(2 − 1) = 1. Looking at a chi-square table for 1 degree of freedom, we see that this is greater than the test statistic at 0.05 (3.84) but less than that at 0.01 (6.64) so $0.05 > p > 0.01$.

Assumptions and Limitations

Assumptions

1. Data are univariate and categorical.
2. Data are from a multinomial population.
3. Data are collected by random, independent sampling.
4. Groups being compared are of approximately same size, particularly for small group sizes.

When to use

1. The data are of a categorical (or frequency) nature.
2. The data fit the earlier assumptions.
3. Goodness-to-fit to a known form of distribution is being tested.
4. Cell sizes are large.

When not to use

1. The data are continuous rather than categorical.
2. Sample sizes are small and very unequal.
3. Sample sizes are too small (e.g., when total *N* is less than 50 or if any expected value is less than 5).
4. Any 2 × 2 comparison is being performed (use Fisher's exact test instead).

R × C Chi-Square Test

The R × C chi-square test can be used to analyze discontinuous (frequency) data as in Fisher's exact test or the 2 × 2 chi-square test; however, in the R × C test, we wish to compare three or more sets of data. An example would be the comparison of the incidence of tumors among mice on three or more oral dosage levels. We can consider the data as positive (tumors) or negative (no tumors). The expected frequency for any box is equal to (row total)(column total)/(N_{total}). As in Fisher's exact test, the initial step is to set up a table (this time an R × C contingency table):

	Positive	Negative	Total
Group I	A_1	B_1	$A_1 + B_1 = N_1$
Group II	A_2	B_2	$A_2 + B_2 = N_2$
	↓	↓	↓
Group R	A_R	B_R	$A_R + B_R = N_R$
Total	N_A	N_B	N_{total}

Using these symbols, the formula for chi-square (χ^2) is

$$\chi^2 = \frac{N_{total}^2}{N_A N_B N_K}\left(\frac{A_1^2}{N_1} + \frac{A_2^2}{N_2} + \cdots + \frac{A_K^2}{N_2} - \frac{N_A^2}{N_{tot}}\right)$$

The resulting χ^2 value is compared to table values [16] according to the number of degrees of freedom, which is equal to (R − 1)(C − 1). If χ^2 is smaller than the table value at the 0.05 probability level, the groups are not significantly different. If the calculated χ^2 is larger, there is some difference among the groups, and 2 × R chi-square or Fisher's exact tests will have to be compared to determine which groups differ from which other groups.

Assumptions and Limitations

1. The test is based on data being organized in a table with *cells* (in the following table, *A*, *B*, *C*, and *D* are cells).
2. None of the expected frequency values is less than 5.0.
3. The chi-square test is always one tailed.
4. Without the use of some form of correction, the test becomes less accurate as the differences between group sizes increase.
5. The results from each additional column (group) are approximately additive. Due to this characteristic, the chi-square test can be readily used for evaluating any R × C combination.
6. The results of the chi-square calculation must be a positive number.
7. The test is weak with either small sample sizes or when the expected frequency in any cell is less than 5 (this latter limitation can be overcome by pooling, or combining, cells).
8. Test results are independent of order of cells, unlike Kolmogorov–Smirnov.
9. The test can be used to test the probability of validity of any distribution.

		Columns (C)		
Rows (R)	**No Effect**	**Control**	**Treated**	**Total**
		A	*B*	*A* + *B*
		C	*D*	*C* + *D*
Total		*A* + *C*	*B* + *D*	*A* + *B* + *C* + *D*

Wilcoxon Rank-Sum Test

The Wilcoxon rank-sum test is commonly used for the comparison of two groups of nonparametric (interval or not normally distributed) data, such as those that are not measured exactly but rather as falling within certain limits (e.g., how many animals died during each hour of an acute study). The test is also used when there is no variability (variance = 0) within one or more of the groups we wish to compare [15].

The data in both groups being compared are initially arranged and listed in the order of increasing value, then each number in the two groups must receive a rank value. Beginning with the smallest number in either group (which is given a rank of 1.0), each number is assigned a rank. If there are duplicate numbers (called *ties*), then each value of equal size will receive the median rank for the entire identically sized group; thus, if the lowest number appears twice, both figures receive a rank of 1.5. This, in turn, means that the ranks of 1.0 and 2.0 have been used and that the next highest number has a rank of 3.0. If the lowest number appears three times, then each is ranked as 2.0, and the next number has a rank of 4.0; thus, each tied number gets a median rank. This process continues until all of the numbers are ranked. Each of the two columns of ranks (one for each group) is totaled, giving the sum of ranks for each group being compared. As a check, we can calculate the value:

$$\frac{(N)(N+1)}{2}$$

where N is the total number of data in both groups. The result should be equal to the sum of ranks for both groups.

The sums of rank values are compared to table values [27] to determine the degree of significant differences, if any. These tables include two limits (an upper and a lower) that are dependent on the probability level. If the number of data is the same in both groups ($N_1 \neq N_2$), then the lesser sum of ranks (smaller N) is compared to the table limits to find the degree of significance. Normally, the comparison of the two groups ends here, and the degree of significant difference can be reported.

Distribution-Free Multiple Comparison

The distribution-free multiple comparison test should be used to compare three or more groups of nonparametric data. These groups are then analyzed two at a time for any significant differences [28]. The test can be used for data similar to those compared by the rank-sum test. We often employ this test for reproduction and mutagenicity studies (such as comparing survival rates of offspring of rats fed various amounts of test materials in the diet).

As shown in Example 9.2, two values must be calculated for each pair of groups: the difference in mean ranks and the probability level value against which the difference will be compared. To determine the difference in mean ranks, we must first arrange the data within each of the groups in the order of increasing values, then we must assign rank values, beginning with the smallest overall figure. Note that this ranking is similar to that in the Wilcoxon test except that it applies to more than two groups. The ranks are then added for each of the groups. As a check, the sum of these values should equal:

$$\frac{(N_{total})(N_{total}+1)}{2}$$

where N_{total} is the total number of figures from all groups. Next, we can find the mean rank (R) for each group by dividing the sum of ranks by the numbers in the data (N) in the group. These mean ranks are then taken in those pairs that we want to compare (usually each test group vs. the control), and the differences are found ($|R_1 - R_2|$). This value is expressed as an absolute figure; that is, it is always a positive number.

The second value for each pair of groups (the probability value) is calculated from the expression:

$$z\left[\frac{a}{K}(K-1)\right]\sqrt{\frac{N_{total}(N_{total}+1)}{12}}\sqrt{\frac{1}{N_1}\frac{1}{N_2}}$$

where

- a is the level of significance for the comparison (usually 0.05, 0.01, 0.001, etc.)
- K is the total number of groups
- z is a figure obtained from a normal probability table and determining the corresponding z-score

The result of the probability value calculation for each pair of groups is compared to the corresponding mean difference $|R_1 - R_2|$. If $|R_1 - R_2|$ is smaller, then there is no significant difference between the groups. If it is larger, then the groups are different, and $|R_1 - R_2|$ must be compared to the calculated probability values for $a = 0.01$ and $a = 0.001$ to find the degree of significance.

Example 9.2

Consider the set of data provided earlier (ranked in increasing order), which could represent the proportion of rats surviving given periods of time during diet inclusion of a test chemical at four dosage levels (survival index).

I		II		III		V	
5.0 mg/kg		2.5 mg/kg		1.25 mg/kg		0.0 mg/kg	
% Value	Rank	% Value	Rank	% Value	Rank	% Value	Rank
40	2.0	40	2.0	50	5.5	60	9.0
40	2.0	50	5.5	50	5.5	60	9.0
50	5.5	80	12.0	60	9.0	80	12.0
100	17.5	80	12.0	100	17.5	90	14.0
		100	17.5	100	17.5	100	17.5
						100	17.5
Sum of ranks	27.0		49.0		55.0		79.0

$N_I = 4$, $N_{II} = 5$, $N_{III} = 5$, $N_{IV} = 6$ $N_{total} = 20$

Check sums of ranks = 210, $(20 \times 21)/2 = 210$
Mean ranks (R)

$$R_1 = \frac{27.0}{4} = 6.75 \qquad R_2 = \frac{49.0}{5} = 9.80$$

$$R_3 = \frac{55.0}{5} = 11.00 \qquad R_4 = \frac{79.0}{6} = 13.17$$

Comparison Groups	R_1–R_2	Probability Test Values
5.0 vs. 0.0	6.42	$\left(\frac{0.05}{4(3)}\right) = Z_{0.00417} = 2.637$
		$\sqrt{\frac{(20)(21)}{12}}\sqrt{\frac{1}{4}+\frac{1}{6}} = 10.07$
2.5 vs. 0.0	3.37	$\left(\frac{0.05}{4(3)}\right) = Z_{0.00417} = 2.637$
		$\sqrt{\frac{(20)(21)}{12}}\sqrt{\frac{1}{5}+\frac{1}{6}} = 9.45$
1.25 vs. 0.0	2.17	$\left(\frac{0.05}{4(3)}\right) = Z_{0.00417} = 2.637$
		$\sqrt{\frac{(20)(21)}{12}}\sqrt{\frac{1}{4}+\frac{1}{6}} = 10.07$

Because each of the $|R_1 - R_2|$ values is smaller than the corresponding probability calculation, the pairs of groups compared are not different at the 0.05 level of significance.

Assumptions and Limitations

1. As with the Wilcoxon rank-sum test, too many tied ranks inflate the false positive.
2. Generally, this test should be used as a post hoc comparison after Kruskal–Wallis.

Mann–Whitney U Test

This is a nonparametric test in which the data in each group are first ordered from lowest to highest values, then the entire set (both control and treated values) is ranked, with the average rank being assigned to tied values. The ranks are then summed for each group, and U is determined according to

$$U_t = n_c n_t + \frac{n_t\left(n_t + 1\right)}{2} - R_t$$

$$U_c = n_c n_t + \frac{n_c\left(n_c + 1\right)}{2} - R_c$$

where

n_c and n_t are the sample sizes for the control and treated groups, respectively

R_c and R_t are the sums of ranks for the control and treated groups, respectively

For the level of significance for a comparison of the two groups, the larger value of U_c or U_t is used. This is compared to critical values as found in tables [29].

With the earlier discussion and methods in mind, we can now examine the actual variables that we encounter in teratology studies. These variables can be readily divided into two groups: measures of lethality and measures of teratogenic effect [30]. Measures of lethality include (1) corpora lutea per pregnant female, (2) implants per pregnant female, (3) live fetuses per pregnant female, (4) percentage of preimplantation loss per pregnant female, (5) percentage of resorptions per pregnant female, and (6) percentage of dead fetuses per pregnant female. Measures of teratogenic effect include (1) percentage of abnormal fetuses per litter, (2) percentage of litters with abnormal fetuses, and (3) fetal weight gain. As demonstrated in Example 9.3, the Mann–Whitney U test is employed for the count data, but which test should be employed for the percentage variables should be decided on the same grounds as described later under reproduction studies.

Example 9.3

In a 2-week study, the levels of serum cholesterol in treatment and control animals are successfully measured and assigned ranks as follows:

Treatment		Control	
Value	**Rank**	**Value**	**Rank**
10	1	19	4
18	3	28	13
26	10.5	29	14.5
31	16	26	10.5
15	2	35	19
24	8	23	7
22	6	29	14.5
33	17	34	18
21	5	38	20
25	9	27	12
Sum of ranks	77.5		132.5

The critical value for one-tailed $p \le 0.05$ is $U \ge 73$. We then calculate

$$U_t = (10)(10) + \frac{10(10+1)}{2} - 77.5$$

$$= 100 + \frac{110}{2} - 77.5 = 77.5$$

$$U_c = (10)(10) + \frac{10(10+1)}{2} - 132.5 = 2.5$$

Because 77.5 is greater than 73, these groups are significantly different at the 0.05 level.

Assumptions and Limitations

1. It does not matter whether the observations are ranked from smallest to largest or vice versa.
2. This test should not be used for paired observations.
3. The test statistics from a Mann–Whitney test are linearly related to those of Wilcoxon. The two tests will always yield the same result. The Mann–Whitney is presented here for historical completeness, as it has been much favored in reproductive and developmental toxicology studies; however, it should be noted that the authors do not include it in the decision tree for method selection (Figure 9.2).

Kruskal–Wallis Nonparametric ANOVA

The Kruskal–Wallis nonparametric one-way ANOVA should be the initial analysis performed when we have three or more groups of data that are by nature nonparametric (not a normally distributed population, data of a discontinuous nature, or all the groups being analyzed not being from the same population) but not of a categorical (or quantal) nature. Commonly, these will be either rank-type evaluation data (such as behavioral toxicity observation scores) or reproduction study data. The analysis is initiated [31] by ranking all the observations from the combined groups to be analyzed. Ties are given the average rank of the tied values (i.e., if two values would tie for 12th rank and therefore are ranked 12th and 13th, then both would be assigned the average rank of 12.5).

The sum of ranks of each group (r_1, r_2, …, r_k) is computed by adding all the rank values for each group. The test value H is then computed as

$$H = \frac{12}{n(n-1)} \sum \frac{r_1^2}{n_1} + \frac{r_2^2}{n_2} + \cdots \frac{r_k^2}{n_k} - 3(n+1)$$

where n_1, n_2, …, n_k are the number of observations in each group. The test statistic is then compared with a table of H values. If the calculated value of H is greater than the table value for the appropriate number of observations in each group, there is significant difference between the groups, but further testing (using the distribution-free multiple comparisons method) is necessary to determine where the difference lies (as demonstrated in Example 9.4).

Example 9.4

As part of a neurobehavioral toxicology study, righting reflex values (whole numbers ranging from 0 to 10) were determined for each of five rats in each of three groups. The values observed and their ranks are as follows:

Control Group		5 mg/kg Group		10 mg/kg Group	
Reflex Score	**Rank**	**Reflex Score**	**Rank**	**Reflex Score**	**Rank**
0	2	1	5	4	11
0	2	2	7.5	4	11
0	2	2	7.5	5	13
1	5	3	9	8	14.5
1	5	4	11	8	14.5
Sum of ranks	16		40		64

From these, the H value is calculated as

$$H = \frac{12}{15(15+1)}\left[\frac{16^2}{5} + \frac{40^2}{5} + \frac{64^2}{5}\right] - 3(15+1)$$

$$= \frac{12}{240}\left[\frac{256 + 1600 + 4096}{5}\right] - 48$$

$$= \frac{1}{20}(1190.4) - 48$$

$$= 59.52 - 48$$

$$= 11.52$$

Consulting a table of values for H, we find that for the case where we have three groups of five observations

each, the test values are 4.56 (for $p = 0.10$), 5.78 ($p = 0.05$), and 7.98 (for $p = 0.01$). As our calculated H is greater than the $p = 0.01$ test value, we have determined that there is a significant difference between the groups at the level of $p < 0.01$ and would now have to continue to a multiple comparisons test to determine where the difference is.

Assumptions and Limitations

1. The test statistic H is used for both small and large samples.
2. When we find a significant difference, we do not know which groups are different. It is not correct to then perform a Mann–Whitney U test on all possible combinations; rather, a multiple comparison method must be used, such as the distribution-free multiple comparisons.
3. Data must be independent for the test to be valid.
4. Too many tied ranks will decrease the power of this test and lead to increased false-positive levels.
5. When $k = 2$, the Kruskal–Wallis chi-square value has 1 degree of freedom. This test is identical to the normal approximation used for the Wilcoxon rank-sum test. As noted in previous sections, a chi-square with 1 degree of freedom can be represented by the square of a standardized normal random variable. In the case of $k = 2$, the H statistic is the square of the Wilcoxon rank-sum z-test (without the continuity correction).
6. The effect of adjusting for tied ranks is to slightly increase the value of the test statistic, H; therefore, omission of this adjustment results in a more conservative test.

Log-Rank Test

The log-rank test is a statistical methodology for comparing the distribution of time until the occurrence of the event in independent groups. In toxicology, the most common event of interest is death or occurrence of a tumor, but it could just as well be liver failure, neurotoxicity, or any other event that occurs only once in an individual. The elapsed time from initial treatment or observation until the *event* is the *event time*, often referred to as *survival time*, even when the event is not death.

The log-rank test provides a method for comparing risk-adjusted event rates, useful when test subjects in a study are subject to varying degrees of opportunity to experience the event. Such situations arise frequently in toxicology studies due to the finite duration of the study, early termination of the animal, or interruption of treatment before the event occurs. Examples where use of the log-rank test might be appropriate include comparing survival times in carcinogenicity bioassay animals that are given a new treatment with those in the control group or comparing times to liver failure for several dose levels of a new NSAID where the animals are treated for 10 weeks or until cured, whichever comes first.

If every animal were followed until the event occurrence, the event times could be compared between two groups using the Wilcoxon rank-sum test; however, some animals may die or complete the study before the event occurs. In such cases, the actual time of the event is unknown because the event does not occur while under study observation. The event times for these animals are based on the last known time of study observation and are referred to as *censored* observations because they represent the lower bound of the true unknown event times. The Wilcoxon rank-sum test can be highly biased in the presence of the censored data.

The null hypothesis tested by the log-rank test is that of equal event time distributions among groups. Equality of the distributions of event times implies similar event rates among groups, not only for the clinical trial as a whole but also for any arbitrary time point during the trial. Rejection of the null hypothesis indicates that the event rates differ among groups at one or more time points during the study.

The idea behind the log-rank test for the comparison of two life tables is simple: If there were no difference between the groups, the total deaths occurring at any time should split between the two groups at that time. So, if the numbers at risk in the first and second groups in, say, the sixth month were 70 and 30, respectively, and 10 deaths occurred in that month, then we would expect

$$10 \times \frac{70}{70+30} = 7$$

of these deaths to have occurred in the first group, and

$$10 \times \frac{30}{70+30} = 3$$

of the deaths to have occurred in the second group.

A similar calculation can be made at each time of death (in either group). By adding together the results of all such calculations for the first group, we obtain a single number, the extent of exposure (E_1), which represents the expected number of deaths in that group if the two groups had the same distribution of survival times. An extent of exposure (E_2) can be obtained for the second group in the same way. Let O_1 and O_2 denote the actual total numbers of deaths in the two groups. A useful arithmetic check is that the total number of deaths ($O_1 + O_2$) must equal the sum of the extents of exposure ($E_1 + E_2$). The discrepancy between the Os and Es can be measured by the quantity

$$x^2 = \frac{\left(|O_1 - E_1| - 1/2\right)^2}{E_1} + \frac{\left(|O_2 - E_2| - 1/2\right)^2}{E_2}$$

For rather obscure reasons, x^2 is known as the log-rank statistic. An approximate significance test of the null hypothesis of identical distributions of survival time in the two groups is obtained by referring x^2 to a chi-square distribution on 1 degree of freedom. This is demonstrated in Example 9.5.

Example 9.5

In a study of the effectiveness of a new monoclonal antibody to treat specific cancer, the times to reoccurrence of the cancer in treated animals in weeks were as follows:

Control Group			Treatment Group		
1	5	11	6	10	22
1	5	12	6	11	23
2	8	12	6	13	25
2	8	15	6	16	32
3	8	17	7	17	32
4	8	22	9	19	34
4	11	23	10	20	35

The table provided later presents the calculations for the log-rank test applied to these times. A chi-square value of 13.6 is significant at the $p < 0.001$ level.

Illustration

$$t = 23, \quad 2\times\frac{6}{7} = 1.7143, \quad 2\times\frac{1}{7} = 0.2857$$

Test of significance

$$x^2 = \frac{\left(|O_1 - E_1| - 1/2\right)^2}{E_1} + \frac{\left(|O_2 - E_2| - 1/2\right)^2}{E_2}$$

$$= \frac{\left(|9 - 19.2| - 1/2\right)^2}{19.2} + \frac{\left(|21 - 10.8| - 1/2\right)^2}{10.8} = 13.6$$

Estimate of relative risk

$$\hat{\theta} = \frac{9/19.2}{21/10.8} = 0.24$$

The log-rank test as presented by Peto et al. [32] uses the product-limit life-table calculations rather than the actuarial estimators shown earlier. The distinction is unlikely to be of practical importance unless the grouping intervals are very coarse.

Peto and Pike [33] suggest that the approximation in treating the null distribution of χ^2 as a chi-square is conservative, as it will tend to understate the degree of statistical significance. In the formula for χ^2, we have used the continuity correction of subtracting 1/2 from $|O_1 - E_1|$ and $|O_2 - E_2|$ before squaring. This is recommended by Peto et al. [32] when, as in nonrandomized studies, the permutational argument does not apply. Peto et al. [32] give further details of the log-rank test and its extension to comparisons of more than two treatment groups and to tests that control for categorical confounding factors.

Assumptions and Limitations

1. The endpoint of concern is, or is defined so it is, right censored; that is, once it happens, it does not recur. Examples are death or a minimum or maximum value of an enzyme or physiologic function (such as respiration rate).
2. The method makes no assumptions on distribution.
3. Many variations of the log-rank test for comparing survival distributions exist. The most common variant has the form

$$x^2 = \frac{\left(O_1 - E_1\right)^2}{E_1} + \frac{\left(O_2 - E_2\right)^2}{E_2}$$

where O_i and E_i are computed for each group, as in the formulas given previously. This statistic also has an approximate chi-square distribution with 1 degree of freedom under H_0. A continuity correction can also be used to reducing the numerators by 1/2 before squaring. Use of such a correction leads to even further conservatism and may be omitted when sample sizes are moderate or large.

Time	At Risk			Relapses			Extent of Exposure		
(*t*)	T	C	Total	T	C	Total	T	C	Total
1	21	21	42	0	2	2	1.0000	1.0000	2
2	21	19	40	0	2	2	1.0500	0.9500	2
3	21	17	38	0	1	1	0.5526	0.4474	1
4	21	16	37	0	2	2	1.1351	0.8649	2
5	21	14	35	0	2	2	1.2000	0.8000	2
6	20.5	12	32.5	3	0	3	1.8923	1.1077	3
7	17	12	29	1	0	1	0.5862	0.4138	1
8	16	12	28	0	4	4	2.2857	1.7143	4
10	14.5	8	22.5	1	0	1	0.6444	0.3556	1
11	12.5	8	20.5	0	2	2	1.2295	0.7705	2
12	12	6	18	0	2	2	1.3333	0.6667	2
13	12	4	16	1	0	1	0.7500	0.2500	1
15	11	4	15	0	1	1	0.7333	0.2667	1
16	11	3	14	1	0	1	0.7857	0.2143	1
17	9.5	3	12.5	0	1	1	0.7600	0.2400	1
22	7	2	9	1	1	2	1.5556	0.4444	2
23	6	1	7	1	1	2	1.7143	0.2857	2
Total				9	21	30	19.2080	10.7920	30
					(O_1)	(O_2)	(E_1)	(E_2)	

4. The Wilcoxon rank-sum test could be used to analyze the event times in the absence of censoring. A generalized Wilcoxon test, sometimes called the *Gehan test*, based on an approximate chi-square distribution, has been developed for use in the presence of censored observations. Both the log-rank and the generalized Wilcoxon tests are nonparametric tests, and they require no assumptions regarding the distribution of event times. When the event rate is greater early in the trial than toward the end, the generalized Wilcoxon test is the more appropriate test because it gives greater weight to the earlier differences.

5. Survival and failure times often follow the exponential distribution. If such a model can be assumed, a more powerful alternative to the log-rank test is the likelihood ratio test. This parametric test assumes that event probabilities are constant over time; that is, the chance that a patient becomes event positive at time t given that he is event negative up to time t does not depend on t. A plot of the negative log of the event times distribution showing a linear trend through the origin is consistent with exponential event times.
6. Life tables can be constructed to provide estimates of the event time distributions. Estimates commonly used are known as the Kaplan–Meier estimates.

HYPOTHESIS TESTING: UNIVARIATE PARAMETRIC TESTS

Univariate case data (where each datum is defined by one treatment and one effect variable) from normally distributed populations generally have a higher information value associated with them, but the traditional hypothesis-testing techniques (which include all the methods described in this chapter) are generally neither resistant nor robust. All the data analyzed by these methods are also, effectively, continuous; that is, at least for practical purposes, the data may be represented by any number, and each such data number has a measurable relationship to other data numbers.

Student's *t*-Test (Unpaired *t*-Test)

Pairs of groups of continuous, randomly distributed data are compared via this test. We can use this test to compare three or more groups of data, but they must be intercompared by examination of two groups taken at time and are preferentially compared by ANOVA. Usually, this means comparison of a test group vs. a control group, although two test groups may be compared as well. To determine which of the three types of *t*-tests described in this chapter should be employed, the *F*-test is usually performed first. This will tell us if the variances of the data are approximately equal, which is a requirement for the use of the parametric methods. If the *F*-test indicates homogeneous variances, and the numbers of data within the groups (N) are equal, then Student's *t*-test is the appropriate procedure [15]. If the *F* is significant (the data are heterogeneous) and the two groups have equal numbers of data, the modified Student's *t*-test is applicable [34].

The value of t for Student's *t*-test is calculated using the following formula:

$$t = \frac{X_1 - X_2}{\sum D_1^2 + \sum D_2^2} \sqrt{\frac{N_1 N_2}{N_1 + N_2}\left(N_1 + N_2 - 2\right)}$$

where

$$\sum D^2 = \frac{N\sum X^2 - \left(\sum X\right)^2}{N}$$

The value of t obtained from the earlier calculations is compared to the values in a *t*-distribution table according to the appropriate number of degrees of freedom (df). If the *F* value is not significant (i.e., variances are homogeneous), the df = $N_1 + N_2 - 2$. If the *F* is significant and $N_1 = N_2$, then the df = $N - 1$. Although this case indicates a nonrandom distribution, the modified *t*-test is still valid. If the calculated value is larger than the table value at $p = 0.05$, it may then be compared to the appropriate other table values in the order of decreasing probability to determine the degree of significance between the two groups. Example 9.6 demonstrates this methodology.

Example 9.6

Suppose we wish to compare two groups (at test and control groups) of dog weights following inhalation of a vapor. First, we would test for homogeneity of variance using the *F*-test. Assuming that this test gave negative (homogeneous) results, we would perform the *t*-test as follows:

	Test Weight		Control Weight	
Dog	X_1 (kg)	X_1^2	X_2 (kg)	X_2^2
1	8.3	68.89	8.4	70.56
2	8.8	77.44	10.2	104.04
3	9.3	86.49	9.6	92.16
4	9.3	86.49	9.4	88.36
Sums	$\sum X_1 = 35.7$	$\sum X_1^2 = 319.31$	$\sum X_2 = 37.6$	$\sum X_2^2 = 355.12$
Means	8.92		9.40	

The difference in means = 9/40 = 8.92 = 0.48.

$$\sum D_1^2 = \frac{4(319.31) - (35.7)^2}{4} = \frac{2.75}{4} = 0.6875$$

$$\sum D_2^2 = \frac{4(355.12) - (37.6)^2}{4} = \frac{6.72}{4} = 1.6800$$

$$t = \frac{0.48}{\sqrt{0.6875 + 1.6800}} \sqrt{\frac{4(4)}{4+4}(4+4)} = 1.08$$

The table value for t at the 0.05 probability level for (4 + 4 – 2), or 6 degrees of freedom, is 2.447; therefore, the dog weights are not significantly different at $p = 0.05$.

Assumptions and Limitations

1. The test assumes that the data are univariate, continuous, and normally distributed.
2. Data are collected by random sampling.
3. The test should be used when the assumptions in 1 and 2 are met and there are only two groups to be compared.
4. Do not use when the data are ranked, when the data are not approximately normally distributed, or when more than two groups are to be compared. Do not use for paired observations.

5. This is the most commonly misused test method, except in those few cases where one is truly comparing only two groups of data and the group sizes are roughly equivalent. It is not valid for multiple comparisons (because of resulting additive errors) or where group sizes are very unequal.
6. The test is robust for moderate departures from normality, and, when N_1 and N_2 are approximately equal, it is robust for moderate departures from homogeneity of variances.
7. The main difference between the *z*-test and the *t*-test is that the *z*-statistic is based on a known SD (σ) while the *t*-statistic uses the sample SD (*s*) as an estimate of σ. With the assumption of normally distributed data, the variance σ^2 is more closely estimated by the sample variance s^2 as *n* gets large. It can be shown that the *t*-test is equivalent to the *z*-test for infinite degrees of freedom. In practice, a large sample is usually considered to be $n \geq 30$.

Cochran *t*-Test

The Cochran test should be used to compare two groups of continuous data when the variances (as indicated by the *F*-test) are heterogeneous and the numbers of data within the groups are not equal ($N_1 \neq N_2$). This is the situation, for example, when the data, although expected to be, are not randomly distributed [3]. Two *t* values are calculated for this test: the observed *t* (t_{obs}) and the expected *t* (t'). The observed *t* is obtained by

$$t_{obs} = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{W_1 + W_2}}$$

where $W = \text{SEM}^2$ (standard error of the mean squared) = S^2/N, where variance *S* can be calculated from

$$S = \frac{N\sum X^2 - \left(\sum X\right)^2}{\left(N/N-1\right)}$$

The value for t' is obtained from

$$t' = \frac{t'_1 W_1 + t'_2 W_2}{W_1 + W_2}$$

where t'_1 and t'_2 are values for the two groups taken from the *t*-distribution table corresponding to $(N - 1)$ degrees of freedom (for each group) at the 0.05 probability level (or such level as one may select).

The calculated t_{obs} is compared to the calculated t' value (or values, if t' values were prepared for more than one probability level). If t_{obs} is smaller than a t', the groups are not considered to be significantly different at that probability level. This procedure is shown in Example 9.7.

Example 9.7

Using the RBC count comparison from the discussion of the *F*-test (with $N_1 = 5$, $N_2 = 4$), the following results were determined:

$$\bar{X}_1 = \frac{37.60}{5} = 7.52 \quad W_1 = \frac{0.804}{5} = 0.1608$$

$$\bar{X}_2 = \frac{29.62}{4} = 7.52 \quad W_2 = \frac{0.025}{4} = 0.1608$$

(Note that S^2 values of 0.804 and 0.025 are calculated using the formula set forth in the section on "Bartlett's Test for Homogeneity of Variance.")

$$t_{obs} = \frac{7.52 - 7.40}{\sqrt{0.1608 + 0.0062}} = 0.29$$

From the *t*-distribution table, we use $t_1 = 2.776$ (df = 4) and $t_2 = 3.182$ (df = 3) for the 0.05 level of significance; there is no statistical difference at $p = 0.05$ between the two groups.

Assumptions and Limitations

1. The test assumes that the data are univariate, continuous, and normally distributed and that group sizes are unequal.
2. The test is robust for moderate departures from normality and very robust for departures from equality of variances.

F-Test

This is a test of the homogeneity of variances between two groups of data [15]. It is used in two separate cases. The first is when Bartlett's test indicates heterogeneity of variances among three or more groups (i.e., it is used to determine which pairs of groups are heterogeneous). Second, the *F*-test is the initial step in comparing two groups of continuous data that we would expect to be parametric (two groups not usually being compared using ANOVA), the results indicating whether the data are from the same population and whether subsequent parametric comparisons would be valid. The *F* is calculated by dividing the larger variance (S_1^2) by the smaller one (S_2^2). S^2 is calculated as

$$S = \frac{N\sum X^2 - \left(\sum X\right)^2}{\left(N/N-1\right)}$$

where

N is the number of data in the group

X represents the individual values within the group

Frequently, S^2 values may be obtained from ANOVA calculations. Use of this is demonstrated in Example 9.8. The

calculated F value is compared to the appropriate number in an F-value table for the appropriate degrees of freedom ($N - 1$) in the numerator (along the top of the table) and in the denominator (along the side of the table). If the calculated value is smaller, it is not significant, and the variances are considered homogeneous (and Student's t-test would be appropriate for further comparison). If the calculated F value is greater, F is significant and the variances are heterogeneous (and the next test would be a modified Student's t-test if $N_1 = N_2$ or the Cochran t-test if $N_1 \neq N_2$; see Figure 9.2 to review the decision tree).

Example 9.8

If we wished to compare the RBC counts of rats receiving a test material in their diet with the RBC counts of control rats, we might obtain the following results:

Test Weight		Control Weight	
X_1 (kg)	X_1^2	X_2 (kg)	X_2^2
8.3	68.89	8.4	70.56
8.8	77.44	10.2	104.04
9.3	86.49	9.6	92.16
9.3	86.49	9.4	88.36
$\sum X_1 = 35.7$	$\sum X_1^2 = 319.31$	$\sum X_2 = 37.6$	$\sum X_2^2 = 355.12$
8.92		940	
Test RBC		**Control RBC**	
X_1	X_1^2	X_2	X_2^2
8.23	67.73	7.22	52.13
8.59	73.79	7.55	57.00
7.51	56.40	7.53	56.70
6.60	46.56	7.32	53.58
6.67	44.49		
$\hat{A}X_1 = 37.60$	$\hat{A}X_1^2 = 285.97$	$\hat{A}X_2 = 29.62$	$\hat{A}X_2^2 = 219.41$

Variance for X_1

$$S_1^2 = \frac{\left(5(285.97) - (37.60)^2/5\right)}{5-1} = 0.804$$

Variance for X_2

$$S_3^2 = \frac{\left(4(219.41) - (29.62)^2/4\right)}{4-1} = 0.804$$

$$F = \frac{0.804}{0.025} = 32.16$$

From a table for F values, for 4 (numerator) vs. 3 (denominator) degrees of freedom, we read the limit of 9.12 at the 0.05 level. As our calculated value is larger (and, therefore, significant), the variances are heterogeneous and the Cochran t-test would be appropriate for comparison of the two groups of data.

Assumptions and Limitations

1. This test could be considered as a two-group equivalent of Bartlett's test.
2. If the test statistic is close to 1.0, the results are (of course) not significant.
3. The test assumes normality and independence of data.

Analysis of Variance

ANOVA is used for the comparison of three or more groups of continuous data when the variances are homogeneous and the data are independent and normally distributed. A series of calculations are required for ANOVA, starting with the values within each group being added (ΣX) and then these sums being added ($\Sigma\Sigma X$). Each figure within the groups is squared, and these squares are then summed (ΣX^2) and these sums added ($\Sigma\Sigma X^2$). Next the correction factor (CF) can be calculated using the following formula:

$$\mathrm{CF} = \frac{\left(\sum_1^K \sum_1^K X\right)^2}{N_1 + N_2 + \cdots + N_k}$$

where

N is the number of values in each group
K is the number of groups

The total sum of squares (SS_{total}) is then determined as follows:

$$\mathrm{SS}_{total} = \sum_1^K \sum_1^N X^2 - \mathrm{CF}$$

In turn, the sum of squares between groups (SS_{bg}) is found from

$$\mathrm{SS}_{bg} = \frac{\left(\sum X_1\right)^2}{N_1} + \frac{\left(\sum X_2\right)^2}{N_2} + \cdots + \frac{\left(\sum X_k\right)^2}{N_k} - \mathrm{CF}$$

The sum of squares within group (SS_{wg}) is then the difference between the last two figures, or

$$\mathrm{SS}_{wg} = \mathrm{SS}_{total} - \mathrm{SS}_{bg}$$

Now, there are three types of degrees of freedom to determine. The first, total degrees of freedom, is the total number of data within all groups under analysis minus one ($N_1 + N_2 + \ldots + N_k - 1$). The second figure (the degrees of freedom between groups) is the number of groups minus one ($K - 1$). The last figure (the degrees of freedom within groups, or error df) is the difference between the first two figures ($\mathrm{df}_{total} - \mathrm{df}_{bg}$).

The next set of calculations requires the determination of the two mean squares (MSs) (MS_{bg} and MS_{wg}). These are the respective sum of square values divided by the corresponding df figures (MS = SS/df). The final calculation is that of the *F* ratio. For this, the MS between groups is divided by the MS within groups ($F = MS_{bg}/MS_{wg}$). A table of the results of these calculations (using data from Example 9.9 at the end of this section) would appear as follows:

	df	SS	MS	*F*
Between groups	3	0.04075	0.01358	4.94
Within groups	12	0.03305	0.00275	
Total	15	0.07380		

For interpretation, the *F* ratio value obtained in the ANOVA is compared to a table of *F* values. If $F \leq 1.0$, the results are not significant and comparison with the table values is not necessary. The degrees of freedom for the greater MS (MS_{bg}) are indicated along the top of the table. Then read down the side of the table to the line corresponding to the degrees of freedom for the lesser MS (MS_{wg}). The figure shown at the desired significance level (traditionally 0.05) is compared to the calculated *F* value. If the calculated number is smaller, there is no significant difference among the groups being compared. If the calculated value is larger, there is some difference, but further (post hoc) testing will be required before we know which groups differ significantly.

Example 9.9

Suppose we want to compare four groups of dog kidney weights, expressed as percentage of body weights, following an inhalation study. Assuming homogeneity of variance (from Bartlett's test), we could complete the following calculations:

	400 ppm	200 ppm	100 ppm	0 ppm
	0.43	0.49	0.34	0.34
	0.52	0.48	0.40	0.32
	0.43	0.40	0.42	0.33
	0.55	0.34	0.40	0.39
ΣX	1.93	1.71	1.56	1.38

$$\Sigma\Sigma X = 1.93 + 1.71 + 1.56 + 1.38 = 6.58$$

Next, these figures are squared:

	400 ppm	200 ppm	100 ppm	0 ppm
	0.1849	0.2401	0.1156	0.1156
	0.2704	0.2304	0.1600	0.1024
	0.1849	0.1600	0.1764	0.1089
	0.3025	0.1156	0.1600	0.1521
ΣX^2	0.9427	0.7461	0.6120	0.4790

$$\Sigma\Sigma X^2 = 0.9427 + 0.7461 + 0.6120 + 0.4790 = 2.7798$$

$$CF = \frac{(6.58)^2}{4+4+4+4} = 2.7060 = 0.04075$$

$$SS_{total} = 2.7798 - 2.7060 = 0.0738$$

$$SS_{bg} = \frac{(1.93)^2}{4} + \frac{(1.71)^2}{4} + \frac{(1.56)^2}{4} + \frac{(1.38)^2}{4} - 2.7060$$

$$= 0.04075$$

$$SS_{wg} = 0.07380 - 0.04075 = 0.03305$$

The total degrees of freedom (df) = 4 + 4 + 4 + 4 − 1 = 15

$$df_{bg} = 4 - 1 = 3$$

$$df_{bg} = 15 - 3 = 12$$

$$MS_{bg} = \frac{0.4075}{3} = 0.01358$$

$$MS_{wg} = \frac{0.03305}{12} = 0.00275$$

$$F = \frac{0.01358}{0.00275} = 4.94$$

Going to a table of *F* values, we find the 3 df_{bg} (greater MS) and 12 df_{wg} (lesser MS), and the 0.05 value of *F* is 3.49. Because our calculated value is greater, there is a difference among groups at the 0.05 probability level. To determine where the difference is, further comparisons by a post hoc test will be necessary.

Assumptions and Limitations

1. What is presented here is the workhorse of toxicology—the one-way ANOVA. Many other forms exist for more complicated experimental designs.
2. The test is robust for moderate departures from normality if the sample sizes are large enough; unfortunately, this is rarely the case in toxicology.
3. ANOVA is robust for moderate departures from equality of variances (as determined by Bartlett's test) if the sample sizes are approximately equal.
4. It is not appropriate to use a *t*-test (or a two-groups-at-a-time version of ANOVA) to identify where significant differences are within the design group. A multiple-comparison post hoc method must be used.

Post Hoc Tests

There is a wide variety of post hoc tests available to analyze data after finding significant results in an ANOVA. Each of these tests has advantages and disadvantages, proponents, and critics. Four of the tests are commonly used in toxicology and will be presented or previewed here. These are Dunnett's *t*-test and Williams's *t*-test. Two other tests that are available in many statistical packages are Turkey's method and the Student–Newman–Keuls method [25]. If ANOVA reveals no

significance, it is not appropriate to proceed to perform a post hoc test in hopes of finding differences. To do so would only be another form of multiple comparisons, increasing the type I error rate beyond the desired level.

Duncan's Multiple Range Test

Duncan's test [35] is used to compare groups of continuous and randomly distributed data (such as body weights or organ weights). The test normally involves three or more groups taken one pair at a time. It should follow only observation of a significant F value in the ANOVA and can serve to determine which group (or groups) differs significantly from which other group (or groups). There are two alternative methods of calculation. The selection of the proper one is based on whether the number of data (N) is equal or unequal in the groups.

Groups with Equal Numbers of Data ($N_1 = N_2$)

Two sets of calculations must be carried out: (1) the determination of the difference between the means of pairs of groups, and (2) the preparation of a probability rate against which each difference in means is compared (as shown in the first of the two examples in this section). The means (averages) are determined (or taken from the ANOVA calculation) and ranked in either decreasing or increasing order. If two means are the same, they take up two equal positions (thus, for four means, we could have ranks of 1, 2, 2, and 4 rather than 1, 2, 3, and 4). The groups are then taken in pairs, and the differences between the means ($\bar{X}_1 - \bar{X}_2$), expressed as positive numbers, are calculated. Usually, each pair consists of a test group and the control group, although multiple test groups may be compared if so desired. The relative rank of the two groups being compared must be considered. If a test group is ranked 2 and the control group is ranked 1, then we say that there are two places between them; if the test group is ranked 3, then there would be three places between it and the control.

To establish the probability table, the SEM must be calculated as presented earlier or as

$$\sqrt{\frac{\text{Error mean square}}{N}} = \sqrt{\frac{\text{Mean square within group}}{N}}$$

where N is the number of animals or replications per dose level. The MS within groups (MS_{wg}) can be calculated from the information given in the ANOVA procedure (refer to the earlier section on "Analysis of Variance"). The SEM is then multiplied by a series of table values [27,36] to set up a probability table. The table values used for the calculations are chosen according to the probability levels (note that the tables have sections for 0.05, 0.01, and 0.001 levels) and the number of means apart for the groups being compared and the number of error degrees of freedom. The error df is the number of degrees of freedom within the groups. This last figure is determined from the ANOVA calculation and can be taken from ANOVA output. For some values of df, the table values are not given and should thus be interpolated. Example 9.10 demonstrates this case.

Example 9.10

Using the data given in Example 9.9 (four groups of dogs, with four dogs in each group), we can make the following calculations:

	Rank			
	1	**2**	**3**	**4**
Concentration (ppm)	0	100	200	400
Mean kidney weight ($\bar{X}$)	0.345	0.390	0.428	0.482

Groups Compared	$\left(\bar{X}_1 - \bar{X}_2\right)$	**No. of Means Apart**	**Probability**
2 vs. 1 (100 vs. 0 ppm)	0.045	2	$p > 0.05$
3 vs. 1 (200 vs. 0 ppm)	0.083	3	$p > 0.05$
4 vs. 1 (400 vs. 0 ppm)	0.137	4	$0.01 > p > 0.001$
4 vs. 2 (400 vs. 100 ppm)	0.092	3	$0.05 > p > 0.01$

The MS within groups from the ANOVA example was 0.00275; therefore, the SEM = $\sqrt{0.00275}$ = 0.02622. The error df (df_{wg}) was 12, so the following table values are used:

No. of Means Apart	**Probability Levels**		
	0.05	**0.01**	**0.001**
2	3.082	4.320	6.106
3	3.225	4.504	6.340
4	3.313	4.662	6.494

When these are multiplied by the SEM, we get the following probability table:

No. of Means Apart	**Probability Levels**		
	0.05	**0.01**	**0.001**
2	0.0808	0.1133	0.1601
3	0.0846	0.1161	0.1662
4	0.0869	0.1212	0.1703

Groups with Unequal Numbers of Data ($N_1 \neq N_2$)

This procedure is very similar to that discussed earlier. As before, the means are ranked, and the differences between the means are determined ($X_1 - X_2$). Next, weighting values (a_{ij} values) are calculated for the pairs of groups being compared in accordance with

$$a_u = \sqrt{\frac{2N_iN_j}{\left(N_i + N_j\right)}} = \sqrt{\frac{2N_1N_2}{\left(N_1 + N_2\right)}}$$

This weighting value for each pair of groups is multiplied by $(\bar{X}_1 - \bar{X}_2)$ for each value to arrive at a t value. This is the t that will later be compared to a probability table.

The probability table is set up as earlier except that instead of multiplying the appropriate table values by SEM, SEM^2 is used. This is equal to $\sqrt{\text{MS}_{wg}}$.

For the desired comparison of two groups at a time, either $(\bar{X}_1 - \bar{X}_2)$ value (if $N_1 = N_2$) is compared to the appropriate probability table. Each comparison must be made according to the number of places between the means. If the table value is larger at the 0.05 level, the two groups are not considered to be statistically different. If the table value is smaller, the groups are different, and the comparison is repeated at lower levels of significance; thus, the degree of significance may be determined. We might have significant differences at 0.05 but not at 0.01, in which case the probability would be represented at $0.05 > p > 0.01$. Example 9.11 demonstrates this case.

Example 9.11

Suppose that the 400 ppm level from the earlier example had only three dogs, but that the mean for the group and the MS within groups were the same. To continue Duncan's test, we would calculate the weighting factors as follows:

100 ppm vs. 0 ppm

$$200\,\text{ppm vs. } 0\,\text{ppm } N_1 = 4; \quad N_2 = 4a_r = \sqrt{\frac{2(4)(4)}{4+4}} = 2.00$$

$$400\,\text{ppm vs. } 0\,\text{ppm } N_2 = 4; \quad N_4 = 4a_r = \sqrt{\frac{2(3)(4)}{3+4}} = 1.852$$

400 ppm vs. 100 ppm

Using the $(\bar{X}_1 - \bar{X}_2)$ from the earlier example, we can set up the following tables:

Concentration (ppm)	No. of Means Apart	$(\bar{X}_1 - \bar{X}_2)$	a_u	$(\bar{X}_1 - \bar{X}_2)a_u$
100 vs. 0	2	0.045	2.000	2.000(0.045) = 0.090
200 vs. 0	3	0.083	2.000	2.000(0.083) = 0.166
400 vs. 0	4	0.137	1.852	1.852(0.137) = 0.254
400 vs. 100	3	0.092	1.852	1.852(0.092) = 0.170

Next we calculate SEM^2 as being 0.00275 = 0.05244. This is multiplied by the appropriate table values chosen for 11 degrees of freedom (df_{wg} for this example). This gives the following probability table:

No. of Means Apart	Probability Levels 0.05	0.01	0.001
2	0.1632	0.2303	0.3291
3	0.1707	0.2401	0.3417
4	0.1753	0.2463	0.3501

Comparing the t values with the probability table values, we get the following:

Comparison	Probability
100 ppm vs. 0 ppm	$p > 0.05$
200 ppm vs. 0 ppm	$p > 0.05$
400 ppm vs. 0 ppm	$0.01 > p > 0.001$
400 ppm vs. 100 m	$0.05 > p > 0.01$

Assumptions and Limitations

1. Duncan's test assures a set alpha level or type I error rate for all tests when means are separated by no more than ordered step increases. Preserving this alpha level means that the test is less sensitive than some others, such as the Student–Newman–Keuls. The test is inherently conservative and not resistant or robust.

Scheffe's Multiple Comparisons

Scheffe's test is another post hoc comparison method for groups of continuous and randomly distributed data. It also normally involved three or more groups [37,38]. It is widely considered a more powerful significance test than Duncan's test. Each post hoc comparison is tested by comparing an obtained test value (F_{contr}) with the appropriate critical F value at the selected level of significance—the table F value multiplied by $(K - 1)$ for an F with $(K - 1)$ and $(N - K)$ degrees of freedom, where K is the number of groups being compared. F_{contr} is computed as follows:

1. Compute the mean for each sample (group).
2. Denote the residual MS by MS_{wg}.
3. Compute the test statistic as

$$F_{contr} = \frac{\left(C_1\bar{X}_1 + C_2\bar{X}_2 + \cdots + C_k\bar{X}_k^{12}\right)}{(K-1)\text{MS}_{wg}\left(C_1^2/n_1 + \cdots + C_k^2/n_k\right)}$$

where C_k is the comparison number such that the sum of C_1, C_2, …, $C_k = 0$. This is demonstrated in Example 9.12.

Example 9.12

At the end of a short-term feeding study, the following body weight changes were recorded:

	Group 1	Group 2	Group 3
	10.2	12.2	9.2
	8.2	10.6	10.5
	8.9	9.9	9.2
	8.0	13.0	8.7
	8.3	8.1	9.0
	8.0	10.8	
		11.5	
Totals	51.6	76.1	46.6
Means	8.60	10.87	9.32

$MS_{wg} = 1.395$.

To avoid logical inconsistencies with pairwise comparisons, we compare the group having the largest sample mean (group 2) with that having the smallest sample mean (group 1), then with the group having the next smallest sample mean, and so on. As soon as we find a nonsignificant comparison in this process (or no group with a smaller sample mean remains), we replace the group having the largest sample mean with that having the second largest sample mean and repeat the comparison process.

Accordingly, our first comparison is between groups 2 and 1. We set $C_1 = -1$, $C_2 = 1$, and $C_3 = 0$ and calculate our test statistic:

$$F_{contr} = \frac{(10.87 - 8.60)^2}{(3-1)(1.395)(1/6 + 1/7)} = 5.97$$

The critical region for F at $p \le 0.05$ for 2 and 11 degrees of freedom is 3.98; therefore, these groups are significantly different at this level. We next compare groups 2 and 3 using $C_1 = 0$, $C_2 = 1$, and $C_3 = -1$:

$$F_{contr} = \frac{(10.87 - 9.32)^2}{(3-1)(1.395)(1/7 + 1/5)} = 2.51$$

This is less than the critical region value, so these groups are not significantly different.

Assumptions and Limitations

1. The Scheffe procedure is robust to moderate violations of the normality and homogeneity of variance assumptions.
2. It is not formulated on the basis of groups with equal numbers (as one of Duncan's procedures is), and if $N_1 \neq N_2$, there is no separate weighting procedure.
3. It tests all linear contrasts among the population means (the other three methods confine themselves to pairwise comparison, except they use a Bonferroni-type correlation procedure).
4. The Scheffe procedure is powerful because of its robustness, yet it is very conservative. The type I error (the false-positive rate) is held constant at the selected test level for each comparison.

Dunnett's *t*-Test

Dunnett's *t*-test [39,40] has, as its starting point, the assumption that what is desired is a comparison of each of several means with one other mean and only one other mean; in other words, that one wishes to compare each and every treatment group with the control group but not compare treatment groups with each other. The problem here is that, in toxicology, one is frequently interested in comparing treatment groups with other treatment groups. However, if one does want to compare treatment groups with only a control group, then Dunnett's is a useful approach. In a study with K groups (one of them being the control), we will wish to make $(K - 1)$ comparisons. In such a situation, we want to have a P level for the entire set of $(K - 1)$ decisions (not for each individual decision). Dunnett's distribution is predicated on this assumption. The parameters for utilizing a Dunnett's table, such as found in his original article, are K (as earlier) and the number of degrees of freedom for the MS within groups (MS_{wg}). The test value is calculated as

$$t = \frac{|T_j - T_i|}{\sqrt{2MS_{wg/n}}}$$

where

- n is the number of observations in each of the groups; the MS within group (MS_{wg}) value is as we have defined it previously
- T_j is the control group mean
- T_i is the mean of, in order, each successive test group observation

Note that one uses the absolute value of the positive number resulting from subtracting T_i from T_j. This is to ensure a positive number for our final t. Example 9.13 demonstrates this test, again with the data from Example 9.9.

Example 9.13

Assume that the means, N values, and sums for the groups previously presented in Example 9.3 are as follows:

	Control	100 ppm	200 ppm	400 ppm
Sum (ΣX)	1.38	1.56	1.71	1.93
N	4	4	4	4
Mean	0.345	0.39	0.4275	0.4825

The MS_{wg} was 0.00275, and our test t for four groups and 12 degrees of freedom is 2.41. Substituting in the equation, we calculate our t for the control vs. the 400 ppm to be

$$= \frac{|0.345 - 0.4825|}{\sqrt{2(0.00275)/4}} = \frac{0.1375}{\sqrt{0.001375}} = \frac{0.1375}{0.037081} = 3.708$$

which exceeds our test value of 2.41, showing that these two groups are significantly different at $p \le 0.05$. The values for the comparisons of the control vs. the 200 and

100 ppm groups are then found to be 2.225 and 1.214, respectively. Both of these are less than our test value; therefore, the groups are not significantly different.

Assumptions and Limitations

1. Dunnett's test seeks to ensure that the type 1 error rate will be fixed at the desired level by incorporating CFs into the design of the test value table.
2. Treated group sizes must be approximately equal.

Williams's *t*-Test

Williams's *t*-test [41,42] is popular, although its use is quite limited in toxicology. It is designed to detect the highest level (in a set of dose/exposure levels) at which there is no significant effect. It assumes that the response of interest (such as change in body weights) occurs at higher levels but not at lower levels and that the responses are monotonically ordered so $X_0 \le X_1 \ldots \le X_k$. This is, however, frequently not the case. Williams's technique handles the occurrence of such discontinuities in a response series by replacing the offending value and the value immediately preceding it with weighted average values. The test also is adversely affected by any mortality at high dose levels. Such moralities "impose a severe penalty, reducing the power of detecting an effect not only at level K but also at all lower doses" [42, p. 529]. Accordingly, it is not generally applicable in toxicology studies.

Analysis of Covariance

ANCOVA is a method for comparing sets of data that consist of two variables (treatment and effect, with our effect variable being the *variate*), when a third variable (the *covariate*) exists that can be measured but not controlled and which has a definite effect on the variable of interest. In other words, it provides an indirect type of statistical control, allowing us to increase the precision of a study and to remove a potential source of bias. One common example of this is in the analysis of organ weights in toxicity studies. Our true interest here is the effect of our dose or exposure level on the specific organ weights, but most organ weights also increase (in the young, growing animals most commonly used in such studies) in proportion to increases in animal body weight. Here, as we are not interested in the effect of this covariate (body weight), we measure it to allow for adjustment. We must be careful before using ANCOVA, however, to ensure that the underlying nature of the correspondence between the variate and the covariate is such that we can rely on it as a tool for adjustments [43,44].

Calculation is performed in two steps. The first is a type of linear regression between the variate Y and the covariate X. This regression, performed as described under the "Linear Regression" section, gives us the following model:

$$Y = a_1 + BX + e$$

which in turn allows us to define adjusted means (Y and X) such that $Y_{1a} = Y_1 - (X_1 - X^n)$.

If we consider the case where K treatments are being compared such that $K = 1, 2, \ldots, K$ and we let X_{ik} and Y_{ik} represent the predictor and predicted values for each individual i in group k, respectively, we can let X_k and Y_k be the means. Then, we define the between-group (for treatment) sum of squares and cross products as

$$T_{xx} = \sum_{k-1}^{k} n_k \left(\bar{X}_k - \bar{X}\right)^2$$

$$T_{yy} = \sum_{k-1}^{k} n_k \left(\bar{Y}_k - \bar{Y}\right)^2$$

$$T_{xy} = \sum_{k-1}^{k} n_k \left(\bar{X}_k - \bar{X}\right)\left(\bar{Y}_k - \bar{Y}\right)$$

In a like manner, within-group sums of squares and cross products are calculated as

$$\sum xx = \sum_{k=1}^{K} \sum_{i} \left(X_{ik} - X_k\right)^2$$

$$\sum yy = \sum_{k=1}^{K} \sum_{i} \left(Y_{ik} - Y_k\right)^2$$

$$\sum xy = \sum_{k=1}^{K} \sum_{i} \left(X_{ik} - X_k\right)\left(Y_{ik} - Y_k\right)$$

where

i indicates the sum from all the individuals within each group
f is the total number of subjects minus number of groups

Also

$$S_{xx} = T_{xx} + \sum xx$$

$$S_{yy} = T_{yy} + \sum yy$$

$$S_{xy} = T_{xy} + \sum xy$$

With these in hand, we can then calculate the residual MSs of treatments (St^2) and error (Se^2):

$$St^2 = \frac{Tyy - \left(S^2xy/Sxx\right) + \left(\sum {}^2xy \Big/ \sum xx\right)}{lc - 1}$$

$$Se^2 = \frac{\left(\sum yy - \left(\sum {}^2y \Big/ \sum xx\right)\right)}{f - 1}$$

These can be used to calculate an F statistic to test the null hypothesis that all treatment effects are equal:

$$F = \frac{St^2}{Se^2}$$

The estimated regression coefficient of Y or X is

$$B = \frac{\sum xy}{\sum xx}$$

The estimated standard error for the adjusted difference between two groups is given by

$$\text{sd} = \text{se}\frac{1}{n_j} + \frac{1}{n_j} + \frac{\left(X_i - X_j\right)}{\sum xx}$$

where n_i and n_j are the sample sizes of the two groups. A test of the null hypothesis that the adjusted differences between the groups is zero is provided by

$$t = \frac{Y_1 - Y_0 - B\left(X_1 - X_0\right)}{\text{sd}}$$

The test value for t is then obtained from the t-table with $f - 1$ degrees of freedom. Computation is markedly simplified if all the groups are of equal size, as demonstrated in Example 9.14.

Example 9.14

An ionophere was evaluated as a potential blood-pressure-reducing agent. Early studies indicated that there was an adverse effect on blood cholesterol and hemoglobin levels, so a special study was performed to evaluate this specific effect. The hemoglobin (Hgb)-level covariate was measured at the start of the study along with the percent changes in serum triglycerides between the start of the study and at the end of the 13-week study. Was there a difference in effects of the two ionopheres?

Ionophere A		Ionophere B	
Hgb x	Serum Triglyceride (% Change) Y	Hgb x	Serum Triglyceride (% Change) Y
7.0	5	5.1	10
6.0	10	6.0	15
7.1	–5	7.2	–15
8.6	–20	6.4	5
6.3	0	5.5	10
7.5	–15	6.0	–15
6.6	10	5.6	–5
7.4	–10	5.5	–10
5.3	20	6.7	–20
6.5	–15	8.6	–40
6.2	5	6.4	–5
7.8	0	6.0	–10
8.5	–40	9.3	–40
9.2	–25	8.5	–20
5.0	25	7.9	–35
		5.0	0
		6.5	–10

To apply ANCOVA using Hgb as a covariate, we first obtain some summary results from the data as follows:

	Ionophere A (Group 1)	Ionophere B (Group 2)	Combined
Σx	112.00	119.60	231.60
Σx^2	804.14	821.64	1,625.78
Σy	–65.00	–185.00	–250.00
Σy^2	4,575.00	6,475.00	11,050.00
Σxy	–708.50	–1,506.50	–2,215.00
x	7.000	6.6444	6.8118
y	–4.625	–10.2778	–7.3529
n	16	18	34

We compute for the ionophere A group ($i = 1$):

$$S_{xx(1)} = 804.14 - \frac{(112)^2}{16} = 20.140$$

$$S_{yy(1)} = 4575.00 - \frac{(-65)^2}{16} = 4310.938$$

$$S_{xy(1)} = \quad 708.50 - \frac{(112)(-65)}{16} = \quad 253.500$$

Similarly for the ionophere B group ($i = 2$), we obtain the following:

$$S_{xx(2)} = 26.964$$

$$S_{yy(2)} = 4573.611$$

$$S_{xy(2)} = -277.278$$

Finally, for the combined data (ignoring groups), we compute as follows:

$$S_{xx} = 48.175$$

$$S_{yy} = 9211.765$$

$$S_{xy} = -512.059$$

The sums of squares can now be obtained as

$$\text{TOT}(\text{SS}) = 9211.8$$

$$\left(20.140 + 26.964\right)\left(4310.938 + 4573.611\right)$$

$$\text{SSE} = \frac{-\left[-253.5 - 277.28\right]^2}{\left(20.14 + 26.964\right)} = 2903.6$$

$$\text{SSG} = \frac{\left(48.175\right)\left(9211.765\right) - \left(-512.059\right)^2}{48.175} - 2903.6 = 865.4$$

$$\text{SSC} = \left(4310.938 + 473.611\right) - 2903.6 = 5980.9$$

and the ANCOVA summary table can be completed as

Source	df	SS	MS	*F*
Treatment	1	865.4	865.4	9.2[a]
X (Hgb)	1	5980.9	5980.9	63.8
Error	31	2903.7	93.7	
Total	33	9211.8		

[a] Significant ($p < 0.05$); critical *F* value = 4.16.

The *F* statistics are formed as the ratios of effect MS to the MSE (93.7). Each *F* statistic is compared with the critical *F* value with 1 upper and 31 lower degrees of freedom. The critical *F* value for $\alpha = 0.05$ is 4.16. The significant covariate effect ($F = 63.8$) indicates that the triglyceride response has a significant linear relationship with Hgb. The significant *F* value for treatment indicates that the mean triglyceride response adjusted for hemoglobin effect differs between treatment groups.

Assumptions and Limitations

1. The underlying assumptions for ANCOVA are fairly rigid and restrictive. The assumptions include the following:
   a. The slopes of the regression lines of a *Y* and *X* are equal from group to group. This can be examined visually or formally (i.e., by a test). If this condition is not met, ANCOVA cannot be used.
   b. The relationship between *X* and *y* is linear.
   c. The covariate *X* is measured without error. Power of the test declines as error increases.
   d. There are no unmeasured confounding variables.
   e. The errors inherent in each variable are independent of each other. Lack of independence effectively (but to an immeasurable degree) reduces sample size.
   f. The variances of the errors in groups are equivalent between groups.
   g. The measured data that form the groups are normally distributed. ANCOVA is generally robust to departures from normality.
2. Of the seven assumptions provided earlier, the most serious are the first four.

MODELING

The mathematical modeling of biological systems, restricted even to the field of toxicology, is an extremely large and vigorously growing area. Broadly speaking, modeling is the principal conceptual tool by which toxicology seeks to develop as a mechanistic science. In an iterative process, models are developed or proposed, tested by experiment, reformulated, and so on, in a continuous cycle. Such a cycle could also be described as two related types of modeling: *explanatory* (where the concept is formed) and *correlative* (where data are organized and relationships derived). An excellent introduction to the broader field of modeling of biological systems can be found in Gold [45].

In toxicology, modeling is of prime interest in seeking to relate a treatment variable with an effect variable and, from the resulting model, predict effects at exact points where no experiment has been done (but in the range where we have performed experiments, such as determining LD_{50} values) to estimate how good our prediction is, and, occasionally, simply to determine if a pattern of effects is related to a pattern of treatment.

For use in prediction, the techniques of linear regression, probit/logit analysis (a special case of linear regression), moving averages (an efficient approximation method), and nonlinear regression (for doses where data cannot be made to fit a linear pattern) are presented. For evaluating the predictive value of these models, both the correlation coefficient (for parametric data) and Kendall's rank correlation (for nonparametric data) are given. And, finally, the concept of trend analysis is introduced and a method presented.

When we are trying to establish a pattern between several data points (whether this pattern is in the form of a line or a curve), what we are doing is *interpolating*. It is possible for any given set of points to produce an infinite set of lines or curves that pass near (for lines) or through (for curves) the data points. In most cases, we cannot actually know the real pattern, so we apply a basic principle of science: Occam's razor. We use the simplest explanation (or, in this case, model) that fits the facts (or data). A line is, of course, the simplest pattern to deal with and describe, so fitting the best line (linear regression) is the most common form of model in toxicology.

Linear Regression

Foremost among the methods for interpolating within a known data relationship is regression, which involves the fitting of a line or curve to a set of known data points on a graph and interpolating (estimating) this line or curve in areas where we have no data points. The simplest of these regression models is that of linear regression (which is valid when increasing the value of one variable changes the value of the related variable in a linear fashion, either positively or negatively). This is the case we will explore here, using the method of least squares.

Given that we have two sets of variables, *x* (say, mg/kg of test material administered) and *y* (say, percentage of animals so dosed that die), it is necessary to solve for *a* and *b* in the equation $Y_i = a + bx_i$, where the uppercase Y_i is the fitted value of y_i at x_i and we wish to minimize $(y_i - Y_i)^2$. So, we solve the following equations:

$$a = \bar{y} - b\bar{x}$$

$$b = \frac{\sum x_1 y_1 - n\bar{x}\bar{y}}{\sum x_1^2 - n\bar{x}^2}$$

where

a is the *y* intercept
b is the slope of the time
n is the number of data points

Use of this is demonstrated in Example 9.13.

Note that, in actuality, dose–response relationships are often not linear, and instead we must use either a transform (to linearize the data) or a nonlinear regression method [46]. Note also that we can use the correlation test statistic (to be described in the "Correlation Coefficient" section) to determine if the regression is significant (and, therefore, valid at a defined level of certainty). A more specific test for significance would be the linear regression ANOVA [31]. To do so, we start by developing the appropriate ANOVA table and then proceed to perform the linear regression portion of the ANOVA as shown in Example 9.15.

Example 9.15

From a short-term toxicity study, we have the following results:

Dose Administered (mg/kg)	Percent Animals Dead (%)		
x_1	x_1^2	y_1	x_1y_1
1	1	10	10
3	9	20	60
4	16	18	72
5	25	20	100
Sums $x_1 = 13$	$x_1^2 = 51$	$y_1 = 68$	$x_1y_1 = 242$

$$\bar{x} = 3.25 \quad \text{and} \quad \bar{y} = 17$$

$$a = 17 - (2.4)(3.25) = 9.20$$

$$b = \frac{242 - (4)(3.25)(17)}{51 - (4)(10.5625)} = \frac{21}{8.75} = 2.40$$

We therefore see that our fitted line is $Y = 9.2 + 2.4X$. These ANOVA table data are then used as shown in Example 9.12.

Linear Regression ANOVA

Source of Variation (1)	Sum of Squares (2)	Degrees of Freedom (3)	MS (=2/3) (4)
Regression	$b_1^2\left(\sum x_1^2 - n\bar{x}^2\right)$	1	By division
Residual	By difference	$n - 2$	By division
Total	$\sum y_1^2 - n\bar{y}^2$	$n - 1$	

We then calculate $F_{1,n-2}$ = (regression MS)/(residual MS). This is demonstrated in Example 9.16.

Example 9.16

We desire to test the significance of the regression line in Example 9.11:

$$\sum y_1^2 = 10^2 + 20^2 + 18^2 + 20^2$$

$$\text{Regression SS} = (2.4)^2\left[51 - 4(3.25)^2\right] = 50.4$$

$$\text{Total SS} = 1224 - 4\left(17^2\right) = 68$$

$$\text{Residual SS} = 68 - 50.4 = 17.6$$

$$F_{1,2} = \frac{50.4}{8.8} = 5.73$$

This value is not significant at the 0.05 level; therefore, the regression is not significant. A significant F value (as found in an F distribution table for the appropriate degrees of freedom) indicates that the regression line is an accurate prediction of observed values at that confidence level. Note that the portion of the total sum of squares explained by the regression is called the *coefficient of correlation*, which in the earlier example is equal to 0.86^2 (or 0.74). Calculation or the correlation coefficient is described later in this chapter.

Finally, we might wish to determine the CIs for our regression line; that is, given a regression line with calculated values for Y_i given x_i, within what limits may we be certain (with, say, a 95% probability) what the real value of Y_i is? If we denote the residual MS in the ANOVA by s^2, then the 95% confidence limits for a (denoted by A, the notation for the true, as opposed to estimated, value for this parameter) are calculated as

$$t_{n-2} = \frac{a - A}{\sqrt{\dfrac{s^2\left(\sum x^2\right)}{n\sum x_1^2 - n^2\bar{x}^2}}}$$

$$= \frac{9.2 - A}{\sqrt{\dfrac{8.8(51)}{4(51) - (16)(10.562)}}} = \frac{9.2 - A}{\sqrt{\dfrac{448}{35.008}}}$$

$$9.2 - A = -15.405$$

$$= 9.2 - 15.405$$

Assumptions and Limitations

1. All the regression methods are for interpolation, not extrapolation; that is, they are valid only in the range for which we have data—the experimental region—not beyond.
2. The method assumes that the data are independent and normally distributed, and it is sensitive to outliers. The x-axis (or horizontal) component plays an extremely important part in developing the least-squares fit. All points have equal weight in determining the height of a regression line, but extreme x-axis values unduly influence the slope of the line.

3. A good fit between a line and a set of data (i.e., a strong correlation between treatment and response variables) does not imply any causal relationship.
4. It is assumed that the treatment variable can be measured without error, that each data point is independent, that variances are equivalent, and that a linear relationship does not exist between the variables.
5. The many excellent texts on regression, which is a powerful technique, include Draper and Smith [47] and Montgomery et al. [48], which are not overly rigorous mathematically.

Probit/Log Transforms and Regression

As we noted in the preceding section, dose–response problems (among the most common interpolation problems encountered in toxicology) rarely are straightforward enough to make a valid linear regression directly from the raw data. The most common valid interpolation methods are based on probability (probit) and logarithmic (log) value scales, with percentage responses (death, tumor incidence, etc.) being expressed on the probit scale and doses (Y_i) expressed on the log scale. There are two strategies for such an approach. The first is based on transforming the data to these scales, then doing a weighted linear regression on the transformed data. (If one does not have access to a computer or a high-powered programmable calculator, the only practical strategy is not to assign weights.) The second requires the use of algorithms (approximate calculation techniques) for the probit value and regression process and is extremely burdensome to perform manually.

Our approach to the first strategy requires that we construct a table with the pairs of values of x_i and y_i listed in the order of increasing values of Y_i (percentage response). Beside each of these columns, a set of blank columns should be left so the transformed values may be listed. We then simply add the columns described in the linear regression procedure. Log and probit values may be taken from any of a number of sets of tables, and the rest of the table is then developed from these transformed x_i and y_i values (denoted as x_i' and y_i'). A standard linear regression is then performed (see Example 9.17). The second strategy we discussed has been broached by a number of authors [49–52]. All of these methods, however, are computationally cumbersome. It is possible to approximate the necessary iterative process using the algorithms developed by Abramowitz and Stegun [53], but even this merely reduces the complexity to a point where the procedure may be readily programmed on a small computer or programmable calculator.

Example 9.17

(See the following table.) Our interpolated log of the LD_{50} is 1.000539, calculated by using $Y = -0.200591 - 0.240226x$, where x equals 5.000 (the probit of 50%) in the regression equation. When we convert this log value to its linear equivalent, we get an LD_{50} of 10.0 mg/kg. Finally, our calculated correlation coefficient is $r = 0.997$. A goodness-of-fit of the data using chi-square may also be calculated.

Percentage of Animals Killed (x_1)	Probit of $x_1 = x_1'$	Dose of Chemical (mg/kg) (y_1)	Log of $y_1 = y_1'$	$(x_1')^2$	$x_1'y_1'$
2	2.9463	3	0.4771	8.6806	1.40568
10	3.7184	5	0.6990	13.8264	2.59916
42	4.7981	10	1.0000	23.0217	4.79810
90	6.2816	20	1.3010	39.4585	8.17223
98	7.2537	30	1.4771	52.6162	10.4190
	$\sum x_1' =$		$\sum y_1' =$	$\sum (x_1')^2 =$	$\sum x_1'y_1' =$
	24.9981		4.9542	137.6034	27.68974

Assumptions and Limitations

1. The probit distribution is derived from a common error function, with the midpoint (50% point) moved to a score of 5.00.
2. The underlying frequency distribution becomes asymptotic as it approaches the extremes of the range; that is, in the range of 16%–84%, the corresponding probit values change gradually. The curve is relatively linear, but beyond this range, the values change ever more rapidly as they approach either 0% or 100%. In fact, there are no values for either of these numbers.
3. A normally distributed population is assumed, and the results are sensitive to outliers.

Moving Averages

An obvious drawback to the interpolation procedures we have examined to date is that they do take a significant amount of time (although they are simple enough to be done manually), especially if the only result we desire is an LD_{50}, LC_{50}, or LT_{50}. The method of moving averages [54,55] gives a rapid and reasonable accurate estimate of this median effective dose (m) and the estimated SD of its logarithm. Such methodology requires that the same number of animals be used per dosage level and that the spacing between successive dosage exposure levels be geometrically constant (e.g., levels of 1, 2, 4, and 8 mg/kg or 1, 3, 9, and 27 ppm). Given this and access to a table for the computation of moving averages, one can readily calculate the median effective dose with the following formula (illustrated for dose):

$$\log m = \log D + \frac{d(K-1)}{2} + df$$

where

m is the median effective dose or exposure
D is the lowest dose tested
d is the log of the ratio of successive doses/exposures
f is a table value taken from Gad [23] for the proper K (the total number of levels tested minus 1)

Example 9.18 demonstrates the use of this method and the new tables.

Example 9.18

As part of an inhalation study, we exposed four groups of five rats each to levels of 20, 40, 80, and 160 ppm of a chemical vapor. These exposures killed 0, 1, 3, and 5 animals, respectively. From the $N = 5$, $K = 3$ tables on the r value 0, 1, 3, 5 line, we get an f of 0.7 and an α_f^4 of 0.31623. We can then calculate the LC_{50} to be

$$\log LC_{50} = 1.30130 + \frac{0.30103(2)}{2} + 0.30103(0.7)$$

$$= 1.30103 + 0.51175$$

$$= 1.81278$$

$$\therefore LC_{50} = 65.0 \text{ ppm with 95\% CIs of } \pm 2.179\, d\sigma_f$$

or

$$\pm 2.179(0.30103) \times (0.31623) = \pm 0.20743$$

Therefore, the log confidence limits are 1.81278 ± 0.20743 = 1.60535–2.02021; on the linear scale, 40.3–104.8 ppm.

Assumptions and Limitations

1. A common misconception is that the moving-average method cannot be used to determine the slope of the response curve. This is not true. Weil has published a straightforward method for determining slope in conjunction with a moving-average determination of the LD_{50} [56].
2. The method also provides CIs.

Nonlinear Regression

More often than not in toxicology, we find that our data demonstrate a relationship between two variables (such as age and body weight) that is not linear; that is, a change in one variable (e.g., age) does not produce a directly proportional change in the other (e.g., body weight), but some form of relationship between the variables is apparent. If understanding such a relationship and being able to predict unknown points are of value, we have a pair of options available to us. The first, which was discussed and reviewed earlier, is to use one or more transformations to linearize our data and then to make use of linear regression. This approach, although most commonly used, has a number of drawbacks. Not all data can be suitably transformed. Sometimes the transformations necessary to linearize the data require a cumbersome series of calculations, and the resulting linear regression is not always sufficient to account for the differences among sample values; there might be significant deviations around the linear regression line (i.e., a line may still not give us a good fit to the data or do an adequate job of representing the relationship between the data). In such cases, we have a second option available—fitting the data to some nonlinear function such as some form of the curve. This is, in general form, nonlinear regression and may involve fitting data to an infinite number of possible functions, but most often, we are interested in fitting curves to a polynomial function of the general form:

$$Y = a + bx + cx^2 + dx^2 + \cdots$$

where x is the independent variable. As the number of powers of x increases, the curve becomes increasingly complex and will be able to fit a given set of data increasingly well. Generally in toxicology, however, if we plot the log of a response (such as body weight) vs. a linear scale of our dose or stimulus, we get one of four types of nonlinear curves [16]:

- *Exponential growth*, where $\log Y = A(B^x)$, such as the growth curve for the log phase of a bacterial culture
- *Exponential decay*, where $\log Y = A(B^{-x})$, such as a radioactive decay curve
- *Asymptotic regression*, where $\log Y = A - B(p^x)$, such as a first-order reaction curve
- *Logistic growth curve*, where $\log Y = A/(1 + Bp^x)$, such as a population growth curve

In all of these cases, A and B are constants and p is a log transform. These curves are illustrated in Figure 9.7.

All four types of curves are fit by iterative processes; that is, best-guess numbers are initially chosen for each of the constants, and after a fit is attempted, the constants are modified to improve the fit. This process is repeated until an acceptable fit has been generated. ANOVA or ANCOVA can be used to objectively evaluate the acceptability of it. Needless to say, the use of a computer generally accelerates such a curve-fitting process.

Assumptions and Limitations

1. The principle of using least squares may still be applicable in fitting the best curve, if the assumptions of normality, independence, and reasonably error-free measurement of response are valid.
2. Growth curves are best modeled using a nonlinear method.

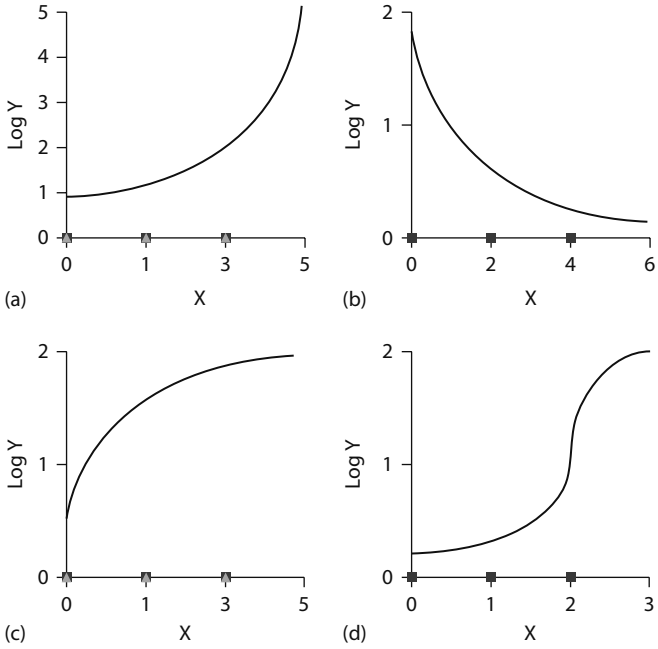


FIGURE 9.7 Common curvilinear curves. (a) Exponential growth law Log Y = A(B^X), (b) exponential decay law Log Y = A(B^{-X}), (c) asymptotic regression Log Y = A – B(ψ^X), (d) logistic growth law Log Y = A/(1 + Bψ^X).

Correlation Coefficient

The correlation procedure is used to determine the degree of linear correlation (direct relationship) between two groups of continuous (and normally distributed) variables; it will indicate whether there is any statistical relationship between the variables in the two groups. For example, we may wish to determine if the liver weights of dogs on a feeding study are correlated with their body weights. Thus, we will record the body and liver weights at the time of sacrifice and then calculate the correlation coefficient between these pairs of values to determine if there is some relationship. A formula for calculating the linear correlation coefficient (r_{xy}) is as follows:

$$r_{xy} = \frac{N\sum XY - \left(\sum X\right)\left(\sum Y\right)}{\sqrt{N\sum X^2 - \left(\sum X\right)^2}\sqrt{N\sum Y^2 - \left(\sum Y\right)^2}}$$

where

- X is each value for one variable (such as the dog body weights in the earlier example)
- Y is the matching value for the second variable (the liver weights)
- N is the number of pairs of X and Y

Once we have obtained r_{xy}, it is possible to calculate t_r, which can be used for more precise examination of the degree of significant linear relationship between the two groups. This value is calculated as follows:

$$t_r = \frac{r_{zy}\sqrt{N-2}}{\sqrt{1-r_{zy}^2}}$$

This calculation is also equivalent to r = sample covariance/(S_xS_y), as was seen earlier under ANCOVA.

The value obtained for r_{xy} can be compared to table values [16] for the number of pairs of data involved minus two. If the r_{xy} is smaller (at the selected test probability level, such as 0.05), the correlation is not significantly different from zero (no correlation). If r_{xy} is larger than the table value, there is a positive statistical relationship between the groups. Comparisons are then made at lower levels of probability to determine the degree of relationship (note that if r_{xy} equals either 1.0 or –1.0, there is complete correlation between the groups). If r_{xy} is a negative number and the absolute value is greater than the table value, there is an inverse relationship between the groups; that is, a change in one group is associated with a change in the opposite direction in the second group of variables. Both computations are demonstrated in Example 9.19.

Because the comparison of r_{xy} with the table values may be considered a somewhat weak test, it is perhaps more meaningful to compare the t_r value with values in a t-distribution table for (N – 2) degrees of freedom (df), as is done for Student's t-test. This will give a more exact determination of the degree of statistical correlation between the two groups. Note that this method examines only possible linear relationships between sets of continuous normally distributed data.

Example 9.19

If we computed the dog body weight vs. dog liver weight for a study, we could obtain the following results:

Dog	Body Weight (kgj) X	X^2	Liver Weight (g) Y	Y^2	XY
1	8.4	70.56	243	59,049	2,041.2
2	8.5	72.25	225	50,625	1,912.5
3	9.3	86.49	241	58,081	2,241.3
4	9.5	90.25	263	69,169	2,498.5
5	10.5	110.25	256	65,536	2,688.0
6	8.6	73.96	266	70,756	2,287.6
Sum	ΣX = 54.8	ΣX^2 = 503.76	ΣY = 1,494	ΣY^2 = 373,216	ΣXY = 13,669.1

$$r_{xy} = \frac{6(13,669.1) - (54.8)(1,494)}{\left(\sqrt{6(503.76) - (54.8)^2}\right)\left(\sqrt{6(373,216) - (1,494)^2}\right)}$$

The table value for six pairs of data (read beside the [$N - 2$] value, or $6 - 2 = 4$) is 0.811 at a 0.05 probability level. Thus, there is a lack of statistical correlation (at $p = 0.05$) between the body weights and liver weights for this group of dogs. The t_r value for these data would be calculated as follows:

$$t_r = \frac{0.381\sqrt{6-2}}{\sqrt{1-(0.381)^2}}$$

The value for the *t*-distribution table for 4 degrees of freedom at the 0.05 level is 2.776; therefore, this again suggests a lack of significant correlation at $p = 0.05$.

Assumptions and Limitations

1. A strong correlation does not imply that a treatment causes an effect.
2. The distances of data points from the regression line are the portions of the data not explained by the model. These are called *residuals*. Poor correlation coefficients imply high residuals, which may be due to many small contributions (variations of data from the regression line) or a few large ones. Extreme values (outliers) greatly reduce correlation.
3. *X* and *Y* are assumed to be independent.
4. Feinstein [57] has provided a fine discussion of the difference between correlation (or association of variables) and causation.

Kendall's Coefficient of Rank Correlation

Kendall's rank correlation, represented by τ (tau), should be used to evaluate the degree of association between two sets of data when the nature of the data is such that the relationship may not be linear. Most commonly, this is when the data are not continuous or normally distributed. An example of such a case is when we are trying to determine if there is a relationship between the length of hydra and their survival time (in hours) in a test medium, as is presented in Example 9.18. Both of our variables here are discontinuous, yet we suspect a relationship exists. Another common use is in comparing the subjective scoring done by two different observers.

Tau is calculated at $\tau = N/n(n-1)$, where n is the sample size and N is the count of ranks, calculated as $N = 4({}^nC_i) - n(n-1)$, with the computing of ${}^nC_i$ being demonstrated in the example. If a second variable Y_2 is exactly correlated with the first variable Y_1, then the variates Y_2 should be in the same order as the Y_1 variates; however, if the correlation is less than exact, the order of the variates Y_2 will not correspond entirely to that of *Y*. The quantity N measures how well the second variable corresponds to the order of the first. It has a maximum value of $n(n-1)$ and a minimum value of $-n(n-1)$.

A table of data is set up with each of the two variables being ranked separately. Tied ranks are assigned as demonstrated earlier under the Kruskal–Wallis test. From this point, disregard the original variates and deal only with the ranks. Place the ranks of one of the two variables in rank order (from lowest to highest), paired with the rank values assigned for the other variable. If one (but not the other) variable has tied ranks, order the pairs by the variables without ties [15]. The most common way to compute a sum of the counts is also demonstrated in Example 9.20. The resulting value of tau will range from –1 to +1, as does the familiar parametric correlation coefficient, *r*.

Example 9.20

During the validation of an in vitro method, it was noticed that larger hydra seem to survive longer in test media than do small individuals. To evaluate this, 15 hydra of random size were measured (mm), then placed in test media. How many hours each individual survives was recorded over a 24 h period. These data are presented in the following text, along with ranks for each variable.

Length	Rank (R_1)	Survival	Rank (R_2)
3	6.5	19	9
4	10	17	7
6	15	11	1
1	1.5	25	15
3	6.5	18	8
3	6.5	22	12
1	1.5	24	14
4	10	16	6
4	10	15	5
2	3.5	21	11
5	13	13	3
5	13	14	4
3	6.5	20	10
2	3.5	23	13
5	13	12	2

We then arrange this based on the order of the rank of survival time (there are no ties here). We then calculate our counts of ranks. The conventional method is to obtain a sum of counts C_i, as follows: examine the first value in the column of ranks paired with the ordered column. In the following case, this is rank 15. Count all ranks subsequent to it that rank greater than 15. There are 14 ranks following the 2 and all of them are less than 15; therefore, we count a score of $C_1 = 0$. We repeat this process for each subsequent rank of R_1, giving us a final score of 1. By this point, it is obvious that our original hypothesis—that larger hydrae live longer in test media than do small individuals—was in error.

R_2	R_1	Following R_2 Ranks Greater than R_1	Counts (C_i)
1	15	—	$C_1 = 0$
2	13	—	$C_2 = 0$
3	13	—	$C_3 = 0$
4	13	—	$C_4 = 0$
5	10	—	$C_5 = 0$
6	6.5	10	$C_6 = 0$
7	10	—	$C_7 = 0$
8	6.5	—	$C_8 = 0$
9	6.5	—	$C_9 = 0$
10	6.5	—	$C_{10} = 0$
11	3.5	6.5	$C_{11} = 0$
12	6.5	—	$C_{12} = 0$
13	3.5	—	$C_{13} = 0$
14	1.5	—	$C_{14} = 0$
15	1.5	—	$C_{15} = 0$
			$C_i = 1$

Our count of ranks (N) is then calculated as follows:

In other words, there is a strong negative correlation between our variables.

$$N = 4(1) - 15(5-1)$$
$$= 4 - 15(14)$$
$$= -206$$

We can then calculate tau as

$$= \frac{-206}{15(15-1)} = \frac{-206}{210} = -0.9810$$

Assumption and Limitation

1. This is a very robust estimator that does not assume normality, linearity, or minimal error of measurement.

Trend Analysis

Trend analysis is a collection of techniques utilized by toxicology since the mid-1970s [58]. The actual methodology dates back to the mid-1950s [59]. Trend analysis methods are a variation on the theme of regression testing. In the broadest sense, the methods are used to determine whether a sequence of observations taken over an ordered range of a variable (most commonly time) exhibits some form of pattern of change (an increase or upward trend) associated with another variable of interest (in toxicology, some form or measure of dosage and/or exposure). Trend corresponds to sustained and/or systematic variations over a long period of time. It is associated with the structural causes of the phenomenon in question—for example, population growth, technological progress, new ways of organization, or capital accumulation.

The identification of trend has always posed a serious statistical problem. The problem is not one of mathematical or analytical complexity but of conceptual complexity. This problem exists because the trend as well as the remaining components of a time series are latent (nonobservables) variables so, therefore, assumptions must be made on their behavioral pattern. The trend is generally thought of as a smooth and slow movement over the long term. The concept of *long* in this connection is relative, and what is identified as a trend for a given series span might well be part of a long cycle once the series is considerably augmented. Often, a long cycle is treated as a trend because the length of the observed time series is shorter than one complete face of this type of cycle. The ways in which data are collected in toxicology studies frequently serve to complicate trend analysis, as the length of time for the phenomena underlying a trend to express themselves is frequently artificially censored.

To avoid the complexity of the problem posed by a statistically vague definition, statisticians have resorted to two simple solutions: one consists of estimating trend and cyclical fluctuations together (the *trend cycle*); the other consists of defining the trend in terms of the series length (the *longest nonperiodic movement*).

Trend Models

Within the large class of models identified for trend, we can distinguish two main categories: deterministic trends and stochastic trends. Deterministic trend models are based on the assumption that the trend of a time series can be approximated closely by simple mathematical functions of time over the entire span of the series. The most common representation of a deterministic trend is by means of polynomials or of transcendental functions. The time series from which the trend is to be identified is assumed to be generated by a nonstationary process where the nonstationarity results from a deterministic trend. A classical model is the regression or error model [66], where the observed series is treated as the sum of a systematic part or trend and a random part or irregular. This model can be written as

$$Z_t = Y_t + U_t'$$

where U_t is a purely random process, that is, $U_t \sim$ i.i.d. $(0, 2/u)$ (independent and identically distributed with expected value 0 and variance $\sigma(2/u)$).

Trend tests are generally described as k-sample tests of the null hypothesis of identical distribution against an alternative of linear order; in other words, if sample I has distribution function F_i, $i = 1$, then the null hypothesis H_0: $F_1 = F_2 - \ldots = F_k$ is tested against the alternative: H_1: $F_1 \geq F_2 \geq \ldots = F_k$ (or its reverse), where at least one of the inequalities is strict. These tests can be thought of as special cases of tests of regression or correlation in which association is sought between the observations and its ordered sample index. They are also related to ANOVA except that the tests are tailored to be powerful against the subset of alternatives H_1 instead of the more general set $\{F_1 \neq F_j$, some $i \neq j\}$.

	Control		Low Doses			High Doses		
Month of Study	Total *X* Animals with Tumors	Change (X_{A-B})	Total *Y* Animals with Tumors	Change (Y_{A-B})	Compared to Control ($Y - X$)	Total *Z* Animals with Tumors	Change (Z_{A-B})	Compared to Control ($Z - X$)
12 (A)	1	NA	0	NA	NA	5	NA	NA
13 (B)	1	0	0	0	0	7	2	(+)2
14 (C)	3	2	1	1	(–)1	11	4	(+)2
15 (D)	3	0	1	0	0	11	0	0
16 (E)	4	1	1	0	(–)1	13	2	(+)1
17 (F)	5	1	3	2	(+)1	14	1	0
18 (G)	5	0	3	0	0	15	1	(+)1
19 (H)	5	0	5	2	(+)2	18	3	(+)3
20 (I)	6	1	6	1	0	19	1	0
21 (J)	8	2	7	1	(–)1	22	3	(+)1
22 (K)	12	4	9	2	(–)2	26	4	0
23 (L)	14	2	12	3	(+)1	28	2	0
24 (M)	18	4	17	5	(+)1	31	3	(–)1
				Sum of signs	4+		Sum of signs	6+
				$Y - X$	4–		$Z - X$	1–
					= 0 (no trend)			= 5

Different tests arise from requiring power against specific elements or subsets of this rather extensive set of alternatives. The most popular trend test in toxicology is currently that presented by Tarone in 1975 [58] because it is the one used by the National Cancer Institute (NCI) in the analysis of carcinogenicity data. A simple, but efficient alternative is the Cox and Stuart test [65], which is a modification of the sign test. For each point at which we have a measure (such as the incidence of animals observed with tumors), we form a pair of observations—one from each of the groups we wish to compare. In a traditional NCI bioassay, this would mean pairing control with low dose and low dose with high dose (to explore a dose-related trend) or each time period observation in a dose group (except the first) with its predecessor (to evaluate a time-related trend). When the second observation in a pair exceeds the earlier observation, we record a plus sign for that pair. When the first observation is greater than the second, we record a minus sign for that pair. A preponderance of plus signs suggests a downward trend, while an excess of minus signs suggests an upward trend. A formal test at a preselected confidence level can then be performed.

More formally put, after having defined what trend we want to test for, we first match pairs as $(X_1 - X_{1+c})$, (X_2, X_{2+c}), …, $(X_{n'-c}, X_{n'})$, where $c = n'/2$ when n' is even and $c = (n' + 1)/2$ when n' is odd (where n' is the number of observations in a set). The hypothesis is then tested by comparing the resulting number of excess positive or negative signs against a sign test table such as are found in Beyer. We can, of course, combine a number of observations to allow ourselves to actively test for a set of trends, such as the existence of a trend of increasing difference between two groups of animals over a period of time. This is demonstrated in Example 9.21.

Example 9.21

In a chronic feeding study in rats, we tested the hypothesis that, in the second year of the study, there was a dose-responsive increase in tumor incidence associated with the test compound. We utilize a Cox–Stuart test for trend to address this question (see the previous table). All groups start the second year with an equal number of animals. Reference to a sign table is not necessary for the low-dose comparison (where there is no trend) but clearly shows the high dose to be significant at the $p \leq 0.5$ level.

Assumptions and Limitations

1. Trend tests seek to evaluate whether there is a monotonic tendency in response to a change in treatment; that is, the dose–response direction is absolute. As the dose goes up, the incidence of tumors increases. Thus, the test loses power rapidly in response to the occurrences of reversals—for example, a low-dose group with a decreased tumor incidence. Methods are available [61] that smooth the bumps of reversals in long data series. In toxicology, however, most data series are short (i.e., there are only a few dose levels).

   Tarone's trend test is most powerful at detecting dose-related trends when tumor onset hazard functions are proportional to each other. For more power against other dose-related group differences, weighted versions of the statistic are also available [62,63]. In 1985, the U.S. *Federal Register* recommended that the analysis of tumor incidence data be carried out with a Cochran–Armitage trend test [64,65]. The test statistic of the Cochran–Armitage test is defined as the term

$$T_{CA} = \sqrt{\frac{N}{(N-r)r}}, \quad \frac{\sum_{i=0}^{k}\left(R_1 - (n_1/N)r\right)d_1}{\sqrt{\sum_{i=0}^{k}(n_i/N)d_1^2\left(\sum_{i=0}^{k}(n_i/N)d_1\right)^2}}$$

with dose scores d_i. Armitage's test statistic is the square of this term (T_{CA}^2). As one-sided tests are carried out for an increase in tumor rates, the square is not considered. Instead, the earlier-mentioned test statistic presented by Portier and Hoel [66] is used. This test statistic is asymptotically standard normal distributed. The Cochran–Armitage test is asymptotically efficient for all monotone alternatives [58], but this result holds only asymptotically. Tumors are rare events, so the binominal proportions are small. In this situation, approximations may become unreliable. As a result, exact tests that can be performed using two different approaches—conditional and unconditional—are considered. In the first case, the total number of tumors r is regarded as fixed; thus, the null distribution of the test statistic is independent of the common probability p. The exact conditional null distribution is a multivariate hypergeometric distribution. The unconditional model treats the sum of all tumors as a random variable, and the exact unconditional null distribution is a multivariate binomial distribution. The distribution depends on the unknown probability.

METHODS FOR THE REDUCTION OF DIMENSIONALITY

Techniques for the reduction of dimensionality are those that simplify the understanding of data, either visually or numerically, while causing only minimal reductions in the amount of information present. These techniques operate primarily by pooling or combining groups of variables into single variables but may also entail the identification and elimination of low-information-content (or irrelevant) variables. Descriptive statistics (calculations of means, SDs, etc.) are the simplest and most familiar form of reduction of dimensionality. Here, we first need to address classification, which provides the general conceptual tools for identifying and quantifying similarities and differences between groups of things that have more than a single linear scale of measurement in common (e.g., which have both been determined to have or lack a number of enzyme activities). We will then consider two collections of methodologies that combine graphic and computational methods: multidimensional/nonmetric scaling and cluster analysis. Multidimensional scaling (MDS) is a set of techniques for quantitatively analyzing similarities, dissimilarities, and distances between data in a display-like manner. Nonmetric scaling is an analogous set of methods for displaying and relating data when measurements are nonquantitative (the data are described by attributes or ranks). Cluster analysis is a collection of graphic and numerical methodologies for classifying things based on the relationships between the values of the variables that they share. The final pair of methods for the reduction of dimensionality that will be tackled in this chapter is Fourier analysis and life-table analysis. Fourier analysis seeks to identify cyclic patterns in data and then either analyze the patterns or the residuals after the patterns are taken out. Life-table analysis techniques are directed toward identifying and quantitating the time course of risks (such as death or the occurrence of tumors).

Classification

Classification is both a basic concept and a collection of techniques that are necessary prerequisites for further analysis of data when the members of a set of data are (or can be) each described by several variables. At least some degree of classification (which is broadly defined as the division of the members of a group into smaller groups in accordance with a set of decision rules) is necessary prior to any data collection. Whether formally or informally, an investigator has to decide which things are similar enough to be counted as the same and develop rules for governing collection procedures. Such rules can be as simple as "measure and record body weights only of live animals on study" or as complex as that demonstrated by the expanded weighting classification presented in Example 9.22. Such a classification also demonstrates that the selection of which variables to measure will determine the final classification of data.

Example 9.22

Is animal of desired species?	Yes/No
Is animal member of study group?	Yes/No
Is animal alive?	Yes/No
Which group does animal belong to?	
Control	
Low dose	
Intermediate dose	
High dose	
What sex is the animal?	Male/Female
Is the measured weight within an acceptable range?	Yes/No

Classifications of data have two purposes [67,68]: *data simplification* (also known as *descriptive function*) and *prediction*. Simplification is necessary because there is a limit to both the volume and the complexity of data that the human mind can comprehend and deal with conceptually. Classification allows us to attach a label (or name) to each group of data, to summarize the data (i.e., assign individual elements of data to groups and to characterize the population of the group) and to define the relationships between groups (i.e., develop a taxonomy).

Prediction, meanwhile, is the use of summaries of data and knowledge of the relationships between groups to develop hypotheses as to what will happen when further data are collected (as when more animals or people are exposed to an agent under defined conditions) and as to the mechanisms that cause such relationships to develop. Indeed, classification is the prime device for the discovery of mechanisms in all of science. A classic example of this was Darwin's realization that there were reasons (the mechanisms of evolution) behind the differences and similarities in species that had caused Linnaeus to earlier develop his initial modern classification scheme (or taxonomy) for animals.

To develop a classification, one first sets bounds wide enough to encompass the entire range of data to be considered but not unnecessarily wide. This is typically done by selecting some global variables (variables every piece of datum has in common) and limiting the range of each so it just encompasses all the cases on hand. Then one

selects a set of local variables (characteristics that only some of the cases have, such as the occurrence of certain tumor types, enzyme activity levels, or dietary preferences) that serves to differentiate between groups. Data are then collected, and a system for measuring differences and similarities is developed. Such measurements are based on some form of measurement of distance between two cases (x and y) in terms of each single variable scale. If the variable is a continuous one, then the simplest measure of distance between two pieces of data is the Euclidean distance, $d(x,y)$, defined as

$$d(x,y) = \sqrt{(x_i - y_i)^2}$$

For categorical or discontinuous data, the simplest distance measure is the matching distance, defined as

$$d(x,y) = \text{Number of times } x_i \neq y_i$$

After we have developed a table of such distance measurements for each of the local variables, some weighting factor is assigned to each variable. A weighting factor seeks to give greater importance to those variables that are believed to have more relevance or predictive value. The weighted variables are then used to assign each piece of data to a group. The actual act of developing numerically based classifications and assigning data members to them is the realm of cluster analysis and will be discussed later in this chapter. Classification of biological data based on qualitative factors has been well discussed; Gordon [68] and Glass [69] do an excellent job of introducing the entire field and mathematical concepts.

Relevant examples of the use of classification techniques range from the simple to the complex. Schaper et al. [70] developed and used a very simple classification of response methodology to identify those airborne chemicals that alter the normal respiratory response induced by CO_2. At the other end of the spectrum, Kowalski and Bender [71] developed a more mathematically based system to classify chemical data (a methodology they termed *pattern recognition*).

Statistical Graphics

The use of graphics in one form or another in statistics is the single most effective and robust statistical tool and, at the same time, one of the most poorly understood and improperly used. Graphs are used in statistics (and in toxicology) for one of four major purposes. Each of the four is a variation on the central theme of making complex data easier to understand and use. These four major functions are exploration, analysis, communication and display of data, and graphical aids. Exploration (which may be simply summarizing data or trying to expose relationships between variables) is determining the characteristics of datasets and deciding on one or more appropriate forms of further analysis, such as the scatterplot. Analysis is the use of graphs to formally evaluate some aspects of the data, such as whether there are outliers present or if an underlying assumption of a population distribution is fulfilled. As long ago as 1960 [72], some 18 graphical methods for analyzing multivariate data relationships had been developed and proposed.

Communication and display of data are the most commonly used functions of statistical graphics in toxicology, whether for internal reports, presentations at meetings, or formal publications in the literature. When communicating data, graphs should not be used to duplicate data that are presented in tables but rather to show important trends or relationships in the data. Although such communication is most commonly of a quantitative compilation of actual data, it can also be used to summarize and present the results of statistical analysis. The fourth and final function of graphics is one that is largely becoming outdated as microcomputers become more widely available. Graphical aids to calculation include nomograms (the classic example in toxicology of a nomogram is that presented by Litchfield and Wilcoxon for determining median effective doses) and extrapolating and interpolating data graphically based on plotted data.

There are many forms of statistical graphics (a partial list, classified by function, is presented in Table 9.10), and a number of these (such as scatterplots and histograms) can be used for each of a number of possible functions. Most of these plots are based on a Cartesian system (i.e., they use a set of rectangular coordinates), and our review of construction and use will focus on these forms of graphs.

Construction of a rectangular graph of any form starts with the selection of the appropriate form of graph followed by the laying out of the coordinates (or axes). Even graphs that are going to encompass multivariate data (i.e., more than two variables) generally have two major coordinates as their starting point. The vertical axis or ordinate (also called the y-axis) is used to present an independent variable. Each of these axes is scaled in the units of measure that will most clearly present the trends of interest in the data. The range covered by the scale of each axis is selected to cover the entire region for which data are presented. The actual demarking of the measurement scale along an axis should allow for easy and accurate assessment of the coordinates of any data point, yet should not be cluttered.

Actual data points should be presented by symbols that present the appropriate indicators of location, and if they represent a summary of data from a normal data population, it would be appropriate to present a symbol for the mean and some indication of the variability (or error) associated with that population, commonly by using error bars, which present the SD (or standard error) from the mean. If, however, the data are not normal or continuous, it would be more appropriate to indicate location by the median and present the range or semiquartile distance for variability estimates. The symbols that are used to present data points can also be used to present a significant amount of additional information. At the simplest level, clearly distinct symbols (circles, triangles, squares, etc.) are very commonly used to provide a third dimension of data (most commonly the treatment group). But by clever use of symbols, all sorts of additional information can be presented. Using a method such as Chernoff's faces [73], in which faces are used as symbols of the data points (and various aspects of the faces present additional data, such as the presence or absence of eyes denoting the presence or absence

TABLE 9.10
Forms of Statistical Graphics (by Function)

Exploration		
Data Summary	**Two Variables**	**Three or More Variables**
Box and whisker plot	Autocorrelation plot	Biplot
Histogram	Cross-correlation plot	Cluster trees
Dot-array diagram	Scatterplot	Labeled scatterplot
Frequency polygon	Sequence plot	Glyphs and metroglyphs
Ogive		Face plots
Stem and leaf diagram		Fourier plots
		Similarity and preference maps
		Multidimensional scaling displays
		Weathervane plot

Analysis		
Distribution Assessment	**Model Evaluation and Assumption Verification**	**Decision Making**
Probability plot	Average vs. standard deviation	Control chart
Q–Q plot	Component-plus-residual plot	Cusum chart
P–P plot	Partial residual plot	Half-normal plot
Hanging histogram	Residual plots	Ridge trace
Rootagram		Youden plot
Poissonness plot		

Communication and Display of Data		
Quantitative Graphics	**Summary of Statistical Analyses**	**Graphical Aids**
Line chart	Means plot	Confidence limits
Pictogram	Sliding reference distribution	Graph paper
Pie chart	Notched box plot	Power curves
Contour plot	Factor space/response	Nomographs
Stereogram	Interaction plot	Sample–size curves
Color map	Contour plot	Trilinear coordinates
Histogram	Predicted response plot	
	Confidence region plot	

of a secondary pathological condition), it is possible to present a large number of different variables on a single graph.

The three other forms of graphs that are commonly used in toxicology are histograms, pie charts, and contour plots. Histograms are graphs of simple frequency distribution. Commonly, the abscissa is the variable of interest (such as lifespan or litter size) and is generally shown as classes or intervals or measurements (such as age ranges of 0–10 and 10–20 weeks). The ordinate, meanwhile, is the incidence or frequency of observations. The result is a set of vertical bars, each of which represents the incidence of a particular set of observations. Measures of error or variability about each incidence are reflected by some form of error bar on top of or in the frequency bars, as shown in Figure 9.8. The size of class intervals may be unequal (in effect, one can combine or pool several small class intervals), but it is proper in such cases to vary the width of the bars to indicate differences in interval size.

Pie charts are the only common form of quantitative graphic technique that is not rectangular; rather, the figure is presented as a circle out of which several slices are delimited. The only major use of the pie chart is in presenting a breakdown of the components of a group. Typically, the entire set of data under consideration (such as total body weight) constitutes the pie, and each slice represents a percentage of the whole (such as the percentages represented by each of several organs). The total number of slices in a pie should be small for the presentation to be effective. Variability or error can be readily presented by having a subslice of each sector shaded and labeled accordingly.

Finally, we have the contour plot, which is used to depict the relationships in a three-variable, continuous data system. That is, a contour plot visually portrays each contour as a locus of the values of two variables associated with a constant value of the third variable. An example would be a relief map that gives both latitude and longitude of constant altitude using contour lines.

The most common misuse of graphs is to either conceal or exaggerate the extent of the difference by using inappropriately scaled or ranged axis. Tufte [74] has identified a statistic for evaluating the appropriateness of scale size, the *lie factor*, defined as

$$\text{Lie factor} = \frac{\text{Size of effect shown in graph}}{\text{Size of effect in data}}$$

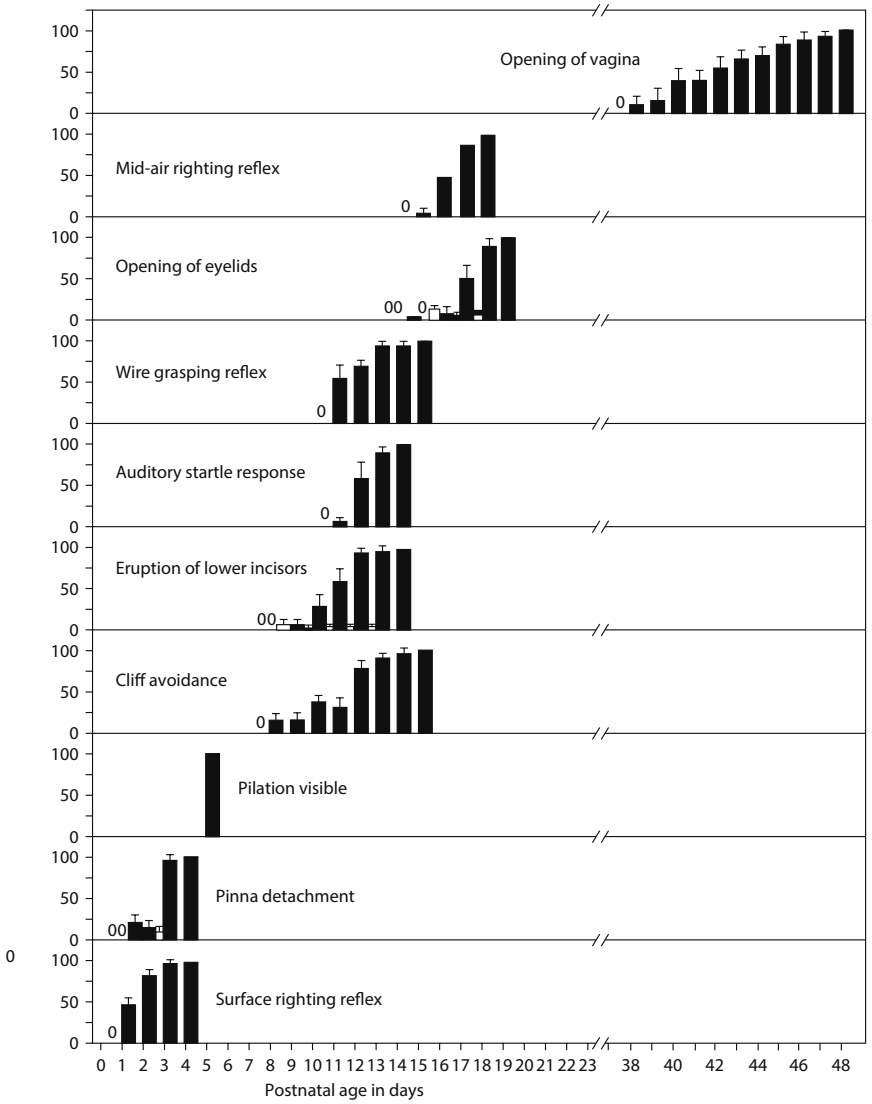


FIGURE 9.8 Acquisitions of postnatal development landmarks in rats.

An acceptable range for the lie factor is from 0.95 to 1.05. Less means the size of an effect is being understated; more means that the effect is being exaggerated. A number of excellent references are available for those who would like to pursue statistical graphics more. Anscombe [10] presents an excellent short overview, while other references [14,74–76] provide a wealth of information.

Multidimensional and Nonmetric Scaling

MDS is a collection of analysis methods for datasets that have three or more variables making up each data point. MDS displays the relationships of three or more dimensional extension of the methods of statistical graphics. MDS presents the structure of a set of objects from data that approximate the distances between pairs of the objects. The data, called *similarities*, *dissimilarities*, *distances*, or *proximities*, must be in such a form that the degree of similarities and differences between the pairs of the objects (each of which represents a real-life data point) can be measured and handled as a distance (remember the discussion of measures of distances under classifications). Similarity is a matter of degree; small differences between objects cause them to be similar (a high degree of similarity), while large differences cause them to be considered dissimilar (a small degree of similarity).

In addition to the traditional human conceptual or subjective judgments or similarity, data can be an objective similarity measure (the difference in weight between a pair of animals) or an index calculated from multivariate data (the proportion of agreement in the results of a number of carcinogenicity studies); however, the data must always represent the degree of similarity of pairs of objects. Each object or data point is represented by a point in a multidimensional space. These plots or projected points are arranged in this space so the distances between pairs of points have the strongest possible relation to the degree of similarity among the pairs of objects. That is, two similar objects are represented by two points that are close together, and two dissimilar objects are

represented by a pair of points that are far apart. The space is usually a two- or three-dimensional Euclidean space, but it may be non-Euclidean and may have more dimensions.

MDS is a general term that includes a number of different types of techniques; however, all seek to allow geometric analysis of multivariate data. The forms of MDS can be classified according to the nature of the similarities in the data [77]. It can be qualitative (nonmetric) or quantitative (metric MDS). The types can also be classified by the number of variables involved and by the nature of the model used; for example, classical MDS (only one data matrix and no weighting factors used on the data), replicated MDS (more than one matrix and no weighting), and weighted MDS (more than one matrix and at least some of the data being weighted). MDS can be used in toxicology to analyze the similarities and differences between effects produced by different agents in an attempt to gain an understanding of the mechanisms underlying the actions of one agent to determine the mechanisms of the other agents. Actual algorithms and a good intermediate-level presentation of MDS can be found in Davison [78].

Nonmetric scaling is a set of graphic techniques closely related to MDS and definitely useful for the reduction of dimensionality. Its major objective is to arrange a set of objects (each object, for our purposes, consisting of a number of related observations) graphically in a few dimensions while retaining the maximum possible fidelity to the original relationships between members (i.e., values that are most different are portrayed as most distant). It is not a linear technique; it does not preserve linear relationships (i.e., *A* is not shown as twice as far from *C* as *B*, even though its value difference may be twice as much). The spacings (interpoint distances) are kept such that, if the distance of the original scale between members *A* and *B* is greater than that between *C* and *D*, then the distances on the model scale will likewise be greater between *A* and *B* than between *C* and *D*. Figure 9.5, presented earlier, uses a form of this technique to add a third dimension by using letters to present degrees of effect on the skin. This technique functions by taking observed measures of similarity or dissimilarity between every pair of *M* objects, then finding a representation of the objects as points in Euclidean space such that the interpoint distances in some sense match the observed similarities or dissimilarities by means of weighting constants.

Cluster Analysis

Cluster analysis is a quantitative form of classification. It serves to help develop decision rules and then use these rules to assign a heterogeneous collection of objects to a series of sets. This is almost entirely an applied methodology (as opposed to theoretical). The final result of cluster analysis is one of several forms of graphic displays and a methodology (set of decision-classifying rules) for the assignment of new members into the classifications. The classification procedures used are based on either density of population or distance between members. These methods can serve to generate a basis for the classification of large numbers of dissimilar variables, such as behavioral observations and compounds with distinct but related structures and mechanisms [79,80], or to separate tumor patterns caused by treatment from those caused by old age [27].

The five types of clustering techniques are [81] as follows:

- Hierarchical techniques—Classes are subclassified into groups, with the process being repeated at several levels to produce a tree that gives sufficient definition to groups.
- Optimizing techniques—Clusters are formed by optimization of a clustering criterion. The resulting classes are mutually exclusive; the objects are clearly partitioned into sets.
- Density or mode-seeking techniques—Clusters are identified and formed by locating regions in a graphic representation that contains concentrations of data points.
- Clumping techniques—These are variations of density-seeking techniques in which assignment to a cluster is weighted on some variables so clusters may overlap in graphic projections.
- Others—These methods do not clearly fall into the other classes.

Romesburg [82] provides an excellent step-by-step guide to cluster analysis.

Fourier or Time Analysis

Fourier analysis [83] is most frequently a univariate method used for either simplifying data (which is the basis for its inclusion in this chapter) or for modeling. It can, however, also be a multivariate technique for data analysis. In a sense, it is like trend analysis; it looks at the relationship of sets of data from a different perspective. In the case of Fourier analysis, the approach is to resolve the time dimension variable in the dataset. At the simplest level, it assumes that many events are periodic in nature, and if we can remove the variation in other variables because of this periodicity (by using Fourier transforms), then we can better analyze the remaining variation from other variables. The complications to this are that (1) there may be several overlying cyclic time–based periodicities and (2) we may be interested in the time-cycle events for their own sake.

Fourier analysis allows one to identify, quantitate, and (if we wish) remove the time-based cycles in data (with their amplitudes, phases, and frequencies) by use of the Fourier transform:

$$nJ_i = x_i \exp\left(-iw_i t\right)$$

where

n is the length
J is the discrete Fourier transform for that case
x is the actual data
i is the increment in the series
w is the frequency
t is the time

A graphic example of the use of Fourier analysis in toxicology is provided in Figure 9.9.

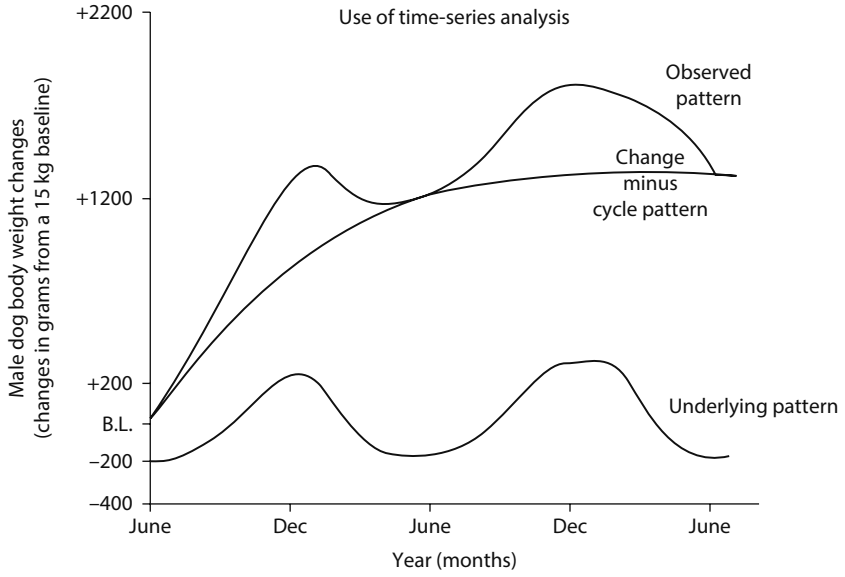


FIGURE 9.9 Use of time-series analysis.

Life Tables

Chronic in vivo toxicity studies are generally the most complex and expensive studies conducted by a toxicologist. Answers to a number of questions are sought in such a study—notably, if a material results in a significant increase in mortality or in the incidence of tumors in those animals exposed to it. But we are also interested in the time course of these adverse effects (or risks). The classic approach to assessing these age-specific hazard rates is the use of life tables (also called *survivorship tables*).

It may readily be seen that during any selected period of time (t_i), we have a number of risks competing to affect an animal. There are risks of natural death, death induced by a direct or indirect action of the test compound, and death due to such occurrences of interest of tumors [84]. Also, we are indeed interested in determining if (and when) the last two of these risks become significantly different than the natural risks (defined as what is seen to happen in the control group). Life-table methods enable us to make such determinations as the duration of survival (or time until tumors develop) and the probability of survival (or of developing a tumor) during any period of time.

We start by deciding the interval length (t_i) we wish to examine within the study. The information we gain becomes more exact as the interval is shortened, but as interval length is decreased, the number of intervals increases and calculations become more cumbersome and less indicative of time-related trends because random fluctuations become more apparent. For a 2-year or lifetime rodent study, an interval length of a month is commonly employed. Some life-table methods, such as the Kaplan–Meyer, have each new event (such as a death) define the start of a new interval.

Having established the interval length, we can tabulate our data [85]. We begin by establishing the following columns in each table, with a separate table being established for each group of animals (i.e., by sex and dose level):

- The interval of time selected (t_i)
- The number of animals in the group that entered that interval of the study alive (l_i)
- The number of animals withdrawn from the study during the interval (such as those taken for an interim sacrifice or that may have been killed by a technician error) (ω_i)
- The number of animals that died during the interval (d_i)
- The number of animals at risk during the interval $l_i = l_i - 1/2\omega_i$, or the number at the start of the interval minus one half the number withdrawn during the interval
- The proportion of animals that died ($D_i = d_i/l_i$)
- The cumulative probability of an animal surviving until the end of that interval of study: $P_i = 1 - D_i$, or 1 minus the number of animals that died during that interval divided by the number of animals at risk
- The number of animals dying until that interval (M_i)
- The number of animals found to have died during the interval (m_i)
- The probability of dying during the interval of the study: $c_i = 1 - (M_i + m_i/l_i)$, or the total number of animals dead until that interval plus the animals discovered to have died during that interval divided by the number of animals at risk through the end of that interval
- The cumulative proportion surviving (p_i) is equivalent to the cumulative product of the interval probabilities of survival (i.e., $p_i = p_1 \cdot p_2 \cdot p_3 \cdot \ldots p_x$)
- The cumulative probability of dying (C_i), equal to the cumulative product of the interval probabilities to that point (i.e., $C_i = c_1 \cdot c_2 \cdot c_3 \cdot \ldots c_x$)

With such tables established for each group in a study (as shown in Example 9.23), we may now proceed to test the hypothesis that each of the treated groups has a significantly

shorter duration of survival or that the treated groups died more quickly (note that plots of total animals dead and total animals surviving will give one an appreciation of the data but can lead to no statistical conclusions).

Now, for these two groups, we wish to determine effective sample size and to compare survival probabilities in the interval months 14–15. For the exposure group, we compute sample size as

$$l_{g14-15} = \frac{0.8400\left(1-0.8400\right)}{\left(0.0367\right)^2} = 99.7854$$

Likewise, we get a sample size of 98.1720 for the control group. The standard error of difference for the two groups here is

$$SD = \sqrt{0.0367^2 + 0.0173^2} = 0.040573$$

The probability of survival differences is $P_D = 0.9697 - 0.8400 = 0.1297$. Our test statistic is then 0.1297/0.040573 = 3.196. From our z value table, we see that the critical values are

$$p \le 0.05 = 1.960$$
$$p \le 0.01 = 2.575$$
$$p \le 0.001 = 3.270$$

As our calculated value is larger than all but the last of these, we find our groups to be significantly different at the 0.01 level ($0.01 > p > 0.001$). A multiplicity of methods is available for testing significance in life tables, with (as is often the case) the power of the tests increasing as does the difficulty of computation [58,86–88].

We begin our method of statistical comparison of survival at any point in the study by determining the standard error of the K interval survival rate as [89]

$$S_K = P_k\sqrt{\sum_1^k\left(\frac{D_i}{1'_x - d_x}\right)}$$

We can also determine the effective sample size (l_1) in accordance with

$$l_1 = \frac{P\left(1-P\right)}{S^2}$$

We can now compute the standard error of difference for any two groups (1 and 2) as

$$S_D = \sqrt{S_1^2 + S_2^2}$$

The difference in survival probabilities for the two groups is then calculated as

$$P_D = P_1 - P_2$$

We can then calculate a test statistic as

$$t' = \frac{P_D}{S_D}$$

This is then compared to the z distribution table. If $t' > z$ at the desired probability level, it is significant at that level.

Test Level 1								
Interval (Months) I_i	**Alive at Beginning of Interval I_i**	**Animals Withdrawn w_i**	**Died during Interval d_i**	**Animals at Risk I_i**	**Proportion of Animals Dead D_i**	**Probability of Survival P_i**	**Cumulative Proportion Surviving P_i**	**Standard Error of Survival S_i**
8–9	109	0	0	109	0	1.0000	1.0000	0.0000
9–10	109	0	2	109	0.0184	0.9816	0.9816	0.0129
10–11	107	0	0	107	0	1.0000	0.9816	0.0128
11–12	107	10	0	102	0	1.0000	0.9816	0.0128
12–13	97	0	1	97	0.0103	0.9897	0.9713	0.0162
13–14	96	0	1	96	0.0104	0.9896	0.9614	0.0190
14–15	95	0	12	95	0.1263	0.8737	0.8400	0.0367
15–16	83	0	2	83	0.0241	0.9759	0.8198	0.0385
16–17	81	0	3	81	0.0370	0.9630	0.7894	0.0409
17–18	78	20	1	68	0.0147	0.9853	0.7778	0.0419
18–19	57	0	2	57	0.0351	0.6949	0.7505	0.0446
Control Level								
11–12	99	0	1	99	0.0101	0.9899	0.9899	0.0100
12–13	98	0	0	98	0	1.0000	0.9899	0.0100
13–14	98	0	0	98	0	1.0000	0.9899	0.0100
14–15	98	0	2	98	0.0204	0.9796	0.9697	0.0172
15–16	96	0	1	96	0.0104	0.9896	0.9596	0.0198
16–17	95	0	0	95	0	1.0000	0.9596	0.0198
17–18	95	20	2	85	0.0235	0.8765	0.9370	0.0249
18–19	73	0	2	73	0.0274	0.9726	0.9113	0.0302

Example 9.23 illustrates the life-table technique for mortality data. With increasing recognition of the effects of time (both as age and as length of exposure to unmeasured background risks), life-table analysis has become a mainstay in chronic toxicology. An example is the reassessment of the ED_{01} study [90] that radically changed interpretation of the results and understanding of underlying methods when adjustment for time on study was made. The increased importance of, and interest in the, analysis of survival data have not been restricted to toxicology, but rather have encompassed all the life sciences. Those with further interest should consult Lee [91] or Elandt-Johnson and Johnson [92], both general in their approach to the subject.

MULTIVARIATE METHODS

In a chapter of this kind, an in-depth explanation of the available multivariate statistical techniques is an impossibility; however, as the complexity of problems in toxicology increases, we can expect to confront more frequently data that are not univariate but rather multivariate (or multidimensional). For example, a multidimensional study might be one in which the animals are being dosed with two materials that interact. Suppose we measure body weight, tumor incidence, and two clinical chemistry values for test material effects and interaction. Our dimensions—or variables—are now A = dose x; B = dose y; W = body weight; C = tumor incidence; D and E, which are levels of clinical chemistry parameters; and possibly also t (length of dosing).These situations are particularly common in chronic studies [93]. Although we can continue to use multiple sets of univariate techniques as we have in the past, we risk significant losses of power, efficiency, and information when doing so, as well as an increased possibility of error [94].

Here we will also look briefly at the workings and uses of each of the most commonly employed multivariate techniques, together with several examples from the literature of their employment in toxicology and the other biological sciences. We will group the methods according to their primary function: hypothesis testing (are these significant or not?), model fitting (what is the relationship between these variables, or what would happen if a population would be exposed to x?), and reduction of dimensionality (which variables are most meaningful?). It should be noted (and will soon be obvious), however, that most multivariate techniques actually combine several of these functions.

The most fundamental concept in multivariate analysis is that of a multivariate population distribution. At this point, it is assumed that the reader is familiar with the univariate random variable and with such standard distributions as the normal distribution. Here, we extend these to the multivariate normal distribution.

Multivariate data are virtually never processed and analyzed other than by computer. One must first set up an appropriate database file and then enter the data, coding some of them to meet the requirements of the software being utilized; for example, if only numerical data are analyzed, sex may have to be coded as 1 for male and 2 for females. Having recorded the data, it is then essential to review for suspect values and errors of various kinds. There are many different types of suspect values, and it is helpful to distinguish among them:

- *Outliers*—These are defined to be observations that appear to be inconsistent with the rest of the data. They may be caused by gross recording or entering errors, but it is important to realize that an apparent outlier may occasionally be genuine and indicate a nonnormal distribution or valuable data point.
- *Inversions*—A common type of error occurs when two consecutive digits are interchanged at the recording, coding, or entering stage. The error may be trivial if, for example, 56.74 appears as 56.47, but it may generate an outlier if 56.74 appears as 65.74.
- *Repetitions*—At the coding or entering stage, it is quite easy to repeat an entire number in two successive rows or columns of a table, thereby omitting one number completely.
- *Values in the wrong column*—It is also easy to get numbers into the wrong columns.
- *Other errors and suspect values*—Many other types of errors are possible, including misrecording of data of a minor nature.

The general term used to denote procedures for detecting and correcting errors is *data editing*. This includes checks for completeness, consistency, and credibility. Some editing can be done at the end of the data entry stage. In addition, many routine checks can be made by the computer itself, particularly those for gross outliers. An important class of such checks are range tests. For each variable, an allowable range of possible values is specified, and the computer checks that all observed values lie within the given range. Bivariate and multivariate checks are also possible; for example, one may specify an allowable range for some functions of two of more variables. Checks called *if–then* checks are also possible; for example, if both age and date of birth are recorded for each animal, then one can check that the answers are consistent. If the date of birth is given, then one can deduce the corresponding age. In fact, in this example, the age observation is redundant. It is sometimes a good idea to include one or two redundant variables as a check on accuracy. Various other general procedures for detecting outliers are described by Barnett and Lewis [95].

When a questionable value or error is detected, the toxicologist must decide what to do about it. One may be able to go back to the original data source and check the observation. Inversions, repetitions, and values in the wrong column can often be corrected in this way. Outliers are more difficult to handle, particularly when they are impossible to check or have been misrecorded in the first place. It may be sensible to treat them as missing values and try to insert a value guessed in an appropriate way (e.g., by interpolation or by prediction from other variables). Alternatively, the value may have to

be left as unrecorded, and then either all observations for the given individual will have to be discarded or one will have to accept unequal numbers of observations for the different variables. With a univariate set of observations, the analysis usually begins with the calculation of two summary statistics—namely, the mean and SD. In the multivariate case, the analysis usually begins with the calculation of the mean and SD for each variable, and, in addition, the correlation coefficient for each pair of variables is usually calculated. These summary statistics are vital in providing a preliminary look at the data.

The sample mean of the *j*th variable is given by

$$\bar{x}_j = \sum_{r=1}^{n} \frac{x_{rj}}{n}$$

and the sample mean vector, x, is given by $x^T = [x_1, x_2, \ldots, x_n]$. If the observations are a random sample from a population with mean x, then the sample mean vector x is usually the point estimate of x, and this estimate can easily be shown to be unbiased. The SD of the *j*th variable is given by

$$S_j = \sqrt{\left[\sum_{r=1}^{n} \frac{\left(x_{rj} - \bar{x}_j^2\right)}{\left(n-1\right)}\right]}$$

The correlation coefficient of variables i and j is given by

$$r_{ij} = \sum_{r=1}^{n} \frac{\left(x_{ri} - \bar{x}_j\right)\left(x_{rj} - \bar{x}_j\right)}{\left(n-1\right)s_i s_j}$$

These coefficients can be conveniently assembled in the sample correlation matrix (R), which is given by

$$R = \begin{bmatrix} 1 & r_{12} & \cdots & r_{1n} \\ r_{21} & 1 & \cdots & r_{2n} \\ \vdots & & & \\ r_n^1 & r_n^2 & \cdots & 1 \end{bmatrix}$$

Note that the diagonal terms are all unity.

The interpretation of means and SDs is straightforward. It is worth looking to determine if, for example, some variables have much higher scatter than others. It is also worth looking at the form of the distribution of each variable and considering whether any of the variables must be transformed; for example, the logarithmic transformation is often used to reduce positive skewness and produce a distribution that is closer to normal. One may also consider the removal of outliers at this stage.

Three significant multivariate techniques have hypothesis testing as their primary function: *MANOVA*, *MANCOVA*, and *factor analysis*. Multivariate analysis of variance (MANOVA) is the multidimensional extension of the ANOVA process we explored before. It can be shown to have grown out of Hotelling's T^2 [96], which provides a means of testing the overall null hypothesis that two groups do not differ in their means on any of p measures. MANOVA accomplishes its comparison of two (or more) groups by reducing the set of p measures on each group to a simple number applying the linear combining rule $W_i = w_j X_{ij}$ (where w_j is a weighting factor) and then computing a univariate F ratio on the combined variables. New sets of weights (w_j) are selected in turn until the set that maximizes the F ratio is found. The final resulting maximum F ratio (based on the multiple discriminant functions) is then the basis of the significance test. As with ANOVA, MANOVA can be one way or higher order, and MANOVA has as a basic assumption a multivariate normal distribution. When Gray and Laskey [97] used MANOVA to analyze the reproductive effects of manganese in the mouse, it allowed the identification of significant effects at multiple sites. Witten et al. [98] utilized MANOVA to determine the significance of the effects of dose, time, and cell division in the action of abrin on the lymphocytes.

Multivariate analysis of covariance (MANCOVA) is the multivariate analog of ANCOVA. As with MANOVA, it is based on the assumption that the data being analyzed are from a multivariate normal population. The MANCOVA test utilizes the two residual matrices using the statistic and is an extension of ANCOVA with two or more uncontrolled variables (or covariables). A detailed discussion can be found in Tatsuoka [99].

Factor analysis is not just a technique for hypothesis testing; it can also serve a reduction of dimensionality function. It seeks to separate the variance unique to particular sets of values from that common to all members in that variable system and is based on the assumption that the intercorrelations among the n original variables are the result of there being some smaller number of variables (factors) that explain the bulk of variation seen in the variables. All of the several approaches to achieving these end results seek a determination of what percentage of the variance of each variable is explained by each factor (a factor being one variable or a combination of variables). The model in factor analysis is $y = Af + xz$, where y is an n-dimensional vector of observable responses, A is a factor loading, an $n \times q$ matrix of unknown parameters, f is a q-dimensional vector of common factor, and z is an n-dimensional vector of unique factor.

Used for the reduction of dimensionality, factor analysis is said to be a linear technique because it does not change the linear relationships between the variables being examined. Joung et al. [100] used factor analysis to develop a generalized water-quality index that promises suitability across the United States and has appropriate weightings for 10 parameters. Factor analysis promises great utility as a tool for developing models in risk analysis where a number of parameters act and interact.

Now, we move on to two multivariate modeling techniques: *multiple regression* and *discriminant analysis*. Multiple regression and correlation seek to predict one (or a few) variable from several others. It assumes that the available variables can be logically divided into two (or more) sets

and serves to establish maximal linear (or some other scale) relationships among the sets. The linear model for the regression is simply

$$Y = b_0 + b_1X_1 + b_2X_2 + \cdots + b_pX_p$$

where

Y is the predicted value
b values are set to maximize correlations between X and Y and Y and Y (the actual observations)

The Xs are independent of predictor variables, and the Ys are dependent variables or outcome measures. One of the outputs from the process will be the coefficient of multiple correlation, which is simply the multivariate equivalent of the correlation coefficient (r).

Schaeffer et al. [101] have neatly demonstrated the utilization of multiple regression in studying the contribution of two components of a mixture to its toxicological action, using quantitative results from an Ames test as an endpoint. Paintz et al. [102] similarly used multiple regression to model the quantitative structure–activity relationships of a series of 14 1-benzoyl-3-methyl-pyrazole derivatives.

Discriminant analysis has for its main purpose to find linear combinations of variables that maximize the differences between the populations being studied, with the objective of establishing a model to sort objects into their appropriate populations with minimal error. At least four major questions are, in a sense, being asked of the data:

- Are there significant differences among the K groups?
- If the groups do exhibit statistical differences, how do the central masses (or centroids, the multivariate equivalent of means) of the populations differ?
- What are the relative distances among the K groups?
- How are new (or at this point unknown) members allocated to establish groups? How do you predict the set of responses of characteristics of an as yet untried exposure case?

The discriminant functions used to produce the linear combinations are of the form

$$D_I = d_{i1}X_i + d_{i2}Z_2 + \cdots + d_{ip}Z_p$$

where

D_I is the score on the discriminant function I
d values are weighting coefficients
Z values are standardized values of the discriminating variables used in the analysis

It should be noted that discriminant analysis can also be used for the hypothesis-testing function by the expedient of evaluating how well it correctly classifies members into proper groups (say, control, treatment 1, treatment 2, etc.). Taketomo et al. [103] used discriminant analysis in a retrospective study of gentamycin nephrotoxicity to identify patient risk factors (i.e., variables that contributed to a prediction of a patient being at risk).

Finally, we introduce four techniques whose primary function is the reduction of dimensionality: *canonical correlation analysis*, *principal components analysis*, *biplot analysis*, and *correspondence analysis*. Canonical correlation analysis provides the canonical R, an overall measure of the relationship between two sets of variables (one set consisting of several outcome measures, and the other of several predictor variables). The canonical R is calculated on two numbers for each subject:

$$W_i = \sum w_j X_{ij} \quad \text{and} \quad V_i = \sum v_j Y_{ij}$$

where

X values are predictor variables
Y values are outcome measures
W_j and V_j are canonical coefficients

MANOVA can be considered a special case of canonical correlation analysis. Canonical correlation can also be used in hypothesis testing for testing the association of pairs of sets of weights, each with a corresponding coefficient of canonical correlation, each uncorrelated with any of the preceding sets of weights, and each accounting for successively less of the variation shared by the two sets of variables. For example, Young and Matthews [104] used canonical correlation analysis to evaluate the relationship between plant growth and environmental factors at 12 different sites.

The main purpose of principal components analysis is to describe, as economically as possible, the total variance in a sample in a few dimensions; that is, one wishes to reduce the dimensionality of the original data while minimizing the loss of information. It seeks to resolve the total variation of a set of variables into linearly independent composite variables that successively account for the maximum possible variability in the data. The fundamental equation is $Y = AZ$, where A is a matrix of scaled eigenvectors, Z is the original data matrix, and Y represents the principal components. The concentration here, as in factor analysis, is on relationships within a single set of variables. Note that the results of principal components analysis are affected by linear transformations. Cremer and Seville [105] used principal components analysis to compare the difference in blood parameters resulting from each of two separate pyrethroids. Henry and Hidy [106], meanwhile, used principal components analysis to identify the most significant contributors to air quality problems.

The biplot display [107] of multivariate data is a relatively new technique that promises wide applicability to problems in toxicology. It is, in a sense, a form of EDA, used for data summarization and description. The biplot is a graphical display of a matrix Y_{nmx} of N rows and M columns by means of row and column markers. The display carries one marker for each row and each column. The *bi* in biplot refers to the joint display of rows and columns. Such plots are used primarily

for inspection of data and for data diagnostics when such data are in the form of matrices. Shy-Modjeska et al. [108] illustrated this usage in the analysis of aminoglycoside renal data from beagle dogs, allowing the simultaneous display of relationships among different observed variables and presentation of the relationship of both individuals and treatment groups to these variables.

Correspondence analysis is a technique for displaying the rows and columns of a two-way contingency table as points in a corresponding low-dimensional vector space. As such, it is equivalent to simultaneous linear regression (for contingency table data, such as tumor incidences, which is a very common data form in toxicology), and it can be considered a special case of canonical correlation analysis. The data are defined, described, and analyzed in a geometric framework. This is particularly attractive to such sets of observations in toxicology as multiple endpoint behavioral scores and scored multiple tissue lesions.

A number of good surveys of multivariate techniques are available that are not excessively mathematical [109–111]. More rigorous mathematical treatments on an introductory level are also available [112]. Most of the techniques we have described are available in the better computer statistical packages.

META-ANALYSIS

Meta-analysis, meaning *analysis among* (and actually entailing analysis of multiple existing analyses), is being used increasingly in biomedical research to try to obtain a qualitative or quantitative synthesis of the research literature on a particular issue. The technique is usually applied to the synthesis of several separate but comparable studies to yield a single answer. Though dating back to the 1930s [113], only recently has it become popular. The process of systematic reviews and meta-analysis has three main components: (1) systematic review and selection of studies, plus (2) quantitative and (3) qualitative analyses [114–116].

Selection of Studies for Analysis: Systematic Reviews

The issue of study selection is perhaps the most problematic for those investigators doing meta-analysis. The criteria for selection may vary from project to project; however, several factors concerning selection must be addressed before analyses commence. Each choice made by the investigator must be weighed carefully as to the likely effect of selection bias vs. the perceived bias that the selection was designed to remove:

- *Use of gray literature.* The current dogma among many scientists is that only peer-reviewed literature is valuable for inclusion in reviews and, therefore, by inference systematic reviews. Should studies be limited to those that are peer reviewed or published? It is well known that negative studies, or those that report little or no benefit from following a particular course of action, are less likely to be published than positive studies; therefore, the published literature may be biased toward studies with positive results, and a synthesis of these studies would give a biased estimate of the impact of pursuing some courses of action. When a systematic review is planned, a plethora of industrial, academic, and government research papers have often been prepared that deal with the issue under consideration. Unfortunately, access to this gray literature is limited, although search engines are now available that can be used to attempt to discover this unpublished information. These studies may give a less biased report on the topic in question; however, some of these unpublished studies may be of lower quality than peer-reviewed materials. Sometimes poor research methods can produce reported results that underestimate the impact, hence providing an opposite bias to that described earlier.
- *Peer review.* As mentioned earlier, peer review is considered the primary method for quality control in scientific publishing. Should publications in a systematic review and meta-analysis be limited to peer-reviewed articles and, if so, what journals should be included or excluded? The choice of journal may be used as another filter based on the rigor of review and editor latitude given to fill the journal. Some investigators recommend that only those studies that are published in peer-reviewed publications be considered in meta-analysis. Although this may seem an attractive option, it might produce an even more highly biased selection of studies for systematic review.
- *Quality control.* Peer review is not the only method of providing quality control and quality-assured data for meta-analysis. Additional quality control and assurance criteria may be used to select the best and most reliable data during systematic review. A rhetorical question we could ask is "Should studies be limited to those that meet additional quality control criteria?" If investigators, undertaking a systematic review, impose an additional set of criteria before including a study in the meta-analysis, the average quality of the studies used should be improved. Contrary to the quality issue is the concern about selection bias. In fact, by placing specific quality filters on data, the investigators may introduce more bias than created by the poor quality data. Moreover, different investigators may use different criteria for a valid study and therefore select a different group of studies for meta-analysis. The result is a possible conflicting output of the meta-analysis.
- *Study design.* Some investigators insist that systematic reviews be limited to randomized controlled studies. Such a limitation produces a variant of the potential bias described earlier. At one time,

rigid quality standards were more likely to be met by randomized controlled studies than by observational studies, but this is no longer necessarily the case. Observational methods are currently used to evaluate naturally occurring effects, particularly those that are uncommon. It is quite possible that more important issues, such as combining data from studies performed in different laboratories and using different strains of a single animal species, may result in more systematic error than the study design.

- *Methodology.* Different methodologies can cause differing degrees of systematic bias on output data. This begs the question "Should studies selected for use in meta-analysis be limited to those using identical methods?" This limitation would mean using only separately published studies from the same laboratory in a limited time frame for which the methods were comprehensively monitored and determined to be identical. In practice, application of this filter would massively reduce the number of studies that could be used in the meta-analysis whose power would therefore be decreased greatly. Accordingly, the user must understand the inherent differences between studies and exercise caution and judgment in selecting and rejecting them for use.

Pooled (Quantitative) Analysis

The main purpose of meta-analysis is to provide a quantitative assessment of the similarity of responses in a number of studies. The goal is to develop better overall estimates of the degree of benefit achieved by specific exposure and dosing techniques based on the combining, or pooling, of estimates found in the existing studies of the interventions. This type of meta-analysis is sometimes called a *pooled analysis* because the analyst pools the observations of many studies and then calculates parameters such as risk or odds ratios from the pooled data. Because of the many decisions regarding inclusion or exclusion of studies, different meta-analyses might reach very different conclusions on the same topic. Even after the studies are chosen, many other methodological issues are involved in choosing how to combine means and variances (e.g., what weighting methods should be used). Pooled analysis should report relative risks and risk reductions as well as absolute risks and risk reductions.

Methodological (Qualitative) Analysis

Sometimes the question to be answered is not how much toxicity is induced by the use of a particular exposure but whether there is any biologically significant toxicity. In this case, a qualitative meta-analysis may be done, in which the quality of the research is scored according to a list of objective criteria. The analyst then examines the methodologically superior studies to determine whether the question of toxicity is answered consistently by them. This qualitative approach has been referred to as *methodological analysis* or *quality scores analysis*. In some cases, the methodologically strongest studies agree with one another and disagree with the weaker studies. These weaker studies may be consistent with one another.

Bayesian Inference

Sensitivity and specificity of a test are important to characterize and to understand the accuracy and precision of the data generated. Once a researcher decides to use a certain test to diagnose an illness, two important questions require answers. First, if the test results are positive, what is the probability that the researcher has uncovered the condition of interest? Second, if the test results are negative, what is the probability that the patient does not have the disease? Bayes's theorem provides a method to answer these two questions. The English clergyman after whom it is named first described the theorem centuries ago [117]. It is one of the most imposing statistical formulas in the biomedical sciences. Put in symbols more meaningful for researchers such as toxicologists and pathologists [118], the formula is as follows:

$$P(D|T+) = \frac{p(T+|D+)\,p(D+)}{\left[p(T+|D+)\,p(D+)\right] + \left[p(T+|D-)\,p(D-)\right]}$$

where

p denotes probability
$D+$ means that the animal has the effect in question
$D-$ means that the animal does not have the effect
$T+$ means that a certain diagnostic test for the effect is positive
$T-$ means that the test is negative

Vertical line (|) means *conditional upon* what immediately follows

Most researchers, who have to address sensitivity, specificity, and predictive values, often do not wish to use Bayes's theorem; however, this is a useful formula. Closer examination of the equation reveals that Bayes's theorem is merely the formula for the positive predictive value.

The numerator of Bayes's theorem describes cell a, the true-positive results, in a 2 × 2 table. The probability of being in cell a is equal to the prevalence multiplied by the sensitivity, where $p(D+)$ is the prevalence (i.e., the probability of being in the affected column) and $p(T+|D+)$ is the sensitivity (i.e., the probability of being in the top row, given the fact of being in the affected column). The denominator of Bayes's theorem consists of two terms, the first of which again describes cell a, the true-positive results, and the second of which describes cell b, the false-positive error rate. This rate can be represented by $p(T+|D-)$, which is multiplied by the prevalence of unaffected animals or

$p(D-)$. True-positive results (a) divided by true-positive plus false-positive results ($a + b$) gives $a/(a + b)$, the positive predictive value.

In genetics, a simpler formula for Bayes's theorem is sometimes used. The numerator is the same, but the denominator is $p(T+)$. This makes sense because the denominator in $a/(a + b)$ is equal to all of those who have positive test results, whether they are true-positive or false-positive results.

Bayes's Theorem in the Evaluation of Safety Assessment Studies

In a population with a low prevalence of a particular toxicity, most of the positive results in a screening program for that lesion or effect would be falsely positive. Although this does not automatically invalidate a study or assessment program, it does raise some concerns about cost effectiveness, which can be explored using Bayes's theorem.

An example to illustrate Bayes's theorem is a study employing an immunochemical stain–based test to screen tissues for a specific effect. This test uses small amounts of antibody, and the presence of an immunologically bound stain is considered a positive result. If the sensitivity and specificity of the test and the prevalence of biochemical effect are known, Bayes's theorem can be used to predict what proportion of the tissues with positive test results will have true-positive results (i.e., truly showing the effect).

Figure 9.10 shows how the calculations are made. If the test has a sensitivity of 96% and if the true prevalence is 1%, then only 13.9% of tissues predicted showing a positive test result actually will be true positives. Pathologists and toxicologists can quickly develop a table that lists different levels of test sensitivity, test specificity, and effect prevalence and shows how these levels affect the proportion of positive results that are likely to be true-positive results. Although this calculation is fairly straightforward and is extremely useful, it seldom has been used in the early stages of planning for large studies or safety assessment programs.

Bayes's Theorem and Individual Animal Evaluation

Uncertainty concerning the exact cause of death of an animal is a problem that faces most toxicological pathologists. Suppose a toxicological pathologist is uncertain about an animal's cause of death and has a positive test result, such as in the example given earlier.

Even if the toxicological pathologist knows the sensitivity and specificity of the test in question, interpretation is still problematic. In order to calculate the positive predictive value, it is necessary to know the prevalence of the particular true tissue/effect that the test is designed to detect. The prevalence is thought of as the expected prevalence in the population from which the animal comes. The actual prevalence is usually not known, but usually an estimate is attempted.

PART 1 Initial data

Sensitivity of immunological stain = 96% = 0.96
False-negative error rate of the test = 04% = 0.04
Specificity of the test = 94% = 0.94
False-positive error rate of the test = 06% = 0.06
Prevalence of effect in the tissues = 01% = 0.01

PART 2 Use Bayes's theorem

$$P(D+|T+) = \frac{p(T+|D+)p(D+)}{\left[p(T+|D+)p(D+)\right] + \left[p(T+|D-)p(D-)\right]}$$

$$= \frac{(\text{Sensitivity})(\text{Prevalence})}{\left[(\text{Sensitivity})(\text{Prevalence})\right] + (\text{False}-\text{Positive error rate})(1-\text{Prevalence})}$$

$$= \frac{(0.096)(0.01)}{\left[(0.096)(0.01)\right] + \left[(0.06)(0.99)\right]}$$

$$= \frac{0.0096}{0.0096 + 0.0594} = \frac{0.0096}{0.0690} = 0.139 = 13.9\%$$

PART 3 Use of a 2 × 2 table, with numbers based on the assumption that 10,000 tissues are in the study:

True Disease Status

Test Result	Number Affected	Not Affected	Total
Positive	96 (96%)	594 (6%)	690 (7%)
Negative	4 (4%)	9306 (94%)	9,310 (93%)
Total	100 (100%)	9900 (100%)	10,000 (100%)

Positive predictive value = 96/690 = 0.139 = 13.9%.

FIGURE 9.10 Use of Bayes's theorem or a 2 × 2 table to determine the positive predictive value of a hypothetical tuberculin-screening program.

An example of such a situation is when a pathologist evaluates a male primate observed to have fatigue and signs of kidney stones. No other clinical signs of parathyroid disease are detected on physical examination. The toxicological pathologist considers the possibility of hyperparathyroidism and arbitrarily decides that its prevalence is perhaps 2%, reflecting that in 100 such primates, probably only 2 of them would have the disease. This probable disease prevalence is referred to as the *prior probability*, reflecting the fact that it is estimated prior to the performance of laboratory tests.

This probability is based on the estimated prevalence of a particular pathology among primates with similar signs and symptoms. Although the toxicological pathologist believes that the probability of hyperparathyroidism is low, he considers the serum calcium concentrations to rule out the diagnosis. Somewhat to his surprise, the results of the test are positive, with an elevated level of calcium of 12.2 mg/dL. The pathologist could order other tests for parathyroid disease, but some test results may be positive and some negative for a number of reasons.

PART 1 Initial data

Sensitivity of the first test = 90% = 0.90
Specificity of the first test = 95% = 0.95

PART 2 Use Bayes's theorem

$$P(D+|T+) = \frac{p(T+|D+)p(D+)}{\left[p(T+|D+)p(D+)\right] + \left[p(T+|D-)p(D-)\right]}$$

$$= \frac{(0.90)(0.02)}{\left[(0.90)(0.02)\right] + (0.05)(0.98)}$$

$$= \frac{0.018}{0.018 + 0.049} = \frac{0.018}{0.067} = 0.269 = 27\%$$

PART 3 Use of a 2 × 2 table

True Disease Status

Test Result	Number Affected	Not Affected	Total
Positive	18 (90%)	49 (5%)	67 (6.7%)
Negative	2 (10%)	931 (95%)	933 (93.3%)
Total	20 (100%)	980 (100%)	1000 (100.0%)

Positive predictive value = 18/67 = 0.269 = 27%.

FIGURE 9.11 Use of Bayes's theorem or a 2 × 2 table to determine posterior probability and positive predictive values.

Under these circumstances, Bayes's theorem could be used to make a second estimate of probability, referred to as the *posterior probability*, reflecting the fact that this determination is made after the test results are known. Calculation of the posterior probability is based on the sensitivity and specificity of the test that was performed, which in this case was elevated serum calcium, and on the prior probability, which in this case was set at 2%. If the serum calcium test had a 90% sensitivity and a 95% specificity, a false-positive error rate of 5% would be expected. Note that specificity plus the false-positive error rate always equals 100%.

When this information is used in Bayes's equation, as shown in Figure 9.11, the result is a posterior probability of 27%. This means that the animal in question is now within a group of primates with a significant possibility of parathyroid disease. In Figure 9.11, note that the result is the same when a 2 × 2 table is used (i.e., 27%).

This is true because the probability based on Bayes's theorem is identical to the positive predictive value.

In light of the 27% posterior probability, the pathologist decides to order a parathyroid hormone radioimmunoassay, even though this test is expensive. If the radioimmunoassay had a sensitivity of 95% and a specificity of 98% and the results turned out to be positive, Bayes's theorem could again be used to calculate the probability of parathyroid disease. This time, however, the posterior probability for the first test (27%) would be used as the prior probability for the second test. The result of the calculation, as shown in Figure 9.12, gives a new probability of 94%. Thus, the primate in all probability did have hyperparathyroidism.

PART 1 Initial data

Sensitivity of the first test = 95% = 0.95
Specificity of the first test = 98% = 0.94
Prior probability of disease = 27% = 0.27

PART 2 Use Bayes's theorem*

$$P(D+|T+) = \frac{p(T+|D+)p(D+)}{\left[p(T+|D+)p(D+)\right] + \left[p(T+|D-)p(D-)\right]}$$

$$= \frac{(0.95)(0.27)}{\left[(0.95)(0.27)\right] + (0.02)(0.73)}$$

$$= \frac{0.257}{0.257 + 0.049} = \frac{0.257}{0.272} = 0.9449^{a} = 94\%$$

PART 3 Use of a 2 × 2 table

True Disease Status

Test Result	Number Affected	Not Affected	Total
Positive	256 (95%)	15 (2%)	271 (27.1%)
Negative	13 (5%)	716 (98%)	729 (72.9%)
Total	269 (100%)	731 (100%)	1000 (100.0%)

Positive predictive value = 256/271 = 0.9446[a] = 94%.

* The slight difference in the results for the two approaches is due to rounding errors. It is not important biologically.

FIGURE 9.12 Use of Bayes's theorem or a 2 × 2 table to determine second posterior probability and second positive predictive values.

The reader may be wondering why the posterior probability increased so much the second time. One reason was that the prior probability was considerably higher in the second calculation compared to the first (27% vs. 2%) based on the fact that the first test yielded positive results. Another reason was that the specificity of the second test was high (98%), which markedly reduced the false-positive error rate and therefore increased the positive predictive value.

Assumptions and Limitations

1. Test results must be independent of each other. This also means that the population remaining after one test must have the same proportional response to the following tests as the original population did.
2. If the calculations are done on an iterative basis, care must be taken to correct for cumulative round-off errors.

DATA ANALYSIS APPLICATIONS IN TOXICOLOGY

Having reviewed basic principles and provided a set of methods for statistical handling of data, the remainder of this chapter addresses the practical aspects and difficulties encountered in day-to-day toxicological work. As a starting point, we present in Table 9.11 an overview of data types

TABLE 9.11
Classification of Data Commonly Encountered in Toxicology

Type of Data	Examples
Continuous normal	Body weights
	Food consumption
	Organ weights: absolute and relative
	Mouse ear swelling test (MEST) measurements
	Pregnancy rates
	Survival rates
	Crown–rump lengths
	Hematology (some)
	Clinical chemistry (some)
Continuous but not normal	Hematology (some; WBC)
	Clinical chemistry (some)
	Urinalysis
Scalar data	Neurobehavioral signs (some)
	PDI scores
	Histopathology (some)
Count data	Resorption sites
	Implantation sites
	Stillborns
	Hematology (some; reticulocyte counts, Howell-Jolly, WBC differentials)
Categorical data	Clinical signs
	Neurobehavioral signs (some)
	Ocular scores
	GP sensitization scores
	Mouse ear swelling test (MEST) sensitization
	Counts
	Fetal abnormalities
	Dose/mortality data
	Sex ratios
	Histopathology data (most)

actually encountered in toxicology, classified by type (as presented earlier). It should be stressed, however, that this classification is of the most frequent measure of each sort of observation (such as body weight) and will not always be an accurate classification. There are now common practices in the analysis of toxicology data, although they are not necessarily the best. They are discussed in the remainder of this chapter, which seeks to review statistical methods on a use-by-use basis and to provide a foundation for the selection of alternatives in specific situations.

Median Lethal and Effective Doses

For many years, the starting point for evaluating the toxicity of an agent was to determine its LD_{50} or LC_{50}, which are the dose or concentration, respectively, of a material at which half of a population of animals would be expected to die. These figures are analogous to the ED_{50} (effective dose for half a population) used in pharmacologic activities and are derived by the same means. To calculate either of these figures we need, at each of several dosage (or exposure) levels, the number of animals dosed and the number that died. If we seek to establish only the median effective dose in a range-finding test, then four or five animals per dose level, using Thompson's method of moving averages, is the most efficient methodology and will give a sufficiently accurate solution. With two dose levels, if the ratio between the high and low doses is 2 or less, even total or no mortality at these two dose levels will yield an acceptably accurate medial lethal dose, although a partial mortality is desirable. If, however, we wish to estimate a number of toxicity levels (LD_{10}, LD_{90}) and are interested in more precisely establishing the slope of the dose/lethality curve, the use of at least 10 animals per dosage level with the probit/log regression technique is the most common approach. Note that in the equation $Y_i = a + bx_1$, b is the slope of the regression line and that our method already allows us to calculate 95% CIs about any point on this line. The CI at any one point will be different from the interval at other points and must be calculated separately. Additionally, the nature of the probit transform is such that toward the extremes—LD_{10} and LD_{90}, for example—the CIs will balloon. That is, they become very wide. Because the slope of the fitted line in these assays has a very large uncertainty, in relation to the uncertainty of the LD_{50} itself (the midpoint of the distribution), much caution must be used with calculated LD_x values other than LD_{50}. The imprecision of the LD_{35}, a value close to the LD_{50}, is discussed by Weil [119], as is that of the slope of the log dose–probit line [120]. Debanne and Haller [121] recently reviewed the statistical aspects of different methodologies for estimating a median effective dose.

There have been questions for years as to the value of LD_{50} and the efficiency of the current study design (which uses large numbers of animals) in determining it. As long ago as 1953, Weil et al. [122] presented forceful arguments that an estimate having only minimally reduced precision could be made using significantly fewer animals. More recently, the last few years have seen an increased level of concern over the numbers and uses of animals in research and testing and have produced additional arguments against existing methodologies for determining the LD_{50} or even the need to make the determination at all [123]. In response, several suggestions for alternative methodologies have been advanced [124–126].

Body and Organ Weights

Among the sets of data commonly collected in studies where animals are dosed with (or exposed to) a chemical are body weight and the weights of selected organs; in fact, body weight is frequently the most sensitive indication of an adverse effect. How to best analyze this and in what form to analyze the organ weight data (as absolute weights, weight changes, or percentages of body weight) have been the subject of a number of articles [127–130].

Both absolute body weights and rates of body weight change (calculated as changes from a baseline measurement

value that is traditionally the animal's weight immediately prior to the first dosing with or exposure to the test material) are almost universally best analyzed by ANOVA followed, if called for, by a post hoc test. Even if the groups were randomized properly at the beginning of a study (no group being significantly different in mean body weight from any other group and all animals in all groups within two SDs of the overall mean body weight), there is an advantage to performing the computationally slightly more cumbersome (compared to absolute body weights) changes in body weight analysis. The advantage is an increase in sensitivity, because the adjustment of starting points (the setting of initial weights as a zero value) acts to reduce the amount of initial variability. In this case, Bartlett's test is performed first to ensure homogeneity of variance, and the appropriate sequence of analysis follows. With smaller sample sizes, the normality of the data becomes increasingly uncertain, and nonparametric methods such as Kruskal–Wallis may be more appropriate [25].

The analysis of relative (to body weight) organ weights is a valuable tool for identifying possible target organs [125]. How to perform this analysis is still a matter of some disagreement, however. Weil [128] presented evidence that organ weight data expressed as percentages of body weight should be analyzed separately for each sex. Furthermore, because the conclusions from organ weight data of males differed so often from those of females, data from animals of each sex should be used in this measurement. Others [129,131–133] have discussed in detail other factors that influence organ weights and must be taken into account.

The two competing approaches to analyzing relative organ weights call for either of the following [130]:

- Calculate organ weights as a percentage of total body weight (at the time of necropsy) and analyze the results by ANOVA.
- Analyze the results by ANCOVA with body weights as the covariates, as discussed previously.

A number of considerations should be kept in mind when these questions are addressed. First, one must keep a firm grasp on the difference between biological significance and statistical significance. In this particular case, we are especially interested in examining organ weights when an organ weight change is not proportional to changes in whole body weights. Second, we are now required to detect smaller and smaller changes while still retaining a similar sensitivity (i.e., the $p < 0.05$ level). Several devices are available to attain the desired increase in power. One is to use larger and larger sample sizes (number of animals), and the other is to utilize the most powerful test we can; however, the use of even currently employed numbers of animals is being vigorously questioned, and the power of statistical tests must, therefore, now assume an increased importance in our considerations.

The biological rationale behind analyzing both absolute body weight and the ratio of organ weight to body weight (this latter as opposed to a covariance analysis of organ weights) is that, in the majority of cases, except for the brain, the organs of interest in the body change weight (except in extreme cases of obesity or starvation) in proportion to total body weight. We are particularly interested in detecting cases where this is not so. Analysis of actual data from several hundred studies (unpublished data) has shown no significant difference in rates of weight change of target organs (other than the brain) compared to total body weight for healthy animals in those species commonly used for repeated dose studies (rats, mice, rabbits, and dogs). Furthermore, it should be noted that ANCOVA is of questionable validity in analyzing body weight and related organ weight changes, because a primary assumption is the independence of treatment—that the relationship of the two variables is the same for all treatments [134]. Plainly, in toxicology, this is not true.

In cases where the differences between the error MSs are much greater, the ratio of F ratios will diverge in precision from the result of the efficiency of covariance adjustment. These cases are where either sample sizes are much larger or where the differences between means themselves are much larger. This latter case is one that does not occur in the designs under discussion in any manner that would leave ANCOVA as a valid approach, because group means start out being very similar and cannot diverge markedly unless there is a treatment effect. As we have discussed earlier, a treatment effect invalidates a prime underpinning assumption of ANCOVA. Shirley and Newman [135] have argued the case for ANCOVA, but without providing answers to arguments presented earlier.

Clinical Chemistry

Several clinical chemistry parameters are commonly determined from the blood and urine collected from animals in chronic, subchronic, and occasionally acute toxicity studies. In the past (and still, in some places), the accepted practice has been to evaluate these data using univariate–parametric methods (primarily t-tests and/or ANOVA); however, this can be shown not to be the best approach on a number of grounds.

First, such biochemical parameters are rarely independent of each other, and our interest often is not focused on just one of the parameters; rather, there are batteries of the parameters associated with toxic actions at particular target organs. For example, increases in creatinine phosphokinase (CPK), γ-hydroxybutyrate dehydrogenase (γ-HBDH), and lactate dehydrogenase (LDH), occurring together, are strongly indicative of myocardial damage. In such cases, we are not just interested in a significant increase in one of these, but in all three. Table 9.12 gives a brief overview of the association of various parameters with actions at particular target organs. A more detailed coverage of the interpretation of such clinical laboratory tests can be found in other references [136–139].

Similarly, the serum electrolytes (sodium, potassium, and calcium) interact with each other; a decrease in one is frequently tied, for instance, to an increase in one of the others. Furthermore, the nature of the data (in the case of some parameters), either

TABLE 9.12
Association of Changes in Biochemical Parameters with Actions at Particular Target Organs

Parameter	Blood	Heart	Lung	Kidney	Liver	Bone	Intestine	Pancreas	Notes
Albumin				↓	↓				Produced by the liver; very significant reductions indicate extensive liver damage
ALP					↑	↑	↑		Elevations usually associated with cholestasis; bone alkaline phosphatase tends to be higher in young animals
ALT (formerly SGPT)					↑				Elevations usually associated with hepatic damage or disease
AST (formerly SGOT)		↑		↑	↑			↑	Present in skeletal muscle and heart and most commonly associated with damage to these
Beta-2-Microglobulin				↑					
Bilirubin (total)	↑				↑				Usually elevated due to cholestasis, due to either obstruction or hepatopathy
BUN				↑	↓				Estimates blood-filtering capacity of the kidneys; does not become significantly elevated until the kidney function is reduced 60%–75%
Calcium				↑					Can be life threatening and result in acute death
Cholinesterase				↑	↓				Found in plasma, brain, and RBC
CPK		↑							Most often elevated due to skeletal muscle damage but can also be produced by cardiac muscle damage; can be more sensitive than histopathology
Creatinine				↑					Also estimates blood-filtering capacity of kidney as BUN does
Glucose								↑	Alterations other than those associated with stress uncommon and reflect an effect on the pancreatic islets or anorexia
GGT					↑				Elevated in cholestasis; this is a microsomal enzyme, and levels often increase in response to microsomal enzyme induction
HBDH		↑			↑				—
KIM-1				↑					
LDH		↑	↑	↑	↑				Increase usually due to skeletal muscle, cardiac muscle, or liver damage; not very specific
Protein (total)				↓	↓				Absolute alterations usually associated with decreased production (liver) or increased loss (kidney); can see increase in case of muscle wasting (catabolism)
SDH					↑↓				Liver enzyme that can be quite sensitive but is fairly unstable; samples should be processed as soon as possible
Trophonin		↑							

Note: ↑, increase in chemistry values; ↓, decrease in chemistry values; ALP, alkaline phosphatase; BUN, blood urea nitrogen; CPK, creatinine phosphokinase; GGT, gamma glutamyl transferase; HBDH, hydroxybutyric dehydrogenase; LDH, lactic dehydrogenase; RBC, red blood cells; SDH, sorbitol dehydrogenase; SGOT, serum glutamic oxaloacetic transaminase (also called AST [aspartate amino transferase]); SGPT, serum glutamic-pyruvic transaminase (also called ALT [alanine amino transferase]).

because of the biological nature of the parameter or the way in which it is measured, is frequently either not normally distributed (particularly because of being markedly skewed) or not continuous in nature. This can be seen in some of the reference data for experimental animals in Mitruka and Rawnsley [140] or Weil [141] in, for example, creatinine, sodium, potassium, chloride, calcium, and blood.

Hematology

Much of what we said about clinical chemistry parameters is also true for the hematologic measurements made in toxicology studies. Which test to perform should be evaluated by use of a decision tree until one becomes confident as to the most appropriate methods. Keep in mind that sets of values and (in some cases) population distribution vary not only between species but also between the commonly used strains of species, and that control or standard values will drift over the course of only a few years.

Again, the majority of these parameters are interrelated and highly dependent on the method used to determine them. RBC count, platelet counts, and mean corpuscular volume (MCV) may be determined using a device such as a Coulter counter to take direct measurements, and the resulting data are usually stable for parametric methods. The hematocrit, however, may actually be a value calculated from the RBC and MCV values and, if so, is dependent on them. If the hematocrit is measured directly, instead of being calculated

TABLE 9.13
Some Probable Conditions behind Hematological Changes

Parameter	Elevation	Depression	Parameter	Elevation	Depression
Red blood cells	Vascular shock Excessive diuresis Chronic hypoxia Hyperadrenocorticism	Anemias: blood loss, hemolysis, low RBC production	Platelets	—	Bone marrow depression Immune disorder
Hematocrit	Increased RBC Stress Shock: trauma, surgery Polycythemia	Anemias Pregnancy Excessive hydration	Neutrophils	Acute bacterial infections Tissue necrosis Strenuous exercise Convulsions Tachycardia Acute hemorrhage	—
Hemoglobin	Polycythemia (increase in the production of RBC)	Anemias Lead poisonings	Lymphocytes	Leukemia Malnutrition Viral infections	—
Mean cell volume	Anemias B_{12} deficiency	Iron deficiency	Monocytes	Protozoal infections	—
Mean corpuscular hemoglobin	Reticulocytosis	Iron deficiency	Eosinophils	Allergy Irradiation Pernicious anemia Parasitism	—
White blood cells	Bacterial infections Bone marrow stimulation	Bone marrow depression Cancer chemotherapy Chemical intoxication Splenic disorders	Basophils	Lead poisoning	—

Sources: Hayes, A.W., ed., *Principles and Method of Toxicology*, 5th edn., Taylor & Francis Group, Philadelphia, PA, 2008; Minckler, J. et al., *Pathology: An Introduction*, Mosby, St. Louis, MO, 1971; Thomas, H.C., *Handbook of Automated Electronic Clinical Analysis*, Reston Publishing, Reston, VA, 1979; Gad, S.C., *Animal Models in Toxicology*, 2nd edn., Marcel Dekker, New York, 2006.

from the RBC and MCV, it may be compared by parametric methods (see Table 9.13).

Hemoglobin is directly measured and is an independent and continuous variable. However, and probably because at any one time a number of forms and conformations (oxyhemoglobin, deoxyhemoglobin, methemoglobin, etc.) of hemoglobin are actually present, the distribution seen is not typically a normal one but rather may be a multimodal one. Here, a nonparametric technique such as the Wilcoxon or multiple rank-sum analysis is called for.

Consideration of the WBC and differential counts leads to another problem. The total WBC is, typically, a normal population amenable to parametric analysis, but differential counts are normally determined by counting, manually, one or more sets of 100 cells each.

The resulting relative percentages of neutrophils are then reported as either percentages or are multiplied by the total WBC count with the resulting count being reported as the absolute differential WBC. Such data, particularly in the case of eosinophils (where the distribution does not approach normality), should usually be analyzed by nonparametric methods. It is widely believed that relative (%) differential data should not be reported because they are likely to be misleading.

Finally, it should always be kept in mind that it is rare for a change in any single hematologic parameter to be meaningful. Rather, because these parameters are so interrelated, patterns of changes in parameters should be expected if a real effect is present, and analysis and interpretation of results should focus on such patterns of changes. Classification analysis techniques often provide the basis for a useful approach to such problems.

Histopathological Lesion Incidence

The last 20 years have seen increasing emphasis placed on histopathological examination of tissues collected from animals in subchronic and chronic toxicity studies. It is not true that only those lesions that occur at a statistically significantly increased rate in treated or exposed animals are of concern, for in some cases, a lesion may be of such a rare type that the occurrence of only one or a few such in treated animals raises a red flag. It is true, however, that in most cases, a statistical evaluation is the only way to determine if what we see in treated animals is significantly worse than what has been seen

in control animals [171]. And although cancer is not our only concern, this category of lesions is that of greatest interest.

Typically, comparison of incidences of any one type of lesion between controls and treated animals are made using the multiple 2 × 2 chi-square test or Fisher's exact test with a modification of the numbers of animals as the denominators. Too often, experimenters exclude from consideration all those animals (in both groups) that died prior to the first animals being found with a lesion at that site. The special case of carcinogenicity bioassays will be discussed in detail in the next chapter.

An option that should be kept in mind is that, frequently, a pathologist can not only identify a lesion as present but also grade those present as to severity. This represents a significant increase in the information content of the data that should not be given up by performing an analysis based only on the perceived quantal nature (present/absent) of the data. Quantal data, analyzed by chi-square or Fisher's exact tests, are a subset (the 2 × 2 case) of categorical or contingency table data. In this case, it also becomes ranked (or ordinal) data; the categories are naturally ordered (e.g., no effect < mild lesion < moderate lesion < severe lesion). This gives a $2 \times R$ table if there are only one treatment and one control group or an $N \times R$ (multiway) table if there are three or more groups of animals.

The traditional method of analyzing multiple, cross-classified data has been to collapse the $N \times R$ contingency table over all but two of the variables, following this with the computation of some measure of association between these variables. For an N-dimensional table, this results in $N(N-1)/2$ separate analyses. The result is crude, giving away information and even (by inappropriate pooling of data) yielding a faulty understanding of the meaning of data. Though computationally more laborious, a multiway ($N \times R$ table) analysis should be utilized.

Reproduction

The reproductive implications of the toxic effects of chemicals are being increasingly important. Because of this, reproduction studies, together with other closely related types of studies (such as teratogenesis, dominant lethal, and mutagenesis studies), are now commonly companion to chronic toxicity studies. One point that must be kept in mind with all reproduction-related studies is the nature of the appropriate sampling unit. What is the appropriate N in such a study: the number of individual pups, the number of litters, the number of pregnant females? Fortunately, it is now fairly well accepted that the first case (using the number of offspring as the N) is inappropriate [129]. The real effects in such studies actually occur in the female that was exposed to the chemical or is mated to a male that was exposed. What happens to her and to the development of the litter she is carrying is biologically independent of what happens to every other female or litter in the stud. This cannot be said for each offspring in each litter; for example, the death of one member of a litter can and will be related to what happens to every other member. Also, the effect on all of the offspring might be similar for all of those from one female and different or lacking for those from another.

As defined by Oser and Oser [142], four primary variables are of interest in a reproduction study. First is the fertility index, which may be defined as the percentage of attempted matings (i.e., each female housed with a male) that resulted in pregnancy, pregnancy being determined by a method such as the presence of implantation sites in the female. Second is the gestation index, which is defined as the percentage of mated females, as evidenced by a vaginal plug being dropped or a positive vaginal smear, that deliver viable litters (i.e., litters with at least one live pup). Two related variables that may also be studied are the mean number of pups born per litter and the percentage of total pups per litter that are stillborn. Third is the viability index, which is defined as the percentage of offspring born that survive at least 4 days after birth. The last in this four-variable system is the lactation index, which is the percentage of animals per litter that survive 4 days and also survive to weaning. In rats and mice, this is classically taken to be 21 days after birth. An additional variable that may reasonably be included in such a study is the mean weight gain per pup per litter.

Given that our N is at least 10, we may test each of these variables for significance using a method such as the Wilcoxon–Mann–Whitney U test or the Kruskal–Wallis nonparametric ANOVA. If N is less than 10, we cannot expect the central limit theorem to be operative and should use the Wilcoxon sum of ranks (for two groups) or the Kruskal–Wallis nonparametric ANOVA (for three or more groups) to compare groups.

Developmental Toxicology

When the primary concern of a reproductive/developmental study is the occurrence of birth defects or deformations (terata, either structural or functional) in the offspring of exposed animals, the study is one of developmental toxicology (teratology). In the analysis of the data from such a study, we must consider several points. First is sample size. Earlier, a method to estimate sufficient sample size was presented. The difficulties with applying these methods here revolve around two points: (1) selecting a sufficient level of sensitivity for detecting an effect and (2) factoring in how many animals will be removed from study (without contributing a datum) by either not becoming pregnant or not surviving to a sufficiently late stage of pregnancy. Experience generally dictates that one should attempt to have 20 pregnant animals per study group if a pilot study has provided some confidence that the pregnant test animals will survive the dose levels selected. Again, it is essential to recognize that the litter, not the fetus, is the basic independent unit for each variable.

A more fundamental consideration, alluded to in the section on "Reproduction," is that as we use more animals, the mean of means (each variable will be such in a mathematical sense)

will approach normality in its distribution. This is one of the implications of the central limit theorem; even when the individual data are not normally distributed, their means will approach normality in their distribution. At a sample size of 10 or greater, the approximation of normality is such that we may use a parametric test (such as a *t*-test or ANOVA) to evaluate results. At sample sizes less than 10, a nonparametric test (Wilcoxon rank-sum or Kruskal–Wallis nonparametric ANOVA) is more appropriate. Other methodologies have been suggested [143,144] but do not offer any prospect of widespread usage. One nonparametric method that is widely used is the Mann–Whitney U test, which was described earlier. Williams and Buschbom [145] further discuss some of the available statistical options and their consequences, and Rai and Ryzin [146] have recommended a dose-responsive model.

Dominant Lethal Assay

The dominant lethal study is essentially a reproduction study that seeks to study the endpoint of lethality to the fetuses after implantation and before delivery. The proper identification of the sampling unit (the pregnant female) and the design of an experiment so that a sufficiently large sample is available for analysis are the primary statistical considerations. The question of sampling unit has been adequately addressed in earlier sections. Sample size is of concern here because the hypothesis-testing techniques that are appropriate with small samples are of relatively low power, as the variability about the mean in such cases is relatively large. With sufficient sample size (e.g., from 30 to 50 pregnant females per dose level per week [147]), variability about the mean and the nature of the distribution allow sensitive statistical techniques to be employed.

The variables that are typically recorded and included in analysis are (for each level/week) (1) the number of pregnant females, (2) live fetuses per pregnancy, (3) total implants per pregnancy, (4) early fetal deaths (early resorptions) per pregnancy, and (5) late fetal deaths per pregnancy.

A wide variety of techniques for the analysis of these data have been used. Most common is the use of ANOVA after the data have been transformed by the arc sine transform [148]. Beta binomial [149,150] and Poisson distributions [151] have also been attributed to these data, and transforms and appropriate tests have been proposed for use in each of these cases (in each case with the note that the transforms serve to stabilize the variance of the data). With sufficient sample size, as defined earlier in this section, the Mann–Whitney U test is recommended for use here. Smaller sample sizes necessitate the use of the Wilcoxon rank-sum test.

Diet and Chamber Analysis

Earlier we presented the basic principles and methods for sampling. Sampling is important in many aspects of toxicology, and here we address its application to diet preparation and the analysis of atmospheres from inhalation chambers. In feeding studies, we seek to deliver doses of a material to animals by mixing the material with their diet. Similarly, in an inhalation study, we mix a material with the air the test animals breathe. In both cases, we must then sample the medium (food or atmosphere) and analyze these samples to determine what levels or concentrations of material were actually present and to assure ourselves that the test material is homogeneously distributed. Having an accurate picture of these delivered concentrations, and how they varied over the course of time, is essential on a number of grounds:

1. The regulatory agencies and sound scientific practice require that analyzed diet and mean daily inhalation atmosphere levels be ±10% of the target level.
2. Excessive peak concentrations, because of the overloading of metabolic repair systems, could result in extreme acute effects that would lead to results in a chronic study that are not truly indicative of the chronic low-level effects of the compound but rather of periods of metabolic and physiologic overload. Such results could be misinterpreted if true exposure or diet levels were not maintained at a relatively constant level.

Sampling strategies are a matter not just of numbers (for statistical aspects) but also of geometry, so the contents of a container or the entire atmosphere in a chamber can be truly sampled; it is also a matter of time, with regard to the stability of the test compound. The samples must be both randomly collected and representative of the entire mass of what one is trying to characterize. In the special case of sampling and characterizing the physical properties of aerosols in an inhalation study, some special considerations and terminology apply. Because of the physiologic characteristics of the respiration of humans and of test animals, our concern is very largely limited to those particles or droplets that are of a respirable size. Unfortunately, respirable size is a complex characteristic based on aerodynamic diameter, density, and physiological characteristics. Although particles with an aerodynamic diameter of less than 10 μm are generally agreed to be respirable in humans (i.e., they can be drawn down to the deep portions of the lungs), 3 μm in aerodynamic diameter is a more realistically value. Typically, it then becomes a matter of calculating measures of central tendency and dispersion statistics, with the identification of those values that are beyond acceptable limits [49].

Genotoxicity

In the last 25 years, a wide variety of tests [152] for genotoxicity have been developed and brought into use. These tests give us a quicker and less expensive (although not as conclusive) way of predicting whether a material of interest is a mutagen, and possibly a carcinogen, than do longer-term whole-animal studies. How to analyze the results of the multitude of tests (Ames, DNA repair, micronucleus, chromosome aberration, cell transformation, and sister chromatid exchange, to name

a few) is an extremely important question. Some workers in the field hold that it is not possible (or necessary) to perform statistical analysis and that the tests can simply be judged to be positive or not positive on the basis of whether or not they achieve a particular increase in the incidence of mutations in the test organism. Quantitations of potency are complicated by the fact that we are dealing with a nonlinear phenomenon; although low doses of most genotoxicants produce a linear response curve with increasing dose, the curve will flatten out (and even turn into a declining curve) as the higher doses provoke an acute response.

Several concepts different from those we have previously discussed need to be examined, for our concern has now shifted from how a multicellular organism acts in response to one of a number of complex actions to how a mutational event is expressed, most frequently by a single cell. Given that we can handle much larger numbers of experimental units in systems that use smaller test organisms, we can seek to detect both weak and strong mutagens.

Conducting the appropriate statistical analysis and utilizing the results of such an analysis properly must begin with an understanding of the biological system involved, and from this understanding, the correct model and hypothesis must be developed. We begin such a process by considering each of five interacting factors [153,154]:

1. α, which is the probability of our committing a type I error (saying an agent is mutagenic when it is not, equivalent to our *p* in such earlier considered designs as Fisher's exact test)—false positive
2. β, which is the probability of our committing a type II error (saying an agent is not mutagenic when it is)—false negative
3. Δ, our desired sensitivity in an assay system (such as being able to detect an increase of 10% in mutations in a population)
4. σ, the variability of the biological system and the effects of chance errors
5. *n*, the single necessary sample size to achieve each of these (we can, by our actions, change only this portion of the equation) as *n* is proportional to σ/α, β, and Δ

The implications of this are, therefore, that (1) the greater σ is, the larger *n* must be to achieve the desired levels of α, β, and Δ; (2) the smaller the desired levels of α, β, and/or Δ (if *n* is constant), the larger our σ is.

What are the background mutation level and the variability in our technique? As any good genetic or general toxicologist will acknowledge, matched concurrent control groups are essential. Fortunately, with these test systems, large *n* values are readily attainable, although there are other complications to this problem, which we will consider later. An example of the confusion that would otherwise result is illustrated in the intralaboratory comparisons on some of these methods done to date, such as that reviewed by Weil [155].

New statistical tests based on these assumptions and upon the underlying population distributions have been proposed, along with the necessary computational background to allow one to alter one of the input variables [α, β, or Δ]. A set that shows particular promise is that proposed by Katz [156,157] in his two articles. He described two separate test statistics: Φ, for when we can accurately estimate the number of individuals in both the experimental and control groups, and θ, for when we do not actually estimate the number of surviving individuals in each group and we can assume that the test material is only mildly toxic in terms of killing the test organisms. Each of these two test statistics is also formulated on the basis of only a single exposure of the organisms to the test chemicals. Given this, then we may compute

$$\Phi = \frac{a\left(M_E - 0.5\right) - Kb\left(M_C + 0.5\right)}{\sqrt{Kab\left(M_E + M_C\right)}}$$

where

- *a* and *b* are the number of groups of control (*C*) and experimental (*E*) organisms, respectively
- $K = N_E/N_C$, where N_C and N_E are the numbers of surviving microorganisms
- M_E and M_C are the numbers of mutations in the experimental and control groups
- μ_e and μ_c are the true (but unknown) mutation rates (as μ_c gets smaller, *N* must increase)

We may compute the second case as

$$\theta = \frac{a\left(M_E - 0.5\right) + \left(M_C + 0.5\right)}{ab\left(M_E - M_C\right)}$$

with the same constituents.

In both cases, at a confidence level for *I* of 0.05, we accept that $\mu_c = \mu_e$ if the test statistic (either Φ or θ) is less than 1.64. If it is equal to or greater than 1.64, we may conclude that we have a mutagenic effect (at $\alpha = 0.05$).

In the second case (θ, where we do not have separate estimates of population sizes for the control and experimental groups), if *K* deviates widely from 1.0 (if the material is markedly toxic), we should use more containers of control organisms (tables for the proportions of each to use given different survival frequencies may be found in Katz [157]). If different levels are desired, tables for θ and Φ may be found in Kastenbaum and Bowman [158].

An outgrowth of this is that the mutation rate per surviving cells (μ_c and μ_e) can be determined. It must be remembered that, if the control mutation rate is so high that a reduction in mutation rates can be achieved by the test compound, these test statistics must be adjusted to allow for a two-sided hypothesis [159]. The α levels may likewise be adjusted in each case or tested for, if we want to assure ourselves that a mutagenic effect exists at a certain level of confidence (note that this is different from disproving the null hypothesis).

It should be noted that numerous specific recommendations have been made for statistical methods designed for individual mutagenicity techniques, such as that of Bernstein et al. [160] for the Ames test.

Behavioral Toxicity

A brief review of the types of studies or experiments conducted in the area of behavioral toxicology and the classification of these into groups is in order. Although a small number of studies do not fit into the following classification, the great majority may be fitted into one of the following four groups. Many of these points have been covered in earlier articles [79,161].

Observational score-type studies are based on observing and grading the response of an animal to its normal environment or to a stimulus that is imprecisely controlled. This type of result is generated by one of two major sorts of studies. Open-field studies involve placing an animal in the center of a flat, open area and counting each occurrence of several types of activities (grooming, moving outside a designated central area, rearing, etc.) or timing until the first occurrence of each type of activity. The data generated are scalar of either a continuous or discontinuous nature but frequently are not of a normal distribution. Tilson et al. [162] presented some examples of this sort.

Observational screen studies involve a combination of observing behavior and evoking a response to a simple stimulus, the resulting observation being graded as normal or as deviating from normal on a graded scale. Most of the data so generated are rank in nature, with some portions being quantal or interval. Irwin [163] and Gad [161] have presented schemes for the conduct of such studies that became the basis of the commonly used functional observational battery. Table 9.14 gives an example of the nature (and of one form of statistical analysis) of such data generated after exposure to one material.

The second type of study is one that generates rates of response as data. The studies are based on the number of responses to a discrete controlled stimulus or are free of direct connection to a stimulus. The three most frequently measured parameters are licking of a liquid (milk, sugar water, ethanol, or a psychoactive agent in water), gross locomotor activity (measured by a photocell or electromagnetic device), or level pulling. Examples of such studies have been published by Annau [164] and Norton [165]. The data generated are most often of a discontinuous or continuous scalar nature and are often complicated by underlying patterns of biological rhythm.

The third type of study generates a variety of data classified as error rate. These are studies based on animals learning a response to a stimulus or memorizing a simple task (such as running a maze or a Skinner-box type of shock-avoidance system). These tests or trials are structured so animals can pass or fail on each of a number of successive trials. The resulting data are quantal, although frequently expressed as a percentage.

The final major type of study is one that results in data that are measures of the time to an endpoint. They are based on animals being exposed to or dosed with a toxicant, and the time taken for an effect to be observed is measured. The endpoint is usually failure to continue to be able to perform a task and can, therefore, be death, incapacitation, or the learning of a response to a discrete stimulus. Burt [166] and Johnson et al. [167] present data of this form. The data are always of a censored nature—that is, the period of observation is always artificially limited as in measuring time to incapacitation in combustion toxicology data, where animals are exposed to the thermal decomposition gases to test materials for a period of 30 min. If incapacitation is not observed during these 30 min, it is judged not to occur. The data generated by these studies are continuous, discontinuous, or rank in nature. They are discontinuous because the researcher may check or may be restricted to checking for the occurrence of the endpoint only at certain discrete points in time. On the other hand, they are rank if the periods to check for occurrence of the endpoint are far enough apart, in which

TABLE 9.14
Functional Observational Battery Parameters Showing Significant Differences between Treated and Control Groups

	Rats (18-Crown-6 Animals Given 40 mg/kg i.p.)				
Parameter	Control Sum of Ranks	N_C	18-Crown-6 Treated Sum of Ranks	N_T	Observed Difference in Treated Animals (Compared to Controls)
Twitches	55.0	10	270.0	15	Involuntary muscle twitches
Visual placing	55.0	10	270.0	15	Less aware of visual stimuli
Grip strength	120.0	10	205.0	15	Considerable loss of strength, especially in hind limbs
Respiration	55.0	10	270.0	15	Increased rate of respiration
Tremors	55.0	10	270.0	15	Marked tremors

Note: All parameters provided earlier are significant at $p < 0.05$.

TABLE 9.15
Overview of Statistical Testing in Behavioral Toxicology

Type of Observation	Most Commonly Used Procedures	Suggested Procedures
Observational scores	Student's *t*-test or one-way ANOVA	Kruskal–Wallis nonparametric ANOVA or Wilcoxon rank-sum
Response rates	Student's *t*-test or one-way ANOVA	Kruskal–Wallis ANOVA or one-way ANOVA
Error rates	ANOVA followed by a post hoc test	Fisher's exact, R × C chi-square, or Mann–Whitney U test
Times to endpoint	Student's *t*-test or one-way ANOVA	ANOVA then a post hoc test or Kruskal–Wallis ANOVA
Teratology and reproduction	ANOVA followed by a post hoc test	Fisher's exact test, Kruskal–Wallis ANOVA, or Mann–Whitney U test

Tests commonly used vs. tests most frequently appropriate.

Note: That these are the most commonly used procedures was established by an extensive literature review that is beyond the scope of this chapter. The reader, however, need only look at the example articles cited in the text of this chapter to verify this fact.

case one may actually only know that the endpoint occurred during a broad period of time—but not where in that period.

There is a special class of test that should also be considered at this point—the behavioral teratology or reproduction study. These studies are based on dosing or exposing either parental animals during selected periods in the mating and gestation process or pregnant females at selected periods during gestation. The resulting offspring are then tested for developmental defects of a neurological and behavioral nature. Analysis is complicated by a number of facts:

1. The parental animals are the actual targets for toxic effects, but observations are made on offspring.
2. The toxic effects in the parental generation may alter the performance of the mother in rearing its offspring, which in turn can lead to a confusion of prenatal and postnatal effects.
3. Different capabilities and behaviors develop at different times.

A researcher can, by varying the selection of the animal model (species, strain, sex), modify the nature of the data generated and the degree of dispersion of these data. In behavioral studies particularly, limiting the within-group variability of data is a significant problem and generally should be a highly desirable goal.

Most, if not all, behavioral toxicology studies depend on at least some instrumentation. Very frequently overlooked here (and, indeed, in most research) is that instrumentation, by its operating characteristics and limitations, goes a long way toward determining the nature of the data generated by it. An activity monitor measures motor activity in discrete segments. If it is a *jiggle cage* type of monitor, these segments are restricted so only a distinctly limited number of counts can be achieved in a given period of time and then only if they are of the appropriate magnitude. Likewise, the technique can also readily determine the nature of the data. In measuring response to pain, for example, one could record it as a quantal measure (present or absent), as a rank score (on a scale of 1–5 for decreased to increased responsiveness, with 3 being normal), or as scalar data (by using an analgesia meter that determines either how much pressure or heat is required to evoke a response).

Study design factors are probably the most widely recognized of the factors that influence the type of data resulting from a study. Number of animals used, frequency of measures, and length of period of observation are three obvious design factors that are readily under the control of the researcher and directly help to determine the nature of the data.

Finally, it is appropriate to review each of the types of studies currently utilized in behavioral toxicology according to the classification presented at the beginning of this section, in terms of which statistical methods are used now and what procedures should be recommended for use. The recommendations, of course, should be viewed with a critical eye. They are intended with current experimental design and technique in mind and can claim to be the best only when one is limited to addressing the most common problems from a library of readily and commonly available and understood tests. Table 9.15 summarizes this review and recommendation process.

Carcinogenesis

In the experimental evaluation of substances for carcinogenesis based on experimental results in a nonhuman species at some relatively high dose or exposure level, an attempt is made to predict the occurrence and level of tumorigenesis in humans at much lower levels. An entire chapter could be devoted to examining the assumptions involved in this undertaking and review of the aspects of design and interpretation of animal carcinogenicity studies. Such is beyond the scope of this effort. The reader is referred to Gad [23] for such an examination. The single most important statistical consideration in the design of carcinogenicity bioassays in the past was based on the point of view that what was being observed and evaluated was a simple quantal response (cancer occurred or it did not) and that a sufficient number of animals needed to be used to have reasonable expectations of detecting such an

effect. Though the single fact of whether or not the simple incidence of neoplastic tumors is increased due to an agent of concern is of interest, a much more complex model must now be considered. The time-to-tumor, patterns of tumor incidence, effects on survival rate, and age at first tumor all must now be included in a model.

Bioassay Design

As presented earlier in the section on "Experimental Design," the first step that must be taken is to clearly state the objective of the study to be undertaken. Carcinogenicity bioassays have two possible objectives. The first objective is to detect possible carcinogens. Compounds are evaluated to determine if they can or cannot induce a statistically detectable increase in tumor rates over background levels, and only by happenstance is information generated that is useful in risk assessment. Most older studies have such detection as their objective. Current thought is that at least two species must be used for detection, although the necessity of a second species (the mouse) is increasingly questioned. The second objective for a bioassay is to provide a range of dose–response information (with tumor incidence being the response) so a risk assessment may be performed. Unlike detection, which requires only one treatment group with adequate survival times (to allow the expression of tumors), dose–response requires at least three treatment groups with adequate survival. We will shortly look at the selection of dose levels for this case; however, given that the species is known to be responsive, only one species of animal needs to be used for this objective.

To address either or both of these objectives, three major types of study designs have evolved. First is the classical skin-painting study, usually performed in mice. A single easily detected endpoint (the formation of skin tumors) is evaluated during the course of the study. Although dose–response can be evaluated in such a study (dose usually being varied by using different concentrations of test material in volatile solvent), most often detection is the objective of such a study. Although others have used different frequencies of application of test material to vary dose, there are data to suggest that this serves to introduce only an additional variable [168]. Traditionally, both test and control groups in such a test consist of 50–100 mice of one sex (males being preferred because of their very low spontaneous tumor rate). This design is also used in tumor initiation/promotion studies.

The second common type of design is the original NCI bioassay. The announced objective of these studies was the detection of moderate to strong carcinogens, although the results have also been used in attempts at risk assessment. Both mice and rats were used in parallel studies. Each study used 50 males and 50 females at each of two dose levels (high and low) plus an equal-sized control group. The National Toxicology Program (NTP) has subsequently moved away from this design because of a recognition of its inherent limitations. More animals per group and more dose groups are now used.

Finally, the standard industrial toxicology design uses at least two species (usually rats and mice) in groups of no fewer that 100 males and females each. Each study has three dose groups and at least one control. Frequently, additional numbers of animals are included to allow for interim terminations and histopathological evaluations. In both this and the NCI design, a long list of organs and tissues are collected, processed, and examined microscopically. This design seeks to address both the detection and dose–response objectives with a moderate degree of success.

Selecting the number of animals to use for dose groups in a study requires consideration of both biological (expected survival rates, background tumor rates, etc.) and statistical factors. The prime statistical consideration is reflected in Table 9.16. It can be seen in this table that, if, for example, we were studying a compound that caused liver tumors and were using mice (with a background or control incidence of

TABLE 9.16
Sample Size Required to Obtain a Specified Sensitivity at $p < 0.05$ Treatment Group Incidence

Background Tumor Incidence	P[a]	0.95	0.90	0.80	0.70	0.60	0.50	0.40	0.30	0.20	0.10
0.30	0.90	10	12	18	31	46	102	389	—	—	—
	0.50	6	6	9	12	22	32	123	—	—	—
0.20	0.90	8	10	12	18	30	42	88	320	—	—
	0.50	5	5	6	9	12	19	28	101	—	—
0.10	0.90	6	8	10	12	17	25	33	65	214	—
	0.50	3	3	5	6	9	11	17	31	68	—
0.05	0.90	5	6	8	10	13	18	25	35	76	464
	0.50	3	3	5	6	7	9	12	19	24	147
0.01	0.90	5	5	7	8	10	13	19	27	46	114
	0.50	3	3	5	5	6	8	10	13	25	56

[a] P is the power for each comparison of treatment group with background tumor incidence.

30%), we would have to use 389 animals per sex per group to be able to demonstrate that an incidence rate of 40% in treatment animals was significant compared to the controls at the $p = 0.05$ level.

Perhaps the most difficult aspect of designing a good carcinogenicity study is the selection of the dose levels to be used. At the start, it is necessary to consider the first underlying assumption in the design and use of animal cancer bioassays—the need to test at the highest possible dose for the longest practical period. The rationale behind this assumption is that, although humans may be exposed at very low levels, detecting the resulting small increase (over background) in the incidence of tumors would require the use of an impractically large number of test animals per group.

This point is illustrated by Table 9.16, where, for example, only 46 animals (per group) are needed to show a 10% increase over a zero background (i.e., a rarely occurring tumor type), but 770,000 animals (per group) would be needed to detect a 0.1% increase above a 5% background. As we increase dose, however, the incidence of tumors (the response) will also increase until it reaches the point where a modest increase (say, 10%) over a reasonably small background level (say 1%) could be detected using an acceptably small-sized group of test animals (in Table 9.17, we see that 51 animals would be needed for this example case). There are, however, at least two real limitations to the highest dose level. First, the test rodent population must have a sufficient survival rate after receiving a lifetime (or 2 years) of regular doses to allow for meaningful statistical analysis. Second, we really want the metabolism and mechanism of action of the chemical at the highest level tested to be the same as at the low levels where human exposure would occur. Unfortunately, toxicologists usually must select the high dose level based only on the information provided by a subchronic or range-finding study (usually 90 days in length), but selection of either too low or too high a dose will make the study invalid for the detection of carcinogenicity and may seriously impair the use of the results for risk assessment.

There are several solutions to this problem. One of these has been the rather simplistic approach of the NTP Bioassay Program, which is to conduct a 3-month range-finding study with sufficient dose levels to establish a level that significantly (10%) decreases the rate of body weight gain. This dose is defined as the maximum tolerated dose (MTD) and is selected as the highest dose. Two other levels, generally one half MTD and one quarter MTD, are selected for testing as the intermediate- and low-dose levels. In many earlier NCI studies, only one other level was used.

The dose range-finding study is necessary in most cases, but the suppression of body weight gain is a scientifically questionable benchmark when dealing with the establishment of safety factors. Physiologic, pharmacologic, or metabolic markers generally serve as better indicators of systemic response than body weight. A series of well-defined acute and subchronic studies designed to determine the chronicity factor and to study the onset of pathology can be more predictive for dose setting than body weight suppression. Also, the NTP's MTD may well be at a level where the metabolic mechanisms for handling a compound at real-life exposure levels have been saturated or overwhelmed, bringing into play entirely artifactual metabolic and physiologic mechanisms [169]. The regulatory response to questioning the appropriateness of the MTD as a high dose level [170] has been to acknowledge that occasionally an excessively high dose is selected but to counter by saying that using lower doses would seriously decrease the sensitivity of detection.

TABLE 9.17
Average Number of Animals Needed to Detect a Significant Increase in the Incidence of an Event (e.g., Tumors, Anomalies) over Background Incidence (Control) at Expected Incidence Levels Using Fisher's Exact Probability Test ($p = 0.05$)

Background Incidence (%)	Expected Increase in Incidence (%)					
	0.01	0.1	1	3	5	10
0	46,000,000[a]	460,000	4,600	511	164	46
0.01	46,000,000	460,000	4,600	511	164	46
0.1	47,000,000	470,000	4,700	520	168	47
1	51,000,000	510,000	5,100	570	204	51
5	77,000,000	770,000	7,700	856	304	77
10	100,000,000	1,000,000	10,000	1100	400	100
20	148,000,000	1,480,000	14,800	1644	592	148
25	160,000,000	1,600,000	16,000	1840	664	166

[a] Number of animals needed in each group, controls as well as treated.

DATA ANALYSIS APPLICATIONS IN TOXICOLOGICAL PATHOLOGY

Having reviewed basic principles and provided a set of methods for the statistical handling of data, the remainder of this chapter addresses the practical aspects and difficulties encountered in preclinical safety assessment in the field of toxicological pathology. Analyses of pathology data are well defined although they may not necessarily use the best methods available. The use of statistical methodology is discussed in the remainder of this chapter. The aim of this section is to review statistical methods on a use-by-use basis and to provide a foundation for the selection of alternatives in specific situations. Meta-analyses and Bayesian approaches are not addressed in detail but should be kept in mind.

Body and Organ Weights

Body weight and the weights of selected organs are usually collected in studies where animals are dosed with, or exposed to, a chemical. In fact, body weight is frequently the most sensitive indication of an adverse treatment effect. How to analyze these data best and in what form to analyze

the organ weight data, such as absolute weights, weight changes, or percentages of body weight, have been the subject of great discussion.

Both absolute body weights and rates of body weight change are best analyzed by ANOVA followed, if called for, by a post hoc test. Body weight change is usually calculated as changes from a baseline measurement value, which is traditionally the animal's weight immediately prior to the first dosing with or exposure to the test material. To standardize body weight, no group should be significantly different in mean body weight from any other group, and all animals in all groups should lie within two SDs of the overall mean body weight. Even if the groups were randomized properly at the beginning of a study, there is an advantage to performing the computationally slightly more cumbersome changes in body weight analysis. The advantage of this calculation is an increase in sensitivity because the adjustment of starting points (i.e., the setting of initial weights as a zero value) reduces the amount of initial variability. In this case, Bartlett's test is performed first to ensure the homogeneity of variance, and the appropriate sequence of analysis follows.

If sample sizes are small or normality of data is uncertain, nonparametric methods, such as Kruskal–Wallis, may be more appropriate. The analysis of relative organ weights is a valuable tool for identifying possible target organs. How to perform this analysis is still a matter of some disagreement. Organ weight data, expressed as percentages of body weight, should be analyzed separately for each sex. Often, conclusions from organ weight data of males differ from those of females; hence, separating these data by gender should always be done. Other factors, such as the effect of stage of the reproductive cycle on uterine weight, may also influence organ weights. These factors must be taken into account both in the stratification of animals and in the interpretation of results.

The two alternative approaches to analyzing relative organ weights call for either calculating organ weights as a percentage of total body weight at the time of necropsy and analyzing the results by ANOVA or analyzing results by ANCOVA, with body weights as the covariates as discussed previously. A number of considerations should be kept in mind when this choice is made. First, one must recognize the difference between biological significance and statistical significance. By evaluating relative body weight, the significance of a weight change that is not proportional to changes in whole body weights must be determined. Second, the toxicological pathologist now must interpret small changes while still retaining a similar sensitivity (i.e., the $p < 0.05$ level).

Several tools can be used to increase the power of the analysis. One is to increase the sample size by increasing the number of animals, and the other is to utilize the most powerful test available that is appropriate to the data. The number of animals used in the groups is currently under debate with respect to the power of detecting a significant change. The power of statistical tests is important in the consideration of animal numbers.

In the majority of cases, except for the brain, the organs of interest change weight in proportion to total body weight, except in extreme cases of obesity or starvation. This change is the biological rationale behind analyzing both absolute body weight and the ratio of organ weight to body weight. Analyses are designed to detect cases where this relative change does not occur. Analysis of data from several hundred studies has shown no significant difference in rates of weight change of target organs, other than the brain, compared to total body weight for healthy animals in rats, mice, rabbits, and dogs used for repeated dose studies. The ANCOVA is of questionable validity in analyzing body weight and related organ weight changes, as a primary assumption is the independence of treatment. In toxicological pathology, the assumption that the relationship of the two variables is the same for all treatments is not true.

In cases where the differences between the error MSs are much greater during the analysis, the ratio of F ratios will diverge in precision from the result of the efficiency of covariance adjustment. These cases occur where either sample sizes are large or where the differences between means themselves are great. This latter case is one that does not occur in the designs under discussion in any manner that would leave ANCOVA as a valid approach because group means are very similar at the beginning of the experiment and cannot diverge markedly unless there is a treatment effect. As discussed earlier, a treatment effect invalidates a prime underpinning assumption of ANCOVA.

Clinical Chemistry

A number of clinical chemistry parameters are commonly determined on the blood and urine collected from animals in chronic, subchronic, and, occasionally, acute toxicity studies. In the past, and currently in some places, the accepted practice has been to evaluate these data using univariate–parametric methods, primarily t-tests and ANOVA; however, this is not the best approach. First, biochemical parameters are rarely independent of each other, and the focus of inquiry is rarely limited to only one of the parameters. Instead, several parameters can change when toxicity is seen in specific organs. For example, simultaneous elevations of creatinine phosphokinase, γ-hydroxybutyrate dehydrogenase, and lactate dehydrogenase are strongly indicative of myocardial damage. In such a case, the clinical importance of these findings is not limited to a significant elevation in one of these enzymes; all three must be considered together. Detailed coverage of the interpretation of such clinical laboratory tests can be found elsewhere.

Second, interaction occurs among parameters; therefore, each parameter is not independent. For example, serum electrolytes (sodium, potassium, and calcium) interact such that a decrease in one is frequently tied to an increase in one of the others. Finally, either because of the biological nature of the parameter or the way in which it is measured, data are frequently skewed or not continuous. This skewness and discontinuous nature of data can be seen in some of the reference data for experimental animals (e.g., creatinine, sodium, potassium, chloride, calcium, and blood).

Carcinogenesis

Inferences about the potential human carcinogenicity of substances are based on experimental results obtained from a nonhuman species given the substance at a high dose or exposure level. The aim of this procedure is to predict the possibility and probability of occurrence of tumorogenesis in humans at much lower levels. An entire textbook could be devoted to examining the assumptions involved in this undertaking and review of the aspects of design and interpretation of animal carcinogenicity studies. Such detail is beyond the scope of this chapter. The reader is referred to Gad [23] for more detail.

In the past, the single most important statistical consideration in the design of carcinogenicity bioassays was based on a simple quantal response: cancer did or did not occur. Experiments were designed so a sufficient number of animals were used so as to have a reasonable expectation of detecting an effect if one occurred. Although the primary objective was to determine whether the incidence of tumors was increased following exposure to the test article of interest, a much more complex model should now be considered to answer other questions pertinent to the extrapolation of experimental results in animals to make inferences about risks to human health.

The time to tumor, patterns of tumor incidence, effects on survival rate, and age at first tumor can now be evaluated. The rationale for including these factors lies in concerns associated with likely planned or unplanned exposure of humans to xenobiotic and naturally occurring substances, and relatively small increases in the incidence of tumors over background would require the use of an impracticably large number of test animals per group.

To illustrate this point, examine the data provided in Table 9.17. Here, only 46 animals per group are required to show a 10% increase over a zero background, where the background included a rarely occurring tumor type. To detect a 0.1% increase above a 5% background, 770,000 animals per group would be needed! As dose increases the incidence of tumors, the response will also increase. This increase occurs until it reaches the point where a modest increase (e.g., 10%) over a reasonably small background level (e.g., 1%) could be detected using an acceptably small-sized group of test animals. Table 9.17 shows that 51 animals would be needed for such a situation. It can be seen that the number of animals required to demonstrate a 1/100,000 increase above a 25% background incidence would be very large.

At least two potential difficulties often occur in the group given the highest dose. First, mortality can be higher than other groups; a sufficient number of rodents must survive to the end of the study to allow for meaningful statistical analysis. Second, toxicological pathologists must select the high dose level based only on the information provided by a subchronic or range-finding study, usually 90 days in length. To predict carcinogenic effects across species, it is necessary that the metabolism and mechanism of action of the chemical at the highest level tested are the same as at the low levels where human exposure would occur. Unfortunately, selection of a dose that is too low may make the study invalid for the detection of carcinogenicity, and selection of a dose that is too high, where toxicokinetics result in different metabolism, may seriously impair the use of the results for risk assessment.

QUESTIONS

9.1 Given that the complexity and volume of data resulting from toxicology studies continue to increase, what methods serve to make results more readily understood while still maintaining accuracy and without losing precision?

9.2 Why are statistical graphics increasingly important?

9.3 Given that safety evaluations of pharmaceuticals and other regulated products involve multiple studies, how might statistics better serve in performing relative risk assessments (read across reviews)?

9.4 Why does controlling (or reducing) variability within group datasets serve to increase the sensitivity of detecting real differences between groups (significant effects)? And how does this contribute to reducing animal usage?

KEYWORDS

Bayesian analysis, Carcinogenicity bioassays, Clinical chemistry, Experimental design, Meta analysis, Modeling, QSAR, Reduction of dimensionality, Statistical graphics, Statistical methods

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10 Practice of Exposure Assessment

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CONTENTS

INTRODUCTION

Health risk assessment is the process wherein toxicology data from animal studies and human epidemiology are evaluated, a mathematical formula is applied to predict the response at low doses, and then information about the degree of exposure is used to predict quantitatively the likelihood that a particular adverse response will be seen in a specific human population.[1–3] More simply, risk assessment is a process by which scientists evaluate the potential for adverse health effects from exposure to naturally occurring or synthetic agents.[4] Regulatory agencies have used the risk assessment process for nearly 50 years, most notably the U.S. Food and Drug Administration (USFDA).[5] However, the difference between assessments performed in the 1950s and 1960s and those performed in the 1980s and 1990s and even more currently is that dose-extrapolation models, quantitative exposure assessments, and quantitative descriptions of uncertainty have been added to the process.[6] Because of increased ability to measure and predict exposures and better quantitative methods for estimating the low-dose response (such as physiologically based pharmacokinetic [PBPK] models), risk assessments conducted today provide more accurate risk estimates than in the past.[3,7,8]

Since 1980, most environmental regulations and some occupational health standards have, at least in part, been based on health risk assessments.[3,9,10] They include standards for pesticide residues in crops, drinking water, ambient air, and food additives, as well as exposure limits for contaminants found in indoor air, consumer products, and other media. Risk managers increasingly rely on risk assessment to decide whether a broad array of risks are significant or trivial—an important task since, for example, more than 400 of the about 2000 chemicals routinely used in industry have been labeled carcinogens in various animal studies.[11–13] In theory, the results of risk assessments in the United States should influence virtually all regulatory decisions involving so-called toxic agents.[14–16]

The risk assessment process has four parts: hazard identification, dose–response assessment, exposure assessment, and risk characterization.[11] Although progress has been made over the past 20+ years in how to conduct and interpret toxicology and epidemiology studies (e.g., hazard identification), and scientists believe that they are doing a better job of dose–response extrapolation than in the past, most significant advances in the risk assessment process have occurred in the field of exposure assessment.[17–19]

Since about 1995, an increasing number of environmental scientists have embraced the view that "toxicology data are important, but they do not mean much without quantitative information about human exposure."[20] For this reason, the toxicology community has shown increasing interest in understanding the exposure assessment field.[21,22] Fortunately, a significant amount of research has been conducted to identify better values for many exposure parameters, and major improvements have been made in applying these exposure factors to various scenarios. This chapter is intended to familiarize toxicologists, risk assessors, and others with this evolving field.

BASIC CONCEPTS

Description of Exposure Assessment

Exposure assessment is the step that quantifies the intake of an agent resulting from contact with various environmental media (e.g., air, water, soil, food).[3,8,23,24] Exposure assessments can address past, current, or future exposures, although uncertainties can become significant when attempting to anticipate what might have happened or what will happen.[8,25–31] Researchers have used a variety of methods to approximate historical and future exposures, including using geographic location, job history, historical records, biomonitoring, and estimates from mathematical models, as a proxy for exposure.[32–37]

Exposure assessment in various forms dates back at least to the early twentieth century, and perhaps earlier, particularly in the fields of epidemiology,[38,39] industrial hygiene,[40,41] and health physics.[42] Exposure assessment combines elements of all three disciplines and relies on aspects of biochemical toxicology (to estimate delivered dose), atmospheric sciences, anthropometry, analytical chemistry, food sciences, physiology, environmental modeling, and others.[43]

Fundamentally, an exposure assessment describes the nature and size of the various populations exposed to a chemical agent and the magnitude and duration of their exposures.[44,45] It determines the degree of contact a person has with a chemical and estimates the magnitude of the absorbed dose.[46] Several factors need to be considered when estimating that dose, including characteristics of the contaminated media, exposure duration, route of exposure, chemical bioavailability from the contaminated media (e.g., soil), and, sometimes, the unique characteristics of the tested population (e.g., hairless mice absorb a greater percent of chemical than other mice). By definition, *duration* is the period of time over which the person is exposed. An *acute* exposure generally involves one contact with the chemical, usually for less than a day. An exposure is considered *chronic* when it takes place over a substantial portion of the person's lifetime. Exposures of intermediate duration are usually called *subchronic*.[43]

Knowledge of the chemical concentration in an environmental medium is essential to determine the magnitude of the absorbed dose. This information is usually obtained by analytical measurements of samples of the contaminated medium (air, water, soil, sediment, food, or house dust). Estimates can also be made using mathematical models, such as models relating air concentrations at various distances from a point of release (e.g., a smoke stack) to factors including release rate, weather conditions, distance, and stability of the agent.[47,48] Needless to say, a significant number of factors need to be considered to quantitatively evaluate a typical, complex contaminated site (Figure 10.1).

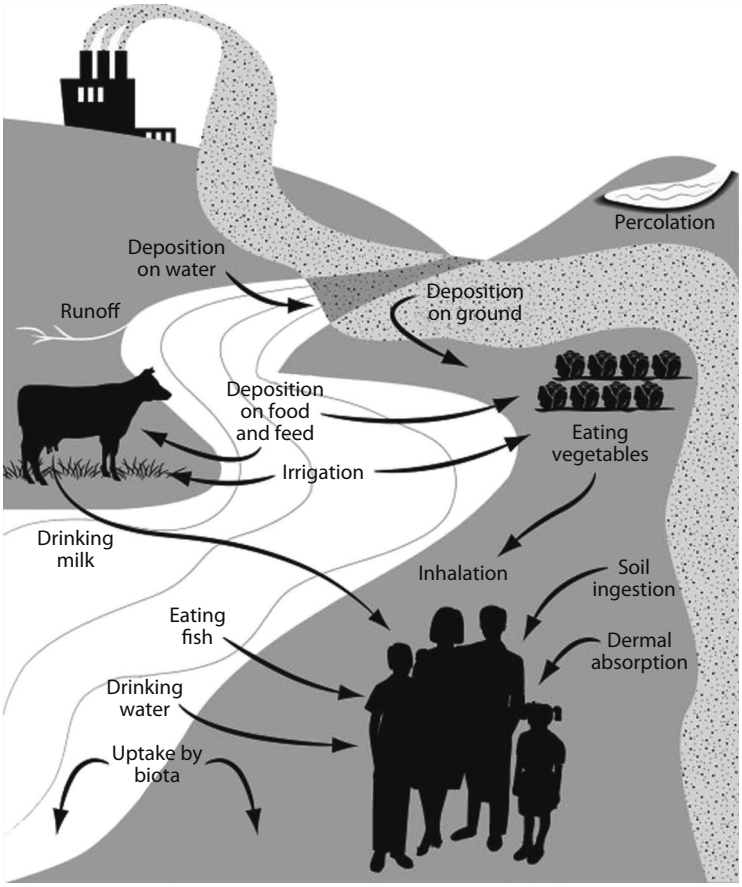

FIGURE 10.1 Illustration of exposure pathways.

In general, exposure assessments have matured to a degree that usually possesses less uncertainty than other steps in the risk assessment. Admittedly, many factors should be considered when estimating exposure; for example, it is a complicated procedure to understand the transport and distribution of a chemical that has been released into the environment.[49] Nonetheless, a number of studies have shown that if the majority of factors are considered, one can do a fairly adequate job of quantifying with confidence the chemical concentrations in various media and the resulting uptake by exposed persons.[8,50,51] No doubt, in the coming years, these estimates will be confirmed or rejected as larger amounts of biomonitoring data become available and are used to relate internal chemical doses to the external environment and the impact on adverse health responses.

The primary routes of human exposure to chemicals in the ambient environment are dust and vapor inhalation, dermal contact with contaminated soils or dusts, and ingestion of contaminated food, water, house dust, or soil. In the workplace, the predominant exposure route usually is inhalation, followed by dermal uptake and, to a lesser extent, dust ingestion due to hand-to-mouth contact.[7] Uncertainty in environmental exposure assessment can be greater than in an occupational exposure assessment. However, in many workplaces, there can be large fluctuations in airborne concentrations, a significant difference in work practices of different persons, and there is real difficulty in measuring dermal uptake and incidental ingestion.[41,52–56]

Scientists in the field of radiological health were the first to quantitatively estimate human uptake of environmental contaminants[57,58]; thus, the published literature in health physics can be a source of valuable information when conducting assessments of chemical contaminants.[59] This work, which was conducted after World War II, provided numerous methodologies for estimating human uptake of environmental contaminants.[59] These have been refined over the past decade.[27,60–62] The availability of information on the degree of exposure associated with various scenarios has increased dramatically in recent years, as evidenced by the recent *EPA Exposure Factors Handbook* and *Child-Specific Exposure Factors Handbook*—documents containing nearly 2000 pages of combined information on exposure assessment.[63,64]

The practice of exposure assessment, at least for regulatory purposes, has changed over time. For example, beginning in the late 1970s, U.S. regulatory policy encouraged or mandated the use of conservative approaches when conducting exposure assessments. This was codified in the Environmental Protection Agency's (EPA) original document entitled *Risk Assessment Guidance for Superfund*, so-called RAGS.[65] At that time, standardization of exposure assessments used to satisfy regulatory agencies was considered prudent because it guaranteed that risks would not be underestimated in order to ensure protection of public health. Beginning in the mid-1980s, concern was expressed that repeated use of conservative exposure factor assumptions was producing unrealistically high estimates of exposure and that the cost of achieving the recommended cleanup levels was becoming enormous.[53,66–73] Thus, to evaluate the accuracy of many of the factors used in these assessments, changes in the process began to occur. Government agencies such as the Office of Management and Budget (OMB) and the U.S. EPA have developed new guidelines and urged risk assessors to do an even better job at eliminating compounding conservatism in their assessments.[74]

Around 1990, risk assessors began to apply Monte Carlo techniques and probabilistic approaches to eliminate the possibility of compounding conservatism and to increase the transparency of the analyses. Application of Monte Carlo techniques to exposure assessment has dramatically improved our understanding of the certainty of exposure estimates thereby altering the field permanently.[37,71,75–87] The EPA and other agencies have now embraced this approach, which is well described in several guidelines as well as the document called RAGS3A (a process for conducting probabilistic risk assessment).[83,88,89]

What Is Exposure?

Over the years, the terminology used in published exposure assessment literature has been inconsistent. Although there is reasonable agreement that human exposure means contact with the chemical or the agent,[24,65,90] there has not yet been widespread agreement as to whether this means contact with (1) the visible exterior of the person (skin and openings in the body, such as mouth and nostrils) or (2) the so-called exchange boundaries where absorption takes place (skin, lungs, gastrointestinal tract).[43] The differing definitions have led to some ambiguity in the use of terms and units for quantifying exposure. For example, the terms dose, uptake, and intake have often been used loosely.

Some scientists find it helpful to think of the human body as having a hypothetical outer boundary that separates the inside of the body from the outside.[43] The outer boundary of the body consists of the skin and openings into the body, such as the mouth, nostrils, or punctures and lesions in the skin. In most exposure assessments, chemical exposure is defined as contact of the chemical with some part of this boundary. An exposure assessment is the quantitative or qualitative evaluation of that contact. It describes the intensity, frequency, and duration of contact, and often quantifies the rate at which the chemical crosses the boundary (chemical intake or uptake rates), the route of the chemical across the boundary (exposure route [e.g., dermal, oral, or respiratory]), the resulting amount of chemical actually crossing the boundary (dose), and the amount of chemical absorbed (internal dose).[24,91] A very workable quantitative definition of exposure is to think of it as "the product of (concentration), (time), and (duration), or rate of transport of toxicant (mg/min)."[52]

Depending on the purpose of the exposure assessment, the numerical output of these analyses may be an estimate of either exposure or dose. If an exposure assessment is being done as part of a risk assessment in support of an epidemiologic study, for example, sometimes only qualitative exposure levels are all that can be provided. In these situations,

categories such as low-, medium-, and high-level exposures may be used (although strongly discouraged by the authors). By contrast, a greater portion of the assessments of environmental or occupational exposure conducted in recent years attempt to quantitatively predict the absorbed dose (mg/kg-day) and, occasionally, the circulating blood level or the concentration of the toxicant in the target organ.[92–98]

Concepts of Exposure, Intake, Uptake, and Dose

The process of a chemical entering the body can be described in two steps—contact (exposure), followed by actual entry (crossing the boundary). Absorption, by crossing the boundary, leads to the availability of an amount of chemical to biologically significant sites within the body (target tissue dose). Although the description of contact with the outer boundary is simple conceptually (e.g., mg benzene/cm^2 skin), estimating the degree to which a chemical crosses this boundary is somewhat more complex.[99]

In the early 1990s, some scientists described the transport of chemicals into the body as involving two separate steps: intake and uptake. Intake involved physically moving the chemical in question through an opening in the outer boundary (usually the mouth or nose), typically via inhalation, eating, or drinking. Normally, the chemical was contained in a medium such as air, food, water, or dust/soil. Here, the key question was the mass inhaled or ingested. Uptake, in contrast to intake, involved absorption of the chemical through the skin or across other barriers.

Today, most scientists tend to lump intake and uptake together, simply calling the amount of chemical entering the body as *intake* or the *absorbed dose*. Some chemicals are absorbed completely, so systemic absorption is the same as that eaten or in contact with the skin. In other cases, the chemical is often contained in a carrier medium, the medium itself typically is not absorbed at the same rate as the *contaminant of interest*, so estimates of the amount of chemical crossing the boundary cannot be made directly. For example, benzene on the surface of a contaminated soil particle will move quickly through the skin, but benzene in the center of the soil particle may never completely reach the surface and, therefore, it is not bioavailable and may never enter the bloodstream. Of course, for many inorganic chemicals such as arsenic or lead in soil, bioavailability can be very low since the chemical is bound to the interstices of the soil particle. Here, absorption can be very low. In short, if a chemical cannot be released, it has no bioavailability and, consequently, since there is no absorbed dose the chemical does not pose a risk.

Dermal absorption is an example of direct uptake across the outer boundary of the body. A chemical uptake rate is the amount of chemical absorbed per unit of time. In this process, mass transfer occurs by diffusion, so uptake will depend on the concentration gradient across the boundary, permeability of the barrier, and other factors.[91,100,101] Chemical uptake rates can be expressed as a function of the exposure concentration, permeability coefficient, and surface area exposed, or as flux.[7]

Bioavailability

The study of the bioavailability of chemicals in various media began around 1980 and continues to be an important area of research.[51,102–120] Most studies are of oral bioavailability, although the dermal and inhalation bioavailabilities of chemicals on various media have also been studied. Bioavailability has been a bit confusing due to the lack of a standard terminology.[121] The review paper by Ruby et al. is probably the most authoritative one on this topic,[102] although the text by Hrudey is also a valuable resource.[121] We suggest that the following definitions be used in future assessments:

Bioavailability: Oral bioavailability is defined as the fraction of an administered dose that reaches the central (blood) compartment from the gastrointestinal tract. Bioavailability defined in this manner is commonly referred to as *absolute bioavailability* and is equal to the oral absorption fraction.

Relative Bioavailability: Relative bioavailability refers to comparative bioavailabilities of different forms of a substance or for different exposure media containing the substance (e.g., bioavailability of a metal from soil relative to its bioavailability from water), expressed in this document as a relative absorption factor (RAF).

Relative Absorption Factor: The RAF describes the ratio of the absorbed fraction of a substance from a particular exposure medium relative to the fraction absorbed from the dosing vehicle used in the toxicity study for that substance (the term relative bioavailability adjustment [RBA] is also used to describe this factor).

Bioaccessibility: The oral bioaccessibility of a substance is the fraction that is soluble in the gastrointestinal environment and is available for absorption. The bioaccessible fraction is not necessarily equal to the RAF (or RBA) but depends on the relation between results from a particular in vitro test system and an appropriate in vivo model.

There are both in vitro and in vivo tests for evaluating bioavailability and many different approaches have been suggested over the past 25 years.[51,102–108,110–125]

As noted by Ruby et al. (1999), a number of in vitro tests have been used to characterize the oral bioavailability of various chemicals in various media.[102] Simple extraction tests have been used for several years to assess the degree of metals dissolution in a simulated gastrointestinal-tract environment. The predecessor of these systems was developed originally to assess the bioavailability of iron from food, for studies of nutrition. In these systems, various metal salts or soils containing metals are incubated in low-pH solution for a period intended to mimic residence time in the stomach. The pH is then increased to near neutral, and incubation continues for a period intended to mimic residence time in the small intestine. Enzymes and organic acids are added to simulate gastric and small-intestinal fluids. The fraction of lead, arsenic, or other metals that dissolve during the stomach and

small-intestinal incubations represents the fraction that is bioaccessible (i.e., is soluble and available for absorption).

A number of in vivo tests have also been used with varying success. For example, gastrointestinal absorption of lead in humans varies with the age, diet, and nutritional status of the subject as well as with the chemical species and the particle size of lead that is administered. Age is a well-established determinant of lead absorption; adults typically absorb 7%–15% of lead ingested from dietary sources, while estimates of lead absorption from dietary sources in infants and children range from 40% to 53%. For the purpose of modeling exposure to lead in soil, the U.S. EPA assumes that the absolute bioavailability of lead in diet and water is 50% and that the absolute bioavailability of lead in soil is 30% for children. This corresponds to a soil RAF of 0.60 (60%) for the bioavailability of soil lead relative to lead in water (i.e., RAF = 0.3/0.5).[102] However, recent findings have revealed that fractional bioaccessibility (bioaccessible compared to total) of lead is only 5%–10% of total lead in urban soils, far lower than the 60% bioavailability of food lead presumed by the U.S. EPA (30% absolute bioavailability used in the integrated exposure uptake biokinetic [IEUBK] model).[126]

The results of bioavailability studies need to be considered in virtually all assessments involving human exposure.[51,102,107,114,116,117,120,121] Often, the effects in uptake will be minor while in other cases, one may find that insignificant quantities of a chemical are absorbed even though the applied dose or exposure is quite high.[122,127]

Applied Dose or Potential Dose

Applied dose has been defined as the amount of chemical available at the absorption barrier (skin, lung, gastrointestinal tract).[43] It is useful to know the applied dose if a relationship can be established between it and the internal dose, a relationship that can sometimes be established experimentally. This can be estimated either through modeling or by direct measurement. For example, years ago, some researchers analyzed phenol concentrations in the blood of volunteers over time after placing their hands in containers of nitrobenzene or benzene in an attempt to quantify the flux rate.[128,129] Usually, it is difficult to measure the applied dose directly, as many of the absorption barriers are internal to the human, and not localized in such a way to make measurement easy. An approximation of applied dose can be made, however, using the concept of potential dose.[43]

Potential dose is simply the amount of chemical that is ingested or inhaled, or the amount of chemical contained in material applied to the skin. It is a useful term or concept in those instances when there is a measurable amount of chemical in a particular medium. The potential dose for ingestion and inhalation is analogous to the administered dose in a dose–response experiment.

For the dermal route, potential dose is the amount of chemical applied or the amount of chemical in the medium applied (e.g., as a small amount of soil deposited on the skin). Note that because all of the chemical in the soil particulate is not contacting the skin, this differs from exposure (the concentration in the particulate times the duration of contact) and applied dose (the amount in the layer actually touching the skin).[43,74,130]

As previously noted, the amount of chemical that reaches the exchange boundaries of the skin, lungs, or gastrointestinal tract may often be less than the potential dose if the material is only partly bioavailable and therefore only partially absorbed. For example, only about 0.001%–1.0% of dioxins or polycyclic aromatic hydrocarbons (PAHs) on fly ash in contact with the skin are likely to penetrate.[125] When bioavailability data are available, adjustments to the potential dose should be made to convert it to the absorbed or internal dose.[121,125]

Internal Dose

The amount of chemical that has been absorbed and is available for interaction with biologically significant receptors (e.g., target organs) is called the internal dose. Estimating internal dose can be difficult but it is one of the primary objectives of a good exposure assessment.[131,132]

Transport models are available to assist in this process.[133] Once absorbed, the chemical can be metabolized, stored, excreted, or transported within the body. The amount transported to an individual organ, tissue, or fluid of interest is termed the delivered dose.[98,134,135] The dose delivered to the target organ may be only a small part of the total internal dose but, by definition, it is the most relevant. For example, although 1 mg of polychlorinated biphenyl (PCB) may be absorbed into the body, at any given time, the amount in the liver (the target organ) may only be 0.001 mg. The time course over which that 0.001 mg is delivered is often equally important to understand. Work to refine the techniques used to estimate delivered dose has been among the most exciting areas of exposure assessment research in recent years. Currently, the best approach to estimate delivered dose is to measure blood or to use PBPK models.[98,132,136–140] Recent research efforts have involved the use of PBPK models and data on polymorphisms in metabolic enzymes to understand the disposition of environmental toxicants in potentially susceptible human populations.[135]

The biologically effective dose (BED), or the amount that actually reaches cells, sites, or membranes where adverse effects occur,[141] may represent only a fraction of the delivered dose, but it is obviously the best one for predicting adverse effects. Understanding the BED is the ultimate goal of exposure assessment. Regrettably, thus far, toxicologists have rarely been able to estimate BED or measure it for most chemicals.[43]

Currently, most risk assessments dealing with environmental chemicals (as opposed to pharmaceutical assessments) rely on dose–response relationships based on the potential (administered) dose or the internal dose, because our understanding of how to estimate the delivered dose or the BED is insufficient for most chemicals. In general, the best method currently available for estimating the dose to the target organ is to use PBPK models. These have been developed for nearly 100 high-volume industrial chemicals (Table 10.1).[142]

Often, it is more convenient in risk assessment to refer to dose rates, or the amount of a chemical dose (applied or

TABLE 10.1
Examples of PBPK Models for Toxic Materials

Benzene	Lead
Benzo[a]pyrene	Methanol
Butoxyethanol	Methoxyethanol (2-ME)
Butoxyethanol	Methyl ethyl ketone (MEK, #1205)
Carbon tetrachloride	Nickel
Chlorfenvinphos	Nicotine
Chloroform	Parathion
Chloropentafluorobenzene	Physostigmine
cis-Dichlorodiammine platinum	PBB
Dichloroethane	PCBs
Dichloromethane	Styrene
Dieldrin	Toluene
Diisopropylfluorophosphate	TCDF
Dimethyloxazolidine dione	TCDD (dioxin)
Dioxane	Tetrachloroethylene
Ethylene oxide	Trichloroethane
Ethyoxy ethanol (2-EE)	Trichloroethylene
Formaldehyde	Trichlorotribluoroethane
Hexane	Vinyl chloride
Hexavalent chromium	Vinylidene fluoride
Kepone	Xylene

Note: This table is an expansion of one presented in a paper by Leung and Paustenbach (1995).[142]

internal) per unit time (e.g., mg/day), or as dose rates on a per-unit-body-weight basis (e.g., mg/kg-day). Most exposure data found in the various editions of EPA's *Exposure Factors Handbook* and *Child-Specific Exposure Factors Handbook* and other guidance documents are presented as dose rates (e.g., grams of fish consumed each day) rather than as absorbed dose.[63,64,143–145]

Exposure and Dose Relationships

Depending on the purpose of the exposure assessment, and the mechanism of action of the chemical, different estimates of exposure and dose may need to be calculated. Often, estimates of uptake will be presented in units so that the dose metric will be the same as that used in the toxicology study, which may not be useful for exposure calculations. When risk is a function of time of exposure, exposure or dose profiles can be very useful. In these profiles, the exposure concentration or dose is plotted as a function of time.[146] Concentration and time are used to depict exposure, while amount and time characterize dose. Such profiles are important for use in risk assessment where the severity of the effect depends on the pattern by which the exposure occurs, rather than on the total (integrated) exposure. For example, a developmental toxicant may only produce effects if exposure occurs during a particular stage of development.[147–149] As shown in Figure 10.2, during the time above a certain dose rate (the shaded portion), there was an increased risk to the fetus of certain birth defects. Similarly, a single acute exposure to very high contaminant

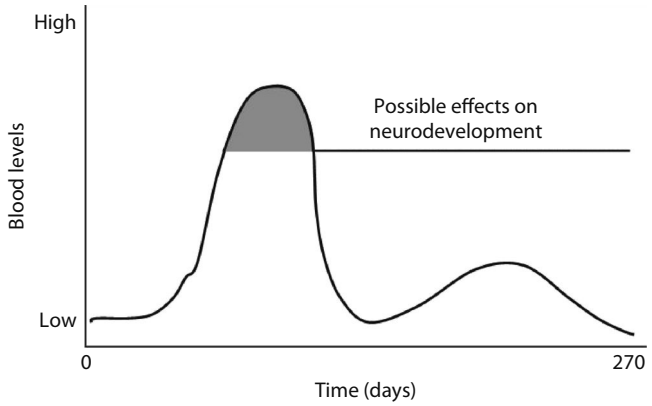

FIGURE 10.2 Time course of exposure to a developmental toxicant. Note that the shaded portion represents the blood concentration of toxicant that is necessary to offer some probability that an inverse effect might occur.

levels may induce adverse effects, even if the average exposure is much lower than apparent no-effect levels. In order to understand hazards posed by most chemicals, it is important to consider its pharmacokinetics. For example, for a chemical that has a long biologic half-life, internal exposure continues long after the chemical is ingested because blood levels remain high until the substance is metabolized and/or eliminated. Conversely, for others, the chemical is inhaled, absorbed, metabolized, and excreted in less than an hour after exposure.

In general, there is a need to consider the time elements of exposure assessment relative to the risk posed by the exposure. Standard approaches to time averaging to estimate long-term daily exposure concentration, in some cases, result in substantial underprediction of short-term variations in exposure. Similarly, the use of short-term measurements may overpredict long-term exposures.[150,151] It is useful to understand the relationship between the biological half-lives of toxicants and the subsequent critical time element of the exposure. Indeed, the appropriate consideration of these elements should dictate the *ATs* for both the exposure limits and exposure assessment.[54,55,152–154]

If a material causes its biological damage quickly and is gone from the body in a short time, then how we test its toxicity is critical. For example, consider a material with a half-life of a few minutes in the body. If we were to test it by spreading or apportioning the daily dose of this material over 24 h using inhalation, it will give very different results than if the animal absorbed the same quantity in a couple of 1 h inhalation exposures. The same dose of this quick-acting material would do much more damage amassed in a bolus dose administered over a few minutes or even an hour or two than if absorbed over 24 h.

Thus, dosing times in animal studies should be commensurate with the biological half-life. We often need to measure the exposure over an appropriately short period of time in which the worst-case exposure may occur. The same logic also holds for the dermal (topically applied) and oral (normally ingested) exposures in that they appear to occur in time frames that are comparable to what we would expect in use. Bolus dosing by

gavage or injection would, of course, be the worst case. Thus, if a material causes its biological damage and is gone from the body in a relatively short period of time, then a long-term measure of exposure will generally be unnecessary.

On the other hand, if the biological half-life of the compound is longer than a few days, then relatively high spikes of exposure over a day or two are not particularly significant from a health impact perspective. What is important from a chronic toxicity perspective for these types of compounds is, of course, the weighted average over a significantly long time period. As such, it would only seem appropriate to use an annual average exposure when you are dealing with a compound with a very long (greater than 90 days) half-life in the body and no evidence of acute toxicity at high short-term dose rate.[153,155,156]

Integrated or aggregate exposure is the sum total of exposure to a chemical via all routes of exposure (and all media). It is now commonplace to add as many as 6–10 different exposure sources per route (e.g., dichlorodiphenyltrichloroethane [DDT] in different fruits and vegetables) and three exposure routes (e.g., DDT via food, air, and dermal contact).[157–160] Modeling software that characterize doses from exposure to multiple chemicals, by multiple routes, and from multiple sources are available.[159] The units of aggregate exposure are concentration times duration. Aggregate exposure has been considered in complex assessments of the past 10 years, for example, incinerators, but it came to the fore with the passage of the Food Quality Protection Act (FQPA) in 1996. An increasing number of guidance documents from regulatory agencies and examples from researchers of how to perform these assessments have been published over the last 5 years.[61,159,161] Some research has been devoted to understanding aggregate environmental exposures, and there have been many attempts to determine the source contribution for different chemicals. These efforts have been the underpinning for studies such as the National Human Exposure Assessment Survey (NHEXAS), Children's Total Exposure to Persistent Pesticides and Other Persistent Organic Pollutants (CTEPP), Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS), and Minnesota Children's Pesticides Exposure Study (MNCPES).[162–170]

Integrated exposure is the total *area under the curve* (AUC) of the exposure profile. An exposure profile (a picture of the exposure concentration over time) is particularly useful when trying to understand occupational exposure because it contains more information than a numerical estimate of the integrated exposure, including the duration and periodicity of exposure, the peak exposure, and the shape of the area under the time–concentration curve. The risk posed by most systemic toxicants with chronic effects is best understood by evaluating the blood concentration versus time relationship.

A common way to characterize exposure is the time-weighted average (TWA). This is particularly relevant when conducting an assessment of a carcinogenic chemical in the workplace. In cancer risk assessments, the time over which exposure is integrated is usually 70 years.[43,171] A TWA dose rate is the total dose divided by the time period of dosing, usually expressed in units of mass per unit time, or mass/time normalized to body weight (e.g., mg/kg-day). TWA dose rates, such as the lifetime average daily dose (LADD), are used in dose–response equations to estimate lifetime risk.

Measures of Dose

For risk assessment purposes, dose estimates should be expressed in a manner that can be compared with available dose–response data from animal or human studies. For example, if data on human exposure are in milligram of lead per deciliter of blood (mg/dL), it would be best to use the blood concentrations in an animal study to predict the risk to humans. Frequently, dose–response relationships are based on potential dose (called administered dose in animal studies), although dose–response relationships are sometimes based on internal dose. These differences need to be accounted for. The measure of dose selected should be based on the mode of action of the adverse effect.[74,146,171–175] For example, to assess a nasal irritant, the airborne concentration of the chemical is a relevant dose and an even better dose metric would be milligram of chemical contacting a square centimeter of nasal mucosa.

Doses may be expressed in several different ways. Solving Equation 10.1, for example, gives the dose rate over the time period of interest. The dose per unit time is the dose rate, which has units of mass/time. The most common dose measure is average daily dose (ADD), which is used to predict or assess the noncarcinogenic effects of a chemical:

$$\mathrm{ADD} = \frac{[\mathrm{C} \cdot \mathrm{IR} \cdot \mathrm{B}]}{[\mathrm{BW} \cdot \mathrm{AT}]} \tag{10.1}$$

where

ADD is the potential average daily dose
BW is the body weight
B is the bioavailability
AT is the time period over which the dose is averaged (days)
C is the mean exposure concentration
IR is the ingestion rate

A typical calculation follows.

Example Calculation 1: Determining the ADD

A typical American eats a certain amount of lettuce over a lifetime (about 2000 kg). Assume that on any given week, the maximum quantity ingested is 0.5 kg, and the maximum on any one day is 0.04 kg/day. Assume that the typical aldrin residue is 4 mg/kg on all lettuce ingested over the person's lifetime. What is the ADD of aldrin for the maximum week? Assume that the oral bioavailability of aldrin in lettuce is 90%.

Given

$$\mathrm{C} = 4 \text{ mg/kg (aldrin)}$$

$$\mathrm{BW} = 70 \text{ kg}$$

$$\mathrm{AT} = 7 \text{ days}$$

$$\mathrm{IR} = 0.5 \text{ kg}$$

$$\mathrm{B} = 0.9$$

Therefore,

$$\text{ADD} = \frac{\left[\text{C} \cdot \text{IR} \cdot \text{B}\right]}{\left[\text{BW} \cdot \text{AT}\right]}$$

$$\text{ADD} = \frac{[4\ \text{mg/kg}] \cdot [0.5\ \text{kg}] \cdot [0.9]}{[70\ \text{kg}] \cdot [7\ \text{days}]}$$

$$\text{ADD} = 0.004\ \text{mg/kg-day}$$

When the primary health risk posed by a chemical is cancer or another chronic effect, then the biological response is usually described in terms of lifetime probabilities (e.g., the increased risk of developing cancer during a 70-year lifetime is 2 in 100,000). In these circumstances, even though exposure does not occur over the entire lifetime, doses are usually presented as LADDs.[43] The LADD takes the form of Equation 10.2, with lifetime (LT) replacing the averaging time (AT):

$$\text{LADD}_{\text{pot}} = \frac{[\text{C} \cdot \text{IR} \cdot \text{B}]}{[\text{BW} \cdot \text{LT}]} \qquad (10.2)$$

Example Calculation 2: Determining the LADD

What is the LADD in Example Calculation 1 involving aldrin in lettuce? Assume that the maximum reasonable lifetime uptake of lettuce (99% person) is 14,000 kg.

Given

$$\text{C} = 4\ \text{mg/kg (aldrin in lettuce)}$$

$$\text{IR} = 14{,}000\ \text{kg}$$

$$\text{B} = 0.9$$

$$\text{BW} = 70\ \text{kg}$$

$$\text{LT} = 70\ \text{years} = 25{,}550\ \text{days}$$

where

$$\text{LADD} = \frac{\left[\overline{\text{C}} \cdot \overline{\text{IR}} \cdot \overline{\text{B}}\right]}{\left[\text{BW} \cdot \text{LT}\right]}$$

Then

$$\text{LADD} = \frac{[4] \cdot [14{,}000] \cdot [0.9]}{[70] \cdot [25{,}550]}$$

$$\text{LADD} = 0.028\ \text{mg/kg-day}$$

Although other measures of chronic dose may be more appropriate for predicting the hazard posed by specific chronic toxicants, such as an area-under-the-blood-concentration (AUC) curve or the peak target tissue concentration, the LADD is the most common dose metric used in carcinogen risk assessment (Figure 10.3).

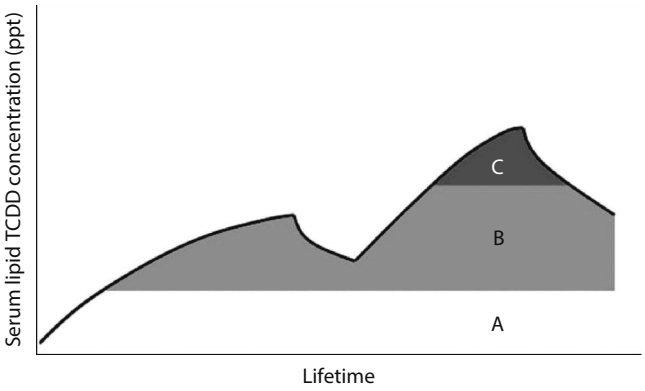

FIGURE 10.3 Theoretical concentration vs. time curve for TCDD illustrating one possible relationship between AUC and response. This figure illustrates the possible combination of AUC and thresholds for production of various responses: area A, no effect; area B, enzyme induction occurs; area C, significant increased cell proliferations. (From Aylward, L.L. et al., *Environ. Sci. Technol.*, 30, 3534, 1996. With permission.)

CONCEPTUAL APPROACHES TO EXPOSURE ASSESSMENT

Quantifying Exposure

Certain methods, such as environmental sensors and geographic information system (GIS), can be used to derive information about external environmental exposures and the personal activity patterns that influence the magnitude, frequency, duration, and pathways of exposure. Other methods, such as biologic sensors, toxicogenomics, and body burden assays, are more frequently being used to derive measurements of internal biologic exposure.[176–179] Although exposure assessments are conducted for a variety of reasons, the process of estimating exposure can be generally approached using one of the following three methods[43]:

1. Direct measurement: The exposure can be measured at the point of contact (the outer boundary of the body) while it is taking place, measuring the exposure concentration and time of contact and integrating them (point-of-contact measurement). An example is the measurement of the amount of contaminated soil on an exposed hand of someone digging a hole to plant a tree. The relevant exposure information would be contaminant concentration in soil (μg/g), surface area of the hand in contact with the soil (100 m^2), and time of exposure (2 h).
2. Exposure scenario: Sometimes one is concerned about an exposure that may or may not occur so a hypothetical exposure scenario is developed. In these assessments, specific data cannot actually be collected, but relevant information can be found. For example, if an incinerator were built, it would not be known today how much of each chemical in the airborne emissions would reach the various compartments in the environment (food, soil,

sediment, surface water), but one can describe what would likely occur (a scenario).

3. Biomonitoring: Sometimes historical exposure can be estimated based on the amount of chemical in the body or being eliminated in breath, urine, or feces. In recent years, doses have been reconstructed through internal indicators (biomarkers, body burden, excretion levels) for persistent organics and several metals. Among the most common historical examples are lead in blood, urinary mercury, volatile hydrocarbons in the breath, and dioxins in blood fat.

These three approaches to exposure quantification (or dose) are independent because each is based on different assumptions and/or data. The fact that they are independent measures is useful in verifying or validating the results of the various approaches. Each of these three has strengths and weaknesses; using them in combination can considerably strengthen the credibility of an exposure assessment.[12,24,180] For example, results of the exposure assessment would be validated if one could mathematically predict the absorbed dose per day of a chemical, estimate the resulting blood concentrations, and confirm these estimates by sampling the blood of the exposed population.[51]

Estimates Based on Direct Measurement

Point-of-contact or direct exposure assessment evaluates the exposure as it occurs. By measuring chemical concentrations at the interface between the person and the environment as a function of time, this yields an exposure profile. The best-known example of point-of-contact measurement is the radiation dosimeter. This small badge-like device measures radiation exposure as it occurs, and provides an integrated exposure estimate for the period of time over which the measurement has been taken.[43] The Total Exposure Assessment Methodology (TEAM) studies conducted by EPA also made use of direct measurements.[181] In the TEAM studies, a small pump with a collector and an absorbent was attached to a person's clothing to measure his or her exposure to airborne solvents or other pollutants as it occurred, just as has been done in industrial hygiene studies of the past 60 years.[182] In both of these examples, the measurements were collected at the interface between the person and the environment while exposure was occurring.

The area of exposure assessment known as agricultural hygiene has developed very sophisticated techniques for estimating the uptake (absorption) of chemicals during the mixing and application of pesticides.[183–185] Macroscale technologies such as laser-based and infrared radiation–based sensors are currently being used for assessing population exposures to sulfur and nitric oxides in industrial-stack effluents. Other microscale sensors, including personal dosimeters, are being used to monitor levels of carbon dioxide, carbon monoxide, volatile organic compounds (VOCs), pesticides, and PAHs in the workplace, household, and personal environment.[176] Recent efforts have focused on automated *lab-on-a-chip* sensing devices for detection environmental agents.[176,186]

A common limitation in exposure assessment is the lack of information about patterns of physical activity and behavior that affect the likelihood of exposure, the frequency and duration of exposure, and the uptake and distribution of environmental agents in the body.[176] GIS approaches have been used for developing individual metrics for exposure to pesticides, drinking water contaminants, and air pollutants, such as nitric oxide, sulfur dioxide, and particulates.[176,187–189] Only recently, researchers have used GIS to derive personal exposure estimates by linking information about personal activity and behavioral patterns with environmental data.[176,189–191] Interestingly, personal dosimetry devices are able to measure individual variables related to activity, such as motion, temperature, pressure, energy use, respiratory function, and heart rate.[176,192–199]

Providing that the measurement devices are accurate, the direct measurement method likely gives the most accurate exposure value for the period of time over which the measurement was taken. It is often expensive, however, to use these techniques to evaluate persons in the community, and measurement devices and techniques do not currently exist for all chemicals (at least at ambient concentrations).

Estimates Based on Exposure Scenarios

Using the exposure scenario approach, the assessor attempts to estimate or predict chemical concentrations in a medium or location, and link this information with the time that individuals or populations are in contact with the chemical. An exposure scenario is the set of assumptions describing how this contact takes place. This is, by far, the most common approach to exposure assessment, and such an approach is necessary when trying to predict the impact of events that may occur in the future, such as building a new manufacturing facility or introducing a new pesticide.[28,200–202]

The first step to building a scenario is to determine the concentration of the contaminated media. This is typically accomplished indirectly by measuring, modeling, or using existing data on concentrations in the media of concern, rather than at the point of contact (e.g., pesticide residues on food or metal emissions on residential soils). Often, we assume that the concentration in the bulk medium is the same as the concentration at the point of exposure. This can be a source of potential error and should be discussed in the uncertainty analysis. For example, over the past 20 years, most assessments of the hazard posed by contaminated soil was based on soil samples collected in the top 6 in. of soil, even though most persons were exposed routinely to the surface soil (usually the top 2.5 in.). Arguments can be made in either direction about the appropriateness of this assumption.

The next step in conducting an exposure scenario is to estimate the contact time, identify who is likely to be exposed, and then develop estimates of the exposure frequency and duration. Like chemical concentration characterization, this is usually done indirectly using demographic data, survey statistics, behavior observation, activity diaries, activity models or, in the absence of more substantive information, assumptions about behavior.[63,64,145]

Chemical concentration and population characterizations are ultimately combined in an exposure scenario. One of the major problems in evaluating dose equations is that the limiting assumptions used to derive them (e.g., steady-state assumptions) do not always hold true. Two approaches to this problem are available: (1) to evaluate the exposure or dose equation under conditions when the limiting assumptions do hold true, or (2) to build a dynamic model that accounts for both accumulation and degradation. The microenvironment method, which is typically used to evaluate air exposures, is an example of the first approach. This method evaluates segments of time and location when the assumption of constant concentration is approximately true, and then sums the time segments to determine the total exposure for the respiratory route, effectively removing some of the uncertainty.[203] In occupational hygiene, this is done by combining time-motion data with short-term air concentration data.[204] While estimates of exposure concentration and time of contact may be estimated in some situations, the concentration and time-of-contact estimates can be measured for each microenvironment. This avoids much of the error due to summing average values in cases where concentration and time of contact vary widely.

In the second approach, a computer model can efficiently predict dose if enough data are available.[39,48,205] When conducting modeling, there are various tools used to describe uncertainty caused by parameter variation, such as Monte Carlo analysis, and these may be necessary in some assessments.[88] Monte Carlo techniques should rarely be helpful when assessing individuals or small populations since actual *by person* data will often be available.

Estimating Exposure Using Biological Monitoring

Exposure can often be estimated after it has taken place. One important factor is whether the biological half-life of the chemical is sufficiently long to allow for accurate measurement. If a total dose is known or can be reconstructed, and information about intake and uptake rates is available, an average past exposure rate can be estimated.[146,206–210] Dose reconstruction relies on measuring biological fluids or other samples (blood, urine, hair, nails, or feces) after exposure and, if intake and uptake have already occurred, these measurements can be used to back-calculate dose.[146] However, data on body burden levels or biomarkers cannot be used directly unless a relationship can be established between these levels (or biomarker indications) and internal dose.

Biological monitoring can be used to evaluate the amount of a chemical in the body by measuring one or more parameters (Table 10.2). In general, if these measurements can be made and the biologic half-life is acceptable, then past

TABLE 10.2
Examples of Types of Measurements to Characterize Exposure-Related Media and Parameters

Type of Measurement (Sample)	Usual Attempts to Characterize (Whole)	Examples	Typical Information Needed to Characterize Exposure
1. Breath	Total internal dose for individuals or population (usually indicative of relatively recent exposures).	Measurement of VOCs, alcohol (usually limited to volatile compounds)	1. Relationship between individuals and population, exposure history (i.e., steady state or not) pharmacokinetics (chemical half-life), possible storage reservoirs within the body 2. Relationship between breath content and body burden
2. Blood	Total internal dose for individuals or population (may be indicative of either relatively recent exposures to fat-soluble organics or long-term body burden for metals).	Lead studies, pesticides, heavy metals (usually best for soluble compounds, although blood lipid analysis may reveal lipophilic compounds)	1. Same as earlier 2. Relationship between blood content and body burden
3. Adipose	Total internal dose for individuals or population (usually indicative of long-term averages for fat-soluble organics).	National Health and Aging Trends Study (NHATS), dioxin studies, PCBs (usually limited to lipophilic compounds)	1. Same as earlier 2. Relationship between adipose content and body burden
4. Nails and hair	Total internal dose for individuals or population (usually indicative of past exposure in weeks to months range; can sometimes be used to evaluate exposure patterns).	Heavy metal studies (usually limited to metals)	1. Same as earlier 2. Relationship between nails, hair content, and body burden
5. Urine	Total internal dose for individuals or population (usually indicative of elimination rates); time from exposure to appearance in urine may vary, depending on chemical.	Studies of tetrachloroethylene and trichloroethylene	1. Same as earlier 2. Relationship between urine content and body burden

exposure estimates can be reasonably accurate. Not all of these can be measured for every chemical and is dependent on several variables[43]:

- The concentration of the chemical itself in biological tissues or sera (blood, urine, breath, hair, adipose tissue, etc.)
- The concentration of the chemical's metabolite(s)
- The biological effect that occurs as a result of human exposure to the chemical (e.g., alkylated hemoglobin [Hb] or changes in enzyme induction)
- The amount of a chemical or its metabolites bound to target molecules

The results of biomonitoring can be used to estimate chemical uptake during a specific interval, if background levels do not mask the marker and the relationship between uptake and the selected marker is known.[211] The sampling time for biomarkers is often critical. Establishing a correlation between exposure and measurement of the marker, including pharmacokinetics, is necessary to properly back-calculate historical exposure.[43,177,178]

The strengths of this method are that it demonstrates exposure and absorption of the chemical that have actually taken place, and theoretically, it can give a good indication of past exposure. The drawbacks are as follows: It will not work for every chemical because of interferences or the reactive nature of the chemical, or because the biological half-life of the agent is too short; the approach has been applied to only a few chemicals; data relating internal dose to exposure are needed; and it may be expensive.

For those chemicals to which biological monitoring can be used to estimate past exposure, the information obtained can be invaluable for conducting retrospective exposure assessments that can be used in epidemiology studies. Some examples of chemicals for which past exposure can reliably be estimated include several metals as well as numerous large organic chemicals (e.g., DDT, chlordane, dioxin, polybrominated biphenyls [PBB], PCB).[146] A more detailed discussion about the current uses and emerging applications of biomonitoring for environmental exposure science is discussed later in this chapter.

INFORMATION UPON WHICH EXPOSURE ASSESSMENTS ARE BASED

Comprehensive exposure assessment of a complex scenario may require several hundred exposure factors to estimate the various chemical concentrations in one of several dozen different media. Among the most complex exposure assessments are those that address the risks posed by airborne emissions from combustors (Figure 10.4).[60,203,212] In order to estimate the concentration, numerous dispersion models, as well as fate and transport models, may be required. In addition, the assessor may need to search the literature to identify relevant studies from as many as 10 related fields of research. Sometimes, hundreds of published papers and government guidance documents need to be evaluated, used, and cited. In short, the exercise can be formidable, especially for assessments involving food chain contamination. Equally difficult and highly complex exposure assessments are those that attempt to estimate the uptake of fish by various members of the angling public.[79]

Obtaining Data on Intake and Uptake

The numerous editions of the *Exposure Factors Handbook* and *Child-Specific Exposure Factors Handbook* present statistical data on many of the factors used to assess exposure, including intake rates, and these provide citations for primary references.[63,64] Today, this series of publications represents the most comprehensive, single source of exposure

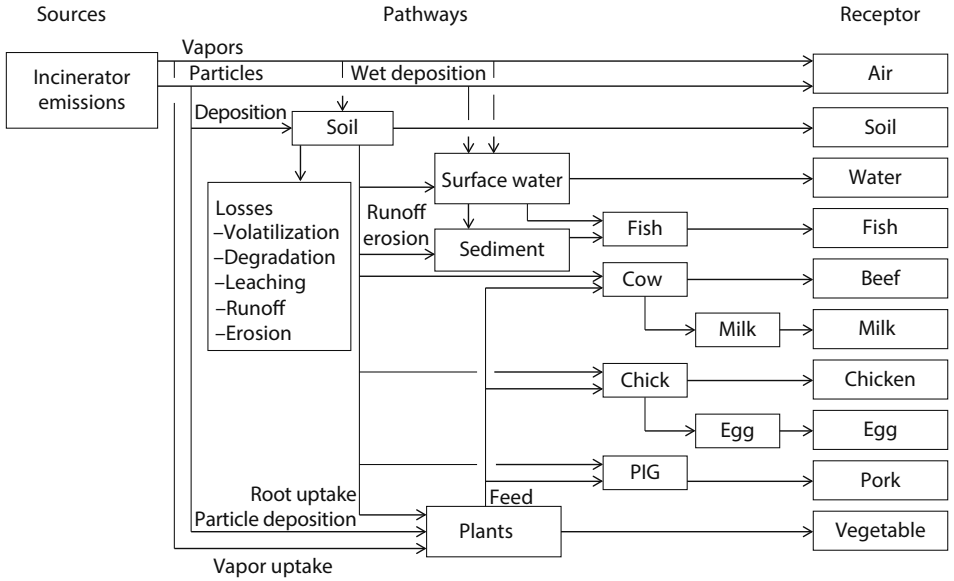

FIGURE 10.4 EPA's conceptual approach to dealing with direct and indirect exposure pathways as illustrated by assessments of incinerator emissions.

assessment information. Some of the many intake factors in the various volumes include the following:

- Drinking water consumption rates
- Breast milk ingestion rates for infants
- Consumption rates for homegrown fruits, vegetables, beef, and dairy products
- Consumption rates for recreationally caught fish and shellfish
- Rates of hand-to-mouth and object-to-mouth activities for children
- Incidental soil ingestion rates
- Pulmonary ventilation rates
- Surface area of various parts of the human body
- Body weight for various age groups
- Duration and frequency in different locations and microenvironments
- Duration and use of consumer product use
- Duration of lifetime

Table 10.3 presents examples of some of the standard or default exposure factors used in risk assessment.

TABLE 10.3
Selected Standard Default Assumptions Used in Exposure Assessment

Variable	Age Category	Mean or Central Tendency	95th Percentile
Drinking Water (mL/day)	1 to <2 years	271	837
	2 to <3 years	317	877
	3 to <6 years	327	959
	6 to <11 years	414	1316
	≥21 years	1043	2958
Soil (ingestion) (mg/day)	1 to <6 years	50	
	6 to 21 years	50	
	Adult	20	
Total Food (g/day)	1 to <2 years	1039	1756
	2 to <3 years	1024	1649
	3 to <6 years	1066	1746
	6 to <11 years	1118	1825
	21 to <40 years	1100	2110
Inhalation Rate (m^3/day)	1 to <2 years	8	12.8
	2 to <3 years	8.9	13.7
	3 to <6 years	10.1	13.8
	6 to <11 years	12	16.6
	11 to <16 years	15.2	21.9
	16 to <21 years	16.3	24.6
	21 to <31 years	15.7	21.3
	31 to <41 years	16	21.4
	41 to <51 years	16	21.2
	51 to <61 years	15.7	21.3
	61 to <71 years	14.2	18.1
	71 to <81 years	12.9	16.6
	≥81 years	12.2	15.7
Body Weight (kg)	1 to <2 years	11.4	14
	3 to <6 years	18.6	26.2
	11 to <16 years	56.8	88.8
	Adult	80	
Lifespan (years)	Male	75	
	Female	80	
Exposed Skin Area (%)	Adult gardening cold months	8	33
	Adult gardening warm months	33	69
Showering (min/day)	1 to <2 years	20	
	2 to <3 years	22	44
	3 to <6 years	17	34
	6 to <11 years	18	41
	≥18 years	17	
Residence Time (years)		13	46

The *Exposure Factors Handbook* is updated routinely to include new research data on previously discussed factors. It also provides default parameter values, which can be used when site-specific data are not available. Obviously, general default values should not be used in place of known, valid data that are more relevant to the assessment being conducted. The *Exposure Factors Handbook*, though substantial, may not contain all available information on exposure factors or relevant studies, so a supplemental literature search should be conducted to ensure that pertinent literature has been identified. As will be discussed later, if a probabilistic or Monte Carlo assessment is to be conducted, the documents titled *Risk Assessment Guidance for Probabilistic Assessment* (2001) and *Options for Development of Parametric Probability Distributions for Exposure Factors* (2000) should be consulted.[88,89]

Concentration Measurements in Environmental Media

Sometimes, the published data are inadequate to conduct a proper site-specific assessment. In these cases, concentration data can be gathered by conducting a new field study, or by evaluating data from past field studies and using them to estimate concentrations. Media measurements taken close to the point of contact are preferable to measurements far removed geographically or temporally. As the distance from the point of contact increases, the certainty of the data at the point of contact usually decreases, and the obligation for the assessor to show relevance of the data to the assessment at hand becomes greater. For example, an outdoor air measurement, no matter how close it is taken to the point of contact, cannot by itself adequately characterize indoor exposure.[213]

Concentrations often vary considerably from place to place, seasonally, and over time due to changing emission and use patterns.[47,53,214] This needs to be considered not only when designing studies to collect new data but also when evaluating the applicability of existing measurements as estimates of exposure concentrations in a new assessment. It is of particular concern when the measurement data will be used to extrapolate to long time periods, such as lifetimes. Transport and dispersion models are frequently used to help answer these questions.[205]

The exposure assessor is likely to encounter several different types of measurements. One type often used to understand concentration trends is outdoor fixed-location monitoring. This measurement is used by EPA and other groups to provide a record of pollutant concentration at one place over time. Nationwide air and water monitoring programs have been established so that baseline values in these environmental media can be documented. Although it is not practical to set up a national monitoring network to gather data for a particular exposure assessment, data from existing networks can be evaluated for relevance to an exposure assessment. These data are often far removed from the point of contact. Adapting data from previous studies usually presents specific challenges.

Indoor air contaminant concentrations can vary as much or more than those in outdoor air.[17,215–219] Consequently, indoor exposure is best represented by measurements taken at the point of contact. However, because pollutants, such as carbon monoxide, can exhibit substantial penetration to the indoor environment, indoor exposure estimates should consider potential outdoor, as well as indoor, sources of the contaminant(s) under evaluation.[22,43,220–222]

Contaminant concentrations in food and drinking water measurements can also be measured. General characterization of these media, such as market basket studies (where representative diets are characterized), shelf studies (where foodstuffs are taken from store shelves and analyzed), or drinking water quality surveys, is usually far removed from the point of contact for an individual but may be useful in evaluating exposure concentrations for a large population. Measurements of tap water or foodstuffs in a home and how they are used are closer to the point of contact. In evaluating the relevance of data from previous studies, variation in the distribution systems must be considered, as well as the space-time proximity.[43]

Consumer or industrial product analysis is sometimes done in order to characterize the chemical concentrations in products. Product formulations can change substantially over time, similar products do not necessarily have similar formulations, and regional differences in product formulation can also occur. These should be considered when determining the relevance of extant data and when setting up sampling plans to gather new data.[43]

Another type of concentration measurement is the microenvironmental measurement. Rather than using measurements to characterize the entire medium, this approach defines specific zones in which the concentration in the medium of interest is thought to be relatively homogeneous and then characterizes the concentration in that zone.[24,223] Typical microenvironments include the home or parts of the home, office, automobile, or other indoor settings. Microenvironments can also be divided into time segments (e.g., kitchen during the day, kitchen during the night). This approach can produce measurements that are closely linked with the point of contact, both in location and time, especially when new data are generated for a particular exposure assessment. The more specific the microenvironment, however, the greater the burden on the exposure assessor to establish that the measurements are representative of the population of interest.

The concentration measurement that provides the closest link to the actual point of contact is personal monitoring. In virtually all cases, if available, this information should be the basis of exposure assessments of individuals. An obvious exception is the work environment where lapel sampling is conducted while the person is wearing a respirator; thus, in this case personal sampling would not reflect genuine exposure.

Models and Their Role

Often the most critical assessment element is estimating pollutant concentrations at exposure points. This is usually

carried out by combining field data and modeling results. In the absence of field data, this process often relies on the results of mathematical models of aerial dispersion, such as Industrial Source Complex Long-Term (ISCLT), or of water movement, such as Modular Three-Dimensional Finite-Difference Groundwater Flow Model (MODFLOW).[224–228] EPA's Science Advisory Board and others have recommended that modeling ideally should be linked with monitoring data in regulatory assessments, although this is not always possible.

A modeling strategy has several aspects, including setting objectives, model selection, obtaining and installing the code, calibrating and running the computer model, and validation and verification. Many of these aspects are analogous to the quality assurance and quality control measures applied to measurements.

Regardless of whether models are extensively used in an assessment or whether a formal modeling strategy is documented in the exposure assessment plan, when computer simulation models, such as fate and transport models, and exposure models are used in exposure assessments, the assessor must be aware of the performance characteristics of the model and state how the exposure assessment requirements are satisfied by the model.[205]

The site must be characterized if models are to be used to simulate pollutant behavior at a specific site. Site characterization for any modeling study includes examining all data on the site, such as source characterization, dimensions and topography of the site, location of receptor populations, meteorology, soils, geohydrology, and ranges and distributions of chemical concentrations. For exposure models that simulate both chemical concentration and time of exposure (through behavior patterns), data on these two parameters must be evaluated.[24,28,229] Criteria are provided by EPA for selecting surface water models and groundwater models, respectively; the reader is referred to these documents for details.[230,231] Similar selection criteria exist for air dispersion models.[232]

A primary consideration in selecting a model is whether to perform a screening study or a detailed evaluation. A screening study makes a preliminary evaluation of a site or a general comparison between several sites. It may be generic to a type of site (i.e., an industrial segment or a climatic region) or may pertain to a specific site for which sufficient data are not available to properly characterize the site. Screening studies can help direct data collection at the site by, for example, providing an indication of the level of detection and quantification that would be required and the distances and directions from a point of release where chemical concentrations might be expected to be highest. An example of a screening-level modeling effort would be to estimate the amount of lead deposited by an incinerator onto local crops using a basic air dispersion model, without considering local geographical or weather conditions. The next level of complexity would consider the presence of mountains, their proximity to the stack, the local weather patterns, and the number of atmospheric inversions per year. A higher level of analysis could incorporate yet other, more subtle factors.

The value of the screening-level analysis is that it is simple to perform and may indicate that no significant contamination exists. Screening-level models are frequently used to get a first approximation of the concentrations that may be present. Often these models use very conservative assumptions; that is, they tend to overpredict concentrations or exposures. If the results of the conservative screening procedure predict concentrations or exposures at less than a predetermined no-concern level, then more detailed analysis is probably not necessary. If the screening estimates are above that level, refinement of the assumptions or a more sophisticated model is necessary to generate a more realistic estimate.[43]

Screening-level models also help the user conceptualize the physical system, identify important processes, and locate available data. The assumptions used in the preliminary analysis should represent conservative conditions, such that the predicted results overestimate potential conditions, limiting false negatives. If the limited field measurements or screening analyses indicate that a contamination problem may exist, then a detailed modeling study may be useful.

By contrast, the purpose of the detailed evaluation is to use the best data available to make the best estimate of spatial and temporal chemical distributions of a specific site. Detailed studies typically require higher-quality data and more sophisticated models.

Accounting for Background Concentrations

Background exposure to xenobiotics, especially environmentally persistent ones, can occur due to natural or anthropogenic sources.[233] In most exposure assessments, background soil concentrations are the focus of attention, but the same issue can be relevant when evaluating sediments, ambient air, groundwater, and vegetation (food stuffs). At some sites, it is important that these so-called *background* concentrations be accounted for because removing the quantity of toxicant due to humans may, in fact, not appreciably change the concentrations or be sufficient to reduce the risk to acceptable levels. For example, naturally occurring concentrations of lead, arsenic, and cadmium, in some locations, may be higher than cleanup levels established by various regulatory agencies.[232,234] The exposure assessor should try to determine local background concentrations by gathering data from nearby locations clearly unaffected by the site under investigation, or by referring to published works that have assessed this issue.[235]

Description of Background Levels

When assessing soils, background levels can be viewed in at least four different ways[224]:

- *Pristine* levels—Some would like to equate background levels with those associated with the *pristine* state, that is, soils or landscapes unaffected by human activity. This rather idealistic situation probably no longer exists; even in Antarctica, mercury

and dioxin concentrations can be detected in some media. Toxic elements mainly associated with the solid phase of some natural material (such as soil dust, plant or volcanic ash, vegetable matter) are relatively mobile in a global sense. For example, Nriagu (1979) has suggested that about 40 million tons of heavy metals have been dispersed atmospherically over the many centuries of human activity.[236]

Increases in pollutant metal concentrations have been measured up to 60 km from smelters, and automotive lead (fine particles) has been measured in soils and rainfall up to about 50 km downwind from major cities. Soil contamination up to 50–100 m from highways by automotive lead (coarser particles) is an example of short-range transport and contrasts with transport of toxic metals on a continental or global scale (e.g., contamination of the Greenland ice sheets from the northern United States, mercury in the Florida Everglades due to aerial releases in South America, the snows of the New Zealand Alps by soil dust from inland Australia).[224]

- *Normal* levels—The question could be asked—are soils contaminated at farms in the higher rainfall areas of the Appalachians, which used to receive automotive exhaust particulates from the New York metropolitan area 200 km away? These soils are not pristine, but the chemical concentrations are perfectly safe for growing food, raising farm animals, and residential living. Soils from such areas would have a range of what is often called *normal* background values. To most exposure assessors, this mosaic of normal soils, which is only affected by the minor pollution of everyday activities associated with modern rural and urban life, should be the basis for defining background values. Statistically, this range of normal background values would constitute a single lognormally distributed population. Obviously, one needs to exclude the outliers or *hot* spots due to a geochemical anomaly, or localized pollution arising from either industrial emissions, disposal of waste products, or intensive (excessive) use of farm chemicals.[224]
- Historically polluted regions—Local community and regulatory policies often affect what are defined as background levels. A community with highly developed environmental consciousness may insist on very low, possibly unreasonable, reference values. Some densely populated areas with historically derived pollution, perhaps from former mining activities, may sustain apparently healthy populations that pragmatically must accept higher *background*-level values. The cities of Philadelphia, Baltimore, and New York, and parts of Japan, for example, may fit in this category.
- Geochemical variation—Background levels of some potentially toxic elements may vary among geographical regions because of differences in soil type. The resulting concentrations are often called naturally occurring levels. An important factor is the composition of rocks and sediments that weather from soils. Some extreme examples are high concentrations of nickel, cobalt, and chromium in igneous rocks such as basalts that cover extensive areas in western Victoria and Tasmania, Australia, and high concentrations of boron in soils on marine sediments in the Riverina, Mallee, and Wimmera districts of South Australia and Victoria, Eyre Peninsula, and parts of western Australia.

Some regulatory agencies have provided written guidance describing how to select soil or sediment cleanup values that account for background chemical concentrations. These have varied significantly, but within the past 5 years, there appears to be some convergence regarding the definition of background, how to measure it, and how it should affect exposure assessment calculations.

ESTIMATING UPTAKE VIA THE SKIN

When attempting to predict chemical risks in the environmental or occupational setting, the dermal exposure route should nearly always be assessed. In most evaluations of hazardous waste sites and ambient air or water contaminants, this is not a major route of exposure. Although the uptake of chemicals via the skin has generally been overlooked in most workplace exposure assessments, it probably represents a substantial portion of the exposure for many occupations. Even though gloves are more frequently used than in years past and training has increased on the possible hazards of dermal exposure, there is still ample evidence to indicate that, in order to conduct a complete exposure assessment, this route deserves attention.[91,92,130,237–240]

In addition to the risks associated with systemic toxicity due to uptake via the skin, it is sometimes necessary to evaluate the allergic contact dermatitis (ACD) hazard. In recent years, techniques have been developed to quantitatively predict the likelihood of elicitation and induction of ACD.[241,242] Some regulatory agencies are concerned with ACD and have developed cleanup standards based on this health endpoint. More recently in the arena of product development, significant improvements have been included in dermal sensitization risk assessments for products such as fragrance ingredients.[243,244] For evaluations of contact sensitization as a critical health endpoint, there is now considerable support for using quantity per unit area of skin (above a minimum application area) rather than other dose metrics such as concentration applied to the skin.[245–251] This dose metric concept has been incorporated in the quantitative risk assessment approach for the global fragrance industry in assessing the risks of dermal sensitizers in fragrance ingredients.[243]

In the workplace, a worker's skin frequently comes into contact with solvents or chemicals mixed in water (aqueous materials). In most environmental settings where persons can be exposed to contaminated soil, dust, or water, dermal uptake must be assessed. Fortunately, a good deal of research

has been conducted to understand the rate at which chemicals pass through the skin. Percutaneous absorption of neat chemicals (e.g., the pure liquid) was often studied in humans until the late 1970s.[129,252–258] Because of the potential toxicity of many chemicals and improved laboratory techniques, in vivo human studies have been largely supplanted by experiments with laboratory animals, in vitro studies, or athymic rodents grafted with human skin.[259] Historical research has shown that, in general, chemical penetration of the human skin is similar to that of a pig or monkey, and much slower than that of the rat and rabbit.[260] Thus, for many chemicals, there is some level of confidence that the rate of dermal uptake of a chemical by humans can be inferred from animal data.

Starting in the 1980s, in vitro studies using human skin began to be conducted on a more routine basis. In these studies, a piece of excised skin is attached to a diffusion apparatus with a top chamber to hold the applied chemical and a temperature-controlled bottom chamber containing saline or other fluids (plus a sampling port to withdraw fractions for analysis).[261] Although human forearm skin is optimal, it is difficult to obtain, so abdominal or breast skin is commonly used. In general, a properly conducted in vitro test can be a reasonably good predictor of the absorption rate in vivo.[262,263] However, due to the fragile nature of the technique, these studies must be carefully interpreted.[264] Often, depending on the conditions of the test, the results are not applicable to humans.

Aside from neat liquids and exposure to contaminated water, dermal exposures can also occur through contact with dust or dirt on surfaces and by way of contact with soil- or dust-bound contaminants.[213] Surface-to-skin transfer of contaminants is a complex process. For example, a pesticide can be transferred to skin during contact with any contaminated exposure medium. Once on the skin, pesticide residues and contaminated particles can be transferred back to the contaminated surface during subsequent contact, loss by dislodgement or washing, or transferred into the body by percutaneous absorption or hand-to-mouth activity.[265] Few studies have directly estimated soil loading on human skin, or measured dermal contact of contaminated equipment by workers.[266–275] However, recent efforts using a fluorescent tracer as a surrogate for pesticide residues and house dust particles have proved informative in understanding the parameters that affect residue transfer from surface to skin, skin to other objects, and skin to mouth.[265,276] In these studies, controlled transfer experiments were conducted by varying contact parameters with each trial. The mass of a tracer transferred was measured and the contact surface area estimated using video imaging techniques. Parameters evaluated included surface type, surface loading, contact motion, pressure, duration, and skin condition. Results have shown that surface loading and skin condition are among the important parameters for characterizing residue transfers.[265]

The available studies probably provide sufficient data to generate point estimates of soil adherence and, perhaps, can provide a reasonable probability density function (PDF) for most persons exposed to contaminated soils. The degree of representativeness of the data to the general population is difficult to assess.[277] Recently, a couple of studies measured the adherence of soil to multiple skin surfaces (hands, forearms, lower legs, faces, and feet) under ambient and recreational conditions.[268,270] Dermal loading on the hands was found to vary over five orders of magnitude and to be dependent on the type of activity. Differences between pre- and postactivity adherence demonstrated the episodic nature of dermal contact with soil. However, because of the activity-dependent nature of soil exposure, data from these studies must be interpreted for their relevance to the type of activity, frequency, duration, and otherwise site-specific nature of exposure. The various studies involving contaminated soil are informative for providing an estimate of exposure; however, they are probably a couple orders of magnitude greater than what might be expected in a chemical plant. Nonetheless, this work is a *starting point* for bracketing potential exposure to dusts in the workplace.

Recently, there has been a reasonable level of research investigating exposure to house dust. The basis for this concern has been increasing evidence that controlling exposure to house dust, especially in homes located near sites with considerable surface soil contamination, is more important for reducing the health hazard than remediating the soil.[213,278–282] Numerous papers in recent years have shown that in-house exposure to toxics is much greater than that encountered due to ambient (so-called environmental) contamination.[22,217,283,284] In household dust studies, the source of the dust sample can provide a historical, as well as recent collection of potential exposures from dust inside the home. Undisturbed surfaces (e.g., top of a refrigerator, attic dust) can be indicative of materials deposited over a long period of time, whereas frequently cleaned surfaces (e.g., kitchen countertops) will be indicative of the most recent deposits.[285]

Along these lines, and of particular interest to those who study indoor exposure, is the recent work to develop standardized approaches for collecting wipe samples and estimating the amount of dust loading on the palm of the hand.[286,287] Using fluorescein-tagged Arizona test dust as a possible surrogate for house dust, particle transfers to both wet and dry skin were quantified for contact events with stainless steel, vinyl, and carpeted surfaces that had been preloaded with the taggest test dust.[276] With these tests, researchers found that (1) only about one-third of the projected hand surface typically came into contact with the smooth test surfaces; (2) the fraction of particles transferred to the skin decreased as the surface roughness increased, with carpeting transfer coefficients averaging only one-tenth those of stainless steel; (3) hand dampness significantly increased the particle mass transfer; and (4) consecutive presses decreased the particle transfer by a factor of three as the skin surface became loaded, requiring about 100 presses to reach an equilibrium transfer rate.[276] Although dermal absorption of toxicants in house dust will almost always pose a relatively low dermal uptake hazard, the uptake of toxicants due to hand-to-mouth contact can be substantial.[213]

Quantitative Description of Dermal Absorption

For the purposes of risk assessment, percutaneous absorption is defined as transport of externally applied chemicals through cutaneous structures and the extracellular medium to the bloodstream.[24,130] In many settings, such as the agricultural workers, platers, mechanics, and others, dermal uptake is the primary route of exposure. The simplest way to describe the rate of skin absorption is to apply Fick's first law of diffusion at steady state[288,289]:

$$J = \frac{dQ}{dt} = \frac{D \cdot k\nabla C}{e} \approx K_p \cdot C \qquad (10.3)$$

where

- J (=dQ/dt) is the chemical flux or rate of chemical absorbed (mg/cm^2-h)
- D is the diffusivity in the stratum corneum (cm^2/h)
- k is the stratum corneum/vehicle partition coefficient of the chemical (unitless)
- ∇C is the concentration gradient (mg/cm^3)
- e is the thickness of the stratum corneum (cm)
- K_p is the permeability coefficient (cm/h)
- C is the applied chemical concentration (mg/cm^3)

The concentration gradient is equal to the difference between the concentrations above and below the stratum corneum. Because the concentration below is small compared to the concentration above, ∇C can be approximated as equal to the applied chemical concentration. From Equation 10.3, it can be seen that the rate of absorption is directly proportional to the applied concentration. The diffusivity represents the rate of migration of the chemical through the stratum corneum. Since the stratum corneum has a nonnegligible thickness, there is a period of transient diffusion (lag time), during which the transfer rate rises to reach a steady state. In these studies, the steady state is maintained indefinitely, provided the system remains constant. Depending on the type of chemical, the lag time can range from minutes to days.[91] From an exposure assessment standpoint, if the exposure duration is shorter than the lag time, it is unlikely that there will be any significant systemic absorption.[288,290]

The partition coefficient (K_p) is one of the key parameters that influences the degree to which a chemical penetrates the skin.[288,290–293] Fatty chemicals tend to accumulate in the stratum corneum. Conversely, the stratum corneum is an effective barrier for hydrophilic substances, which tend to have low skin absorption rates. Because stratum corneum/vehicle partition coefficients are difficult to measure, the three parameters (D, k, and e) are combined to give an overall permeability coefficient (K_p). It is noteworthy that Equation 10.3 only approximates most in vivo exposure situations in which true steady-state conditions are rarely attained. In spite of its limitations, this equation has yielded reasonable estimations of the actual absorption rates of chemicals for many situations (Table 10.4).

Dermal absorption is often reported in experimental studies as fractional absorption; however, this is generally not

TABLE 10.4
Human Cutaneous Permeability Coefficient Values for Some Industrial Chemicals in Aqueous Medium

	MW	K_{ow}	Observed	Calculated[a]
Organic chemicals				
2-Amino-4-nitrophenol	154.13	21.38	0.00066	0.019
4-Amino-2-nitrophenol	154.13	9.12	0.0028	0.0081
Aniline	93.12	7.94	0.041[b]	0.091
Benzene	78.11	134.90	0.11	0.39
p-Bromophenol	173.02	389.05	0.036	0.25
Butane-2,3-diol	90.12	0.12	<0.00005	0.0009
n-Butanol	74.12	7.59	0.0025	0.024
2-Butanone	72.10	1.94	0.0045	0.007
Carbon disulfide	76.14	100.00	0.54[b]	0.3
Chlorocresol	142.58	1258.93	0.055	1.31
S-Chlorophenol	128.56	147.91	0.033	0.19
p-Chlorophenol	128.56	257.04	0.036	0.34
Chloroxylenol	156.61	1621.81	0.059	1.35
m-Cresol	108.13	100.00	0.015	0.18
o-Cresol	108.13	100.00	0.016	0.18
p-Cresol	108.13	85.11	0.018	0.15
Decanol	158.28	37,153.52	0.08	30.11
2,4-Dichlorophenol	163.01	1995.26	0.06	1.5
1,4-Dioxane	88.10	0.38	0.00043	0.0016
Ethanol	46.07	0.49	0.0008	0.0036
2-Ethoxyethanol	90.12	0.29	0.0003	0.0013
Ethylbenzene	106.16	1412.54	1.215[b]	2.65
Ethylether	74.12	6.76	0.016	0.022
p-Ethylphenol	122.17	549.54	0.035	0.79
Heptanol	116.20	257.04	0.038	0.41
Hexanol	102.17	107.15	0.028	0.21
Methanol	32.04	0.17	0.0016	0.0026
Methyl hydroxybenzoate	152.15	91.20	0.0091	0.082
β-Naphthol	144.16	691.83	0.028	0.7
3-Nitrophenol	139.11	100.00	0.0056	0.11
4-Nitrophenol	139.11	81.28	0.0056	0.09
Nitrosodiethanolamine	134.13	0.13	0.0000055	0.0005
Nonanol	144.26	2951.21	0.06	2.99
Octanol	130.22	933.25	0.061	1.19
Pentanol	88.15	36.31	0.006	0.091
Phenol	94.11	32.36	0.0082	0.074
Propanol	60.09	2.00	0.0017	0.0088
Resorcinol	110.11	6.03	0.00024	0.011
Styrene	104.14	891.25	0.635[b]	1.72
Thymol	150.21	1995.26	0.053	1.84
Toluene	92.13	489.78	1.01	1.15
2,4,6-Trichlorophenol	197.46	2344.23	0.059	1.02
3,4-Xylenol	122.16	169.82	0.036	0.25
Inorganic chemicals				
Cobalt chloride	129.84		0.0004	
Lead acetate	325.29		0.0000042[b]	
Mercuric chloride	271.50		0.00093	
Nickel chloride	129.60		0.001	

TABLE 10.4 (continued)
Human Cutaneous Permeability Coefficient Values for Some Industrial Chemicals in Aqueous Medium

	MW	K_{ow}	Observed	Calculated[a]
Nickel sulfate	154.75		<0.000009	
Silver nitrate	169.87		<0.00035[b]	
Sodium chromate	161.97		0.0021[b]	

[a] Permeability coefficients calculated using equation presented in Leung and Paustenbach.[142]

[b] All the observed permeability coefficients were obtained by using in vitro techniques except those denoted with superscript b, which were determined in vivo.

independent of loading conditions. Some studies have suggested that relative dermal absorption of certain chemicals decreases with increasing dermal loading (i.e., amount of substance per unit area of skin).[294,295] Therefore, a calculated internal dose when extrapolating from experimental conditions to an exposure setting of interest, without accounting for dermal loading, may underestimate or overestimate actual risks.[240] This is particularly important when evaluating dermal toxicity studies because if the area of dosing is so small that no effect is observed (even if it is saturated), the apparent chemical toxicity can be artificially reduced by loading more chemical onto the same target area.[296] In a quantitative assessment of dermal absorption experiments, the relationship between dermal loading and relative absorption was compared across 138 different studies and 98 substances.[240] In the majority of cases (63%), an inverse relationship between relative dermal absorption and dermal loading was observed with relative absorption varying by more than factor of a 100 within a dermal loading range that is relevant for occupational scenarios. A likely explanation offered for this trend is the saturation of the absorption capacity of the skin, particularly for the slow rate of absorption of poorly water-soluble substances through the water film of the skin or its saturation at higher dermal loading ranges.[240]

Pharmacokinetic Models for Estimating the Uptake of Chemicals in Aqueous Solution

Pharmacokinetic models predict the uptake of a chemical through the skin based on fundamental thermodynamics. Several different models have been proposed. For example, a four-compartment pharmacokinetic model was developed in 1982.[293] This model, which uses first-order rate constants, describes chemical movement through the compartments representing the various skin structures. It has been used successfully to predict the chemical disposition in the skin and plasma as a function of their physicochemical properties, and when an input rate constant to the skin surface is added to the model, it can be used to assess vehicle effects. A similar model that treats the barrier membrane as a series of spaces filled with immiscible liquids has also been developed[291]; its advantage is that it allows examination of non-steady-state conditions in which Fick's law does not apply.

Under an infinite-dose situation in which the amount of a chemical lost by penetration is too small to alter the applied concentration (e.g., where one is swimming), the rate of absorption is essentially linear once steady state has been reached. In the finite-dose system, however, the chemical solution is applied as a thin film and the concentration decreases as penetration proceeds (e.g., a splash). All other model parameters being the same, penetration is reduced under finite-dose conditions. This is because the chemical concentration is continuously reduced over time, resulting in a decrease in the gradient across the stratum corneum. These modeling results indicate that the mechanism by which fluxes are affected must be considered when extrapolating to non-steady-state conditions.

Although classic pharmacokinetic modeling like that described by Guy et al. (1982) can provide a good mathematical description of the disposition of chemicals,[290] it does not depict exactly the biological processes in the intact animal. Fortunately, pharmacokinetic methods based on physiological principles are feasible alternatives for analysis of in vivo skin penetration studies. PBPK models realistically describe the disposition of the chemical in the intact animal in terms of rates of blood flow, permeability of membranes, and partitioning of chemicals in tissues.[291,297] Characterizing dermal absorption in terms of actual anatomical, physiological, and biochemical parameters facilitates extrapolations to the real species of interest, humans.

PBPK models were developed to describe the percutaneous absorption of volatile organic contaminants in dilute aqueous solutions.[298] The exposure scenario modeled was either hand or full-body immersion into a vessel of solute-contaminated water. Modeling results suggested that chemical uptake in aqueous solutions is most markedly influenced by epidermal blood flow rates, followed by epidermal thickness and lipid content of the stratum corneum. In general, thicker and fattier skin provides a better barrier to dermal penetration of chemicals. These are precisely the principles under which barrier creams offer their protection for increasing the effective thickness and lipophilicity of the skin. This model also predicted that the dose of some volatile organic chemicals in water absorbed through the skin during a 20 min bath may be equivalent to the amount inhaled.[298]

Among the most complex and best validated of the various models for dermal uptake of liquids is that developed by McDougal et al.[100,299–312] These authors have successfully predicted dermal uptake rates of humans for more than a dozen chemicals based on animal data. One advantage of dermal PBPK models over traditional in vivo methods is their ability to accurately describe nonlinear biochemical and physical processes. For example, describing skin penetration based on blood concentrations or excretion rates as *percent absorbed* assumes that all processes have a simple linear relationship with the exposure concentration. This is often not the case. The kinetics become nonlinear when the absorption, distribution, metabolism, or elimination of a chemical is saturated

at high exposure concentrations. This model and other models developed since then are generally reliable methods for estimating dermal uptake for certain classes of chemicals.

Factors Used to Estimate Dermal Uptake

Many factors need to be quantitatively accounted for in order to estimate the likely systemic uptake of a chemical that comes into contact with the skin, either as a liquid or when present in soil or dust.[7,130,313]

Dermal Bioavailability

The typical media of concern for assessing cutaneous contact to environmental chemicals, in contrast to occupational exposure, are house dust, soil, fly ash, and sediment. In the workplace, dermal uptake is due to direct contact with liquids and contact with surfaces contaminated with dirt or liquids. A number of parameters can influence the degree of cutaneous bioavailability of chemicals in complex matrices. These may include aging (time following contamination), soil type (e.g., silt, clay, and sand), type and concentration of co-contaminants (e.g., oil and other organics), and the concentration of the chemical contaminant in the media.[125] The bioavailability of a chemical in soil will usually be affected by its physicochemical properties. Chemicals with high molecular weights tend to bind to soil/dust and be less water soluble, while smaller molecules will frequently be water soluble, less tightly bound, and relatively bioavailable.[101,121] The cutaneous bioavailability of perhaps 20–30 chemicals in soils has been determined in animals.[51,106,115,118,120–122,125,314–317] These studies show that different media and different chemicals can yield dramatically different cutaneous bioavailabilities. The results of these studies, for example, produce values of bioavailability for different chemicals that range from 0.001% to 3% for chemicals in soil. Dioxin and lead are considered classic case studies since there are many human populations that have been exposed to these chemicals in dust or soil yet the blood levels can be very high or very low depending on the bioavailability.

Skin Surface Area

There is an abundance of information about the surface area of different portions of the body. One simple approach is to use the *rule of nines* for estimating the surface area of the human body[318]: the head and neck are 9%, upper limbs are each 9%, lower limbs are each 18%, and the front or back of the trunk is 18%.[69] EPA has estimated an exposed surface area (arms, hands, legs, and feet) of 2900 cm^2 for children 0–2 years old; 3400 cm^2 for children 2–6 years old; and 2940 cm^2 for adults (an adult is assumed to wear pants, an open-neck short-sleeve shirt, shoes, and no hat or gloves).[43] When assessing chemical exposure in the ambient environment, most of the necessary surface area information can be found in EPA's *Exposure Factors Handbook* and *Child-Specific Exposure Factors Handbook*.[63,64] Table 10.5 presents the skin surface areas commonly used when conducting exposure assessments.[63,64,318] A distribution plot of skin area versus body weight has been developed.[319]

TABLE 10.5
Representative Mean Surface Areas of the Human Body (Adult Male 21 years and Older)

Body Portion	Area (cm^2)
Whole body	20,600
Head	1,360
Trunk (includes neck)	8,270
Upper extremities	3,930
Arms	3,140
Upper arms	1,720
Forearms	1,480
Hands	1,070
Lower extremities	8,020
Legs	6,820
Thighs	4,120
Lower legs	2,680
Feet	1,370

Soil Loading on the Skin

A key factor to consider when estimating dermal uptake is the soil-to-skin adherence rate. Values of 0.5–0.6 and 0.2–2.8 mg/cm^2 have been reported for adults and children, respectively, although it is important to carefully consider site-specific information when conducting these assessments.[53,266,272,320] Work by Finley et al. (1994), Kissel et al. (1996), and Holmes et al. (1996) have built on prior studies to show that dermal loading can vary significantly among different activities and different people.[267,268,270] The *Exposure Factors Handbook* and *Child-Specific Exposure Factors Handbook* give considerable attention to this topic.[63,64] One approach to improving dermal uptake calculations is to use area-weighted adherence factors. When assessing the risk to large populations, Monte Carlo techniques are often useful in characterizing distributions of exposure.[37]

Interpreting Wipe Samples

In some workplaces, wipe sampling has been conducted historically to assess the degree of surface contamination. Hospitals were among the first occupational settings, as long ago as 1940, to rely on this method to determine microbial levels in operating rooms. In pharmaceutical manufacturing, wipe sampling has been used as an indicator of hygienic conditions since the 1960s. The health physics profession has utilized wipe samples extensively as an indicator of the need for better housekeeping and decontamination; this group performed most of the early work in quantifying the relationship of wipe sample concentrations to dermal and oral uptakes.

Over the years, few papers have discussed on how to collect and interpret wipe samples.[270,321–325] When the primary effect of a chemical is skin discoloration, ACD, or chloracne, wipe sampling was nearly always the preferred approach for assessing the acceptability of the workplace (rather than relying on air samples). Beginning in the 1980s, a substantial number of wipe samples were collected in office buildings contaminated

with dioxins and furans after electrical transformer fires to estimate the potential human exposure.[326] This approach was recently used to assess exposures to dust generated from the September 11, 2001, terrorist attacks,[285,327–329] as well as household dust in homes surrounding dioxin-contaminated sites in Midland, Michigan.[279–282] Some of the most interesting investigations of surface dust are the use of chemical tracers or signatures to identify sources of contaminated surface dust, such as in the case to evaluate buildings impacted from the World Trade Center dust or to characterize chemical air dispersion and personal exposures in urban environments.[330,331]

Although wipe sampling data have generally been used as an indicator of cleanliness,[321] these data can also be used to estimate systemic uptake of a contaminant if the degree of skin contact with the contaminated surfaces and the bioavailability of the chemical (in that medium) are known. While historical wipe sampling methods were rather imprecise, they were useful for obtaining a rough estimate of the possible exposure, which could be refined later by other means, such as biological monitoring. Based on estimates of the contribution of dust to overall intake of persistent chemicals (e.g., polybrominated diphenyl ethers, polychlorinated biphenyls, and polyaromatic hydrocarbons), it has been suggested that residential dust measurements could be useful in augmenting or replacing questionnaire-based assessments of human exposure in epidemiologic studies.[332]

If one knows that wipe sampling results are representative of what comes into contact with the hands (i.e., actually able to be absorbed), then the procedures for converting wipe sample data to estimates of systemic uptake are straightforward.[333] For example, if one knows the number of times a surface (e.g., valve handle, instrument controller, or drum) is touched, the surface area of the hand touching these items (usually the palm), and the percutaneous chemical absorption rate, then the uptake can be estimated using wipe sample information. The best wipe sampling data were those collected in a reliable and consistent manner, with a focus on the mass per unit area. Hand wash or hand wipe sampling is often more representative of the true hazard than surface wipe samples.[334] The bulk of what is known about how to best evaluate this matter comes from the relatively robust studies of agricultural workers that have been conducted over the past 20 years by hygienists, who specialized in this area (e.g., Kissel, Poppendorf, Spears, Knarr, Knack, and Fenske).

Until recently, no standardized approaches existed for conducting wipe sampling. Although surface sampling has been utilized as a metric to evaluate human health under a variety of settings,[335–338] a number of factors can influence the amount of substance collected and measured. These factors include differences in (1) methodology (wet/dry wipe, collection media, microvacuum), (2) human factors (operator differences in sampling technique), (3) surface characteristics (physical shape, porosity, roughness), and (4) residue characteristics (particle size, dust loading).[339,340] Differences in the use of wetting agent (acetone, methylene chloride, water, saline, isopropanol, and ethanol) and sampling media (paper, cotton, and synthetic fibers) produced drastically different results. In some procedures, especially those that used methylene chloride (in which the paint was concurrently stripped by the solvent), the chemical in the paint matrix was assumed to be bioavailable (a completely unreasonable assumption). Much of the previous work, which measured the amount of chemical released following aggressive scrubbing of the contaminated surfaces with detergent or solvent, did not reflect a realistic exposure scenario. Thus, there has been a need for standard techniques that attempt to mimic the conditions in which a hand comes into contact with a contaminated surface.[325] Some of the techniques have been developed by hygienists involved in agricultural exposure assessment.[184,341–343]

In an attempt to fill this need, fairly sophisticated work to standardize these procedures has been conducted by researchers at Rutgers University. A couple of their wipe sampling procedures and devices have been patented.[286] They have also developed a dry contact sampling device that offers promise for understanding the hazard from surface dusts.[287] The implications from recent wipe sampling research are that (1) a minimum number of samples are needed to have statistical confidence, (2) the pressure applied to the cloth during sample collection should be standardized, (3) neat solvent should not be used as a collection medium, (4) the size of the sample area needs to be sufficient to collect enough contaminant for quantification, and (5) the technique should be validated by using glove analyses. More research will be devoted to this topic in the coming years as it has become quite clear that *in-home* versus outdoor exposures are generally much better predictors of risk since such a large fraction of the day is spent indoors (and because rugs, upholstery, drapes, toys, and other objects tend to accumulate toxicants over time).[344–346]

Estimating the Dermal Uptake of Chemicals in Soil

One of the most frequently occurring exposure scenarios involving environmental exposures is that of contaminated soil.[53] Unfortunately, dermal uptake of chemicals found in soil has infrequently been evaluated experimentally.[91,347] Dermal absorption of chemicals from soil is influenced by a number of chemical and physical factors, including layering (extent and pattern of soil particles across the skin surface), particle size distribution, sorption capacity, soil–chemical contact time (*aging*), and contaminated soil–skin contact time (exposure).[347] There are several key aspects of chemical absorption from soil particles that are important to consider.[347] First, as chemicals have water solubility limits, the capacity of chemicals to sorb to soil particles is finite. Thus, it is important to keep in mind that experimental data representing absorption of the free chemical correspond to the neat chemical and not absorption from soil. Second, sorption of chemicals by soil particles is not instantaneous and depending on the properties of the chemical, some compounds require long periods (e.g., months) to reach equilibrium. Third, compounds move from soil to skin through two primary mechanisms: contact transfer and diffusion. The transfer of contaminants as a result of soil particles directly contacting the skin may occur quickly, whereas diffusion may occur slowly but may

ultimately account for a larger percentage of total absorption.[347] In effort to encourage standardization of methods on soil dermal absorption research, Spalt and colleagues (2009) offered five primary criteria to improve interpretation of investigations on this topic. These criteria include reporting of results in the context of possible layering effects, soil saturation, particle size selection, soil–agent contact time, and assurance of continuous soil–skin contact.[347]

A model to estimate the amount of a chemical in soil that crosses the stratum corneum into the underlying tissue layer has been developed.[101] In order to differentiate this absorptive process from bioavailability, which also includes transport into blood, McKone (1990) refers to the percentage of available chemical as an uptake fraction. The approach is based on the fugacity concept, which measures the tendency of a chemical to move from one phase to another. Because the skin has a fat content of about 10% and soil has an organic carbon content of 1%–4%, a chemical in soil placed on the skin will move from the soil to the underlying adipose layers of the skin. However, this transfer depends on the period of time between deposition on the skin and removal by evaporative processes. The mass-transfer coefficients of the soil-to-skin layer and the soil-to-air layer define the rate at which these competing processes occur.

Results of this model suggest that the chemical uptake fraction in soil varies with the exposure duration, soil deposition rate, and physical properties of the chemical, and is particularly sensitive to the values of the K_{ow}, as well as the mass or depth of soil deposited on the skin. When the amount of soil on the skin is low (<1 mg/cm^2), a high uptake fraction, approaching unity in some cases, is predicted. With higher soil loading (20 mg/cm^2), an uptake of only 0.5% is predicted. Because of the diverse variations of the uptake fraction with soil loading, results obtained from experiments with a single soil loading should be applied with caution to human soil-exposure scenarios.

The dermal uptake of chemicals in soil is a complex process, but its behavior is predictable if the controlling factors are accounted for and quantified.[91,101] In situations involving a relatively thin layer of a chemical on the skin, a few generalizations can be made. First, for chemicals with a high K_{ow} and a low air/water partition coefficient, it is reasonable to assume 100% uptake in 12 h. Second, for chemicals with an air/water partition coefficient greater than 0.01, the uptake fraction is unlikely to exceed 40% in 12 h. Third, for chemicals with an air/water partition coefficient greater than 0.1, one can expect less than 3% uptake in 12 h. In most occupational settings, contaminated soil will rarely be in contact with the skin for greater than 4 h before it is washed off. Consequently, this should be accounted for when attempting to predict systemic uptake.

Dermal Uptake of Contaminants in Soil

In order to estimate chemical uptake, one needs to know the percutaneous absorption rate, the exposed skin area, the chemical concentration, and the exposure duration.[348] One scenario would be a thin film of chemical on the skin. For this finite-dose scenario, the following equation is useful:

$$\text{Uptake (mg)} = (C)(A)(x)(f)(t) \quad (10.4)$$

where
- C is the concentration of the chemical (mg/cm^2)
- A is the skin surface area (cm^2)
- x is the thickness of the film layer (cm)
- f is the absorption rate (%/h)
- t is the duration of exposure (h)

Another scenario would be an excess amount of a chemical on the skin (i.e., infinite dose). In this case, the thickness of the chemical layer is not calculated and steady-state kinetics are assumed. For a chemical in an aqueous or gaseous media,

$$\text{Uptake (mg)} = (C)(A)(K_p)(t)(d) \quad (10.5)$$

where d is the distribution factor. For a neat liquid chemical,

$$\text{Uptake (mg)} = (A)(J)(t) \quad (10.6)$$

where
- K_p is the permeability coefficient (cm/h)
- J is the flux of chemical (mg/cm^2/h)

EPA has suggested using the following equation for estimating percutaneous absorption of chemicals in soil[313]:

$$\text{Uptake (mg)} = (C)(A)(r)(B) \quad (10.7)$$

where
- C is the concentration of the chemical in soil (mg/g)
- A is the skin surface area (cm^2)
- r is the soil-to-skin adherence rate (g/cm^2)
- B is the cutaneous bioavailability (unitless)

Example Calculation 3: Skin Uptake of a Chemical in Soil

A person gardens with soil contaminated on average with 250 ng dioxin per gram of soil (250 ppb). Assuming that the person's hands and lower arms are in contact with the soil, the soil loading is equal to 0.2 mg/cm^2, and the cutaneous bioavailability of dioxin in soil is 1%,[125] what is the plausible uptake of dioxin by this person (using Equation 10.7)? Assume that the person washes his or her hands every 4 h and the exposed area of skin is 1800 cm^2.

$$\text{Uptake (ng)} = (C)(A)(r)(B)$$

where

$$C = 250 \text{ ng/g}$$

$$A = 1800 \text{ cm}^2$$

$$r = 0.2 \text{ mg/cm}^2$$

$$B = 0.01$$

By substitution,

$$\text{Uptake} = \left(\frac{250\text{ ng TCDD}}{1\text{ g soil}}\right)\left(\frac{0.2\text{ mg soil}}{\text{cm}^2\text{ skin}}\right)\left(\frac{1\text{ g}}{10^3\text{ mg}}\right) \times \left(1800\text{ cm}^2\text{ skin}\right)\left(0.01\right) = 0.9\text{ ng TCDD}$$

Note: A preferred method for performing this calculation, if data are available, is to use a flux rate (ng/cm^2-h) for the chemical. Assume that the rate is 500 ng/cm^2-h:

$$\text{Uptake (ng)} = (C)(J)(A)(t)$$

where

$$J = 500\text{ ng/cm}^2\text{-h}$$

$$t = 4\text{ h}$$

By substitution,

$$\text{Uptake} = \left(\frac{250\text{ ng TCDD}}{1\text{ g soil}}\right)\left(\frac{1\text{ g}}{10^9\text{ ng}}\right)\left(1800\text{ cm}^2\text{ skin}\right) \times \left(4\text{ h}\right)\left(500\text{ ng/cm}^2\text{ h}\right) = 0.9\text{ ng TCDD}$$

Uptake of Chemicals in an Aqueous Matrix

Published estimates of dermal uptake of chemicals in water have generally focused on evaluating workplace or environmental exposure. A number of different scenarios have been evaluated.[76,349–353] For example, the possible uptake of a chemical present in water, the amount of chlordane absorbed through the skin by a man swimming for 4 h in water containing 1 ppb chloroform has been estimated.[350,351] Likewise, the amount of chloroform absorbed by a boy swimming for 3 h in water has been calculated.[343] However, about 10 years ago, it was recognized that in the indoor environment, dermal exposure to volatile chemicals present in drinking water will rarely represent the vast majority of the hazard. Specifically, it was found that inhalation exposure due to the release of vapors from liquids to which people were in close contact was a greater contributor to the dose.[354] For example, comparisons have been made of the chloroform concentration in exhaled breath after a shower to that after an inhalation-only exposure.[60,350,351,355–361]

Example Calculation 4: Skin Uptake of a Chemical from Water

A person has filled his swimming pool with shallow well water contaminated with 0.002 mg/mL (2 ppb) toluene. What is the plausible dermal uptake of toluene while swimming in the contaminated water for half an hour? Assume that 18,000 cm^2 of skin is exposed and the K_p is 1.01 cm/h. From Equation 10.5,

$$\text{Uptake} = (C)(A)(K_p)(t)(d)$$

where

$$C = 0.002\text{ mg/mL}$$

$$A = 18{,}000\text{ cm}^2$$

$$K_p = 1.01\text{ cm/h}$$

$$t = 0.5\text{ h}$$

d = distribution factor (1 mL of water covers 1 cm^3).

By substitution,

$$\text{Uptake} = (0.002\text{ mg/mL})\ (18{,}000\text{ cm}^2)\ (1.01\text{ cm/h}) \times (0.5\text{ h})\ (1\text{ mL water/1 cm}^3)$$

$$\text{Uptake} = 18\text{ mg}$$

Percutaneous Absorption of Liquid Solvents

While the percutaneous absorption of chemical solutes generally proceeds by simple diffusion, the skin uptake of neat chemical liquids is not necessarily exclusively governed by Fick's law. Consequently, the uptake of neat liquid through the skin needs to be estimated using direct in vivo skin contact techniques. Table 10.6 presents the percutaneous absorption rates of some neat industrial liquid solvents that have been determined in human volunteer studies.

TABLE 10.6
Absorption Rates of Some Neat Industrial Liquid Chemicals in Human Skin In Vivo

Chemical	Absorption Rate (mg/cm^2-h)
Aniline	0.2–0.7
Benzene	0.24–0.4
2-Butoxyethanol	0.05–0.68
2-(2-Butoxyethoxy)ethanol	0.035
Carbon disulfide	9.7
Dimethylformamide	9.4
Ethylbenzene	22–23
2-Ethoxyethanol	0.796
2-(2-Ethyoxyethoxy)ethanol	0.125
Methanol	11.5
2-Methoxyethanol	2.82
2-(2-Methoxyethoxy)ethanol	0.206
Methyl butyl ketone	0.25–0.48
Nitrobenzene	2
Styrene	9–15
Toluene	14–23
Xylene (mixed)	4.5–9.6
m-Xylene	0.12–0.15

Example Calculation 5: Skin Uptake of a Neat Liquid Chemical

Due to carelessness or a leak, the inside of a glove becomes contaminated with 2-methoxyethanol. How much can be absorbed if a worker wears the contaminated glove on one hand for half an hour? Assume that the surface area of exposed skin is 360 cm^2 and the flux rate is 2.82 mg/cm^2-h. From Equation 10.6,

$$\text{Uptake} = (A)(J)(t)$$

where

$$A = 360\ cm^2$$

$$J = 2.82\ mg/cm^2\text{-h}$$

$$t = 0.5\ h$$

By substitution,

$$\text{Uptake} = (360\ cm^2)\ (2.82\ mg/cm^2\text{-h})\ (0.5\ h)$$

$$\text{Uptake} = 508\ mg$$

In order to understand the relative hazard from skin exposure versus inhalation exposure, the quantity of 2-methoxyethanol absorbed by the same worker via inhalation for 8 h (10 m^3 of air inhaled), assuming a threshold limit value (TLV®) of 16 mg/cm^3, can be estimated and compared to the dose due to inhalation exposure. Assume an 80% inhalation uptake efficiency.

$$\text{Inhalation uptake} = (16\ mg/m^3)(10\ m^3)(0.8)$$

$$= 128\ mg$$

Thus, the uptake of 2-methoxyethanol following 30 min of skin exposure of a single hand can be as much as four times that from inhalation for 8 h at the TLV concentration, a presumably safe level of exposure. From this example, it is clear that the cutaneous route of entry can, in some situations, significantly contribute to the total absorbed dose, especially in the occupational setting.

Percutaneous Absorption of Chemicals in the Vapor Phase

Until the 1990s, it was generally assumed that the plausible dose resulting from vapors absorbed through the skin was too low to pose a hazard. Only a few studies have examined this issue.[255,352,362] A few clinical reports have encouraged some limited in vivo research to evaluate the absorption of several chemicals in the gaseous phase through the human skin (Table 10.7). A chamber system to measure the whole-body percutaneous absorption of chemical vapors in rats has been described by McDougal et al.,[100] and this approach has produced some interesting results.[304] In this system, chemical flux across the skin is determined from the chemical concentration in blood during exposure by using a PBPK model. In most cases, vapor absorption through the skin amounts to less than 10% of the total dose received from a combined skin and inhalation exposure. While there is good agreement between the rat and human in the relative ranking of the permeability coefficients among the chemicals studied, for an individual chemical the rat skin appears to be two to four times more permeable than the human skin. These observations are consistent with previously reported data.[125,129,257,262,264]

TABLE 10.7
Percutaneous Absorption Rates for Chemical Vapors In Vivo

Chemical	Skin Update in Combined Exposure (%)[a]	Permeability Coefficient K_p (cm/h)	
		Rat	Human
Styrene	9.4	1.75	0.35–1.42
m-Xylene	3.9	0.72	0.24–0.26
Toluene	3.7	0.72	0.18
Perchloroethylene	3.5	0.67	0.17
Benzene	0.8	0.15	0.08
Halothane	0.2	0.05	
Hexane	0.1	0.03	
Isoflurane	0.1	0.03	
Methylene chloride		0.28	
Dibromomethane		1.32	
Bromochloromethane		0.79	
Phenol			15.74–17.59
Nitrobenzene			11.1
1,1,1-Trichloroethane			0.01

[a] In combined exposure, rats are simultaneously absorbing chemical vapors by inhalation and by whole-body absorption through the skin.

It is generally not necessary to account for the contribution from percutaneous uptake of vapors when the occupational exposure limit (OEL) is used as a guideline for acceptable exposure, because uptake via this route is usually inherent in the data; that is, the studies of animals or humans from which data were collected were usually exposed via inhalation (whole body) so dermal uptake of the vapor occurred. While good work practices and the law require that persons not be placed in life-threatening atmospheres, sometimes, in emergency situations, airline (supplied air) respirators or self-contained breathing apparatus (SCBA) are worn in environments containing chemical concentrations 10- to 1000-fold greater than the TLV. In these cases, it is can be useful to account for vapor uptake through either exposed or covered skin.

Although nearly all data on vapor absorption involve bare skin, the role of clothing in preventing skin uptake has occasionally been evaluated. For example, a study of workers wearing denim clothing indicated no decreased uptake of phenol vapors[257] but found a 20% and 40% reduction in uptake of nitrobenzene and aniline vapor,[128,252] respectively. Although standard clothing may slightly decrease the amount

of a chemical transferred from air through the skin, it can be a significant source of continuous exposure if the clothing has been contaminated.

Example Calculation 6: Skin Uptake of a Chemical Vapor

Assume that a person needs to repair a leaking pump, so he enters a room wearing an airline respirator. Assume that the room contains 500 mg/m^3 nitrobenzene (100 times the current TLV) and it takes 30 min to repair the pump. How much nitrobenzene might be absorbed through the skin?

The head, neck, and upper limbs are assumed to be exposed (surface area = 4860 cm^2), and the rest of the body (surface area = 13,140 cm^2) is covered with clothing. Assume that the percutaneous K_p of nitrobenzene is 11.1 cm/h and that the clothing has reduced the skin uptake rate of vapors by about 20%.[128]

$$\text{Uptake} = (C)(A)(K_p)(t)$$

$$\begin{aligned}\text{Uptake through exposed skin} &= (500\ \text{mg/m}^3)(4860\ \text{cm}^2) \\ &\quad \times (11.1\ \text{cm/h})\ (0.5\ \text{h}) \\ &\quad \times (1\ \text{m}^3/10^6\ \text{cm}^3) \\ &= 13.5\ \text{mg}\end{aligned}$$

$$\begin{aligned}\text{Uptake through clothing} &= (500\ \text{mg/m}^3)(13{,}140\ \text{cm}^2) \\ &\quad \times (11.1\ \text{cm/h})(0.8)(0.5\ \text{h}) \\ &\quad \times (1\ \text{m}^3/10^6\ \text{cm}^3) \\ &= 29\ \text{mg}\end{aligned}$$

$$\text{Total uptake} = 13.5 + 29 = 42.5\ \text{mg}$$

From this example, it is clear that if one enters an environment containing a high concentration of an airborne contaminant, even if a supplied air respirator is worn, the degree of skin uptake of the vapor may be worthy of evaluation to ensure that the worker is protected. In this example, uptake following 1 day of inhalation exposure at the TLV (5 mg/m^3) results in 50 mg uptake [(10 m^3)(5 mg/m^3)]. These kinds of calculations sometimes have to be conducted in difficult work environments that are in a state of alert (e.g., submarines, chemical plants during emergency situations).

ESTIMATING INTAKE VIA INGESTION

If the appropriate information is available, estimating the intake of various chemicals due to ingestion is a relatively straightforward exercise. In general, one is concerned with the ingestion of the following media: drinking water, other liquids, food, soil, and house dust. Drinking water contamination may occur due to soil contamination from leaking underground storage tanks, landfills, or hazardous waste sites, as well as discharges from contaminated streams or water transport systems. Nearly all foods in Western society contain a number of intentional and unintentional chemicals, including pesticide residues, naturally occurring chemicals, and food additives that serve as preservatives or enhancers of taste or visual appeal. Soils are ingested as a result of eating incompletely washed vegetables, hand-to-mouth contact, and through direct ingestion by children. Soils are also ingested when particles too large to reach the lower respiratory tract are inhaled (and then are swallowed). House dust contaminated with a number of chemicals can be ingested due to contact with foods, toys, upholstery, carpet, and hand-to-mouth activities.[213,363]

Estimating Intake of Chemicals in Drinking Water

Estimating the magnitude of the potential dose of toxics from drinking water requires knowledge of the amount of water ingested, the chemical concentrations in the water, and the chemical bioavailability in the gastrointestinal tract. The amount of water ingested per day varies with each person and is usually related to the amount of physical activity. A good deal of literature has addressed the amount of water ingested by persons engaged in different kinds of activities. Numerous studies cited in EPA's *Exposure Factors Handbook* and *Child-Specific Exposure Factors Handbook* have generated data on drinking water intake rates.[63,64] Many of the studies have reported fluid intake rates for both total fluids and tap water. Total fluid intake is defined as consumption of all types of fluids including tap water, milk, soft drinks, alcoholic beverages, and water intrinsic to purchased foods. Total tap water is defined as water consumed directly from the tap as a beverage or used to prepare foods and beverages (i.e., coffee, tea, frozen juices, soups). Data for both consumption categories are presented in numerous publications. Table 10.8 presents typical information reported from these studies.[63,64,99]

All currently available studies on drinking water intake are based on short-term survey data. Although short-term data may be suitable for obtaining mean intake values that are representative of both short- and long-term consumption patterns, upper-percentile values may be different for short- and long-term data because there is generally more variability in short-term surveys. Most of the currently available drinking water surveys are based on recall, which may be a source of uncertainty in the estimated intake rates because of the subjective nature of this type of survey technique.[63,64,99]

TABLE 10.8
Summary of Tap-Water Intake by Age

	Intake (mL/day)		Intake (mL/kg-day)	
Age Group	Mean	10th–90th Percentiles	Mean	10th–90th Percentiles
Infants (<1year)	302	0–649	43.5	0–100
Children (1–10 years)	736	286–1,294	35.5	12.5–64.4
Teens (11–19 years)	965	353–1,701	18.2	6.5–32.3
Adults (20–64 years)	1,366	559–2,268	19.9	8.0–33.7
Adults (65+ years)	1,459	751–2,287	21.8	10.9–34.7
All ages	1,193	423–2,092	22.6	8.2–39.8

However, recently researchers have looked for ways to better characterize exposures that persons might have experienced as a result of ingestion of contaminated drinking water. These efforts have included the use of GIS software to integrate fate and transport modeling of chemicals in groundwater with the geocoded study population, develop input data for the simulation model, and assign individual exposures to chemicals of interest by linking results of the model to the census block group of residence.[189,364]

To estimate the intake of toxics via direct ingestion of drinking water, the calculation is straightforward:

$$\text{Intake} = (V)\,(C)\,(B)$$

where

V is the volume of water (L/day)
C is the concentration of chemical in water (μg/L)
B is the bioavailability (unitless)

One of the more interesting observations of the past 20 years is that ingestion of contaminated drinking water is sometimes not the primary route of exposure to the toxicant in drinking water. Uptake of volatile chemicals via inhalation can be nearly as great in some homes as ingestion, which is the result of the presence of these chemicals in air due to showering, off-gases from the dishwasher, and other opportunities for volatilization of the chemical.[350,351,354]

Importance of Soil Ingestion When Estimating Human Exposure

Between 1980 and 1995, predicted risks associated with the ingestion of contaminated soil were the primary drivers for remediating many (if not most) hazardous waste sites. As discussed by Paustenbach et al.,[53] there was no better example than the site in Times Beach, Missouri. Billions of dollars can be needed to clean up these kinds of sites to levels that would not pose a significant risk if children actually ate significant quantities of contaminated soil. Because of the expense of remediation, a good deal of research has been conducted over the past 20 years to attempt to quantitatively understand this route of exposure.

Clearly, the ingestion of soil and house dust is a potential source of human exposure to toxicants.[17,279–281,365,366] The potential for contaminant exposure via this source is greater for children because they are more likely to ingest greater quantities of soil than adults. Inadvertent soil ingestion among children may occur through the mouthing of objects or hands, whereas soil ingestion in adults can be driven by occupational contact with soil.[64,367] Mouthing behavior is considered to be a normal phase of childhood development. Adults may also ingest soil or dust particles that adhere to food, cigarettes, or their hands. Deliberate soil ingestion is defined as pica and is considered to be relatively uncommon.[64] Because normal, inadvertent soil ingestion is more prevalent and data for individuals with pica behavior are limited, the focus of most exposure assessments is on normal levels of soil ingestion that occur as a result of mouthing or unintentional hand-to-mouth activity.[43,53,71,368]

Mouthing activities by children, which are generally accepted as normal and commonplace (e.g., Barltrop [1966] estimated that almost 80% of all children at age 1 year exhibited mouthing tendencies),[369] are potential exposure routes to trace amounts of soil and/or dust adhering to fingers, hands, and objects placed in the mouth. The available data indicate that soil exposure occurs through several indirect routes:

1. Soil contributes to house dust (e.g., by local dust deposition and mud and dirt carried in by shoes and pets).
2. House dust (fine particles) adheres to objects and to children's hands.
3. Children ingest dust particles when sucking and mouthing objects and fingers.

Obviously in some situations, exposure may be direct (a child playing outdoors may eat dirt directly). In other situations, oral exposure may occur via contamination of domestic water supplies or contamination of vegetable produce grown onsite. However, the content and concentration of dusts in the indoor environment, which may represent the most important source of indirect exposure to soil, need to be better understood.[213,224] Considerable efforts have been made through large-scale studies, such as the National Children's Study, NHEXAS, and MNCPES, to better characterize indoor exposures to chemicals adhered to household dust, particularly pesticide, lead, and allergen exposures to chlidren.[162,370–372] Several researchers have concluded that the hazard posed by the majority of household pesticides is better detected by dust sampling than by air sampling.[363,366,373–375] Studies designed to characterize children's exposure to pesticides indicate that the largest number of pesticides and the highest concentrations are found in household dust compared to air, soil, and food.[376,377] Other recent research efforts have involved better characterization of personal activities using questionnaire, videotaping, wireless-coupled infrared technologies, and personal digital assistant (PDA) techniques to quantify dermal and ingestion exposures of microactivities, such as hand-to-mouth and object-to-mouth activities.[165,167,170,378,379] Overall, recent studies have shown that children's behavioral and activity patterns that may lead to ingestion through hand-to-mouth activities are better characterized through video assessment rather than questionnaires.[380–382] This has been further confirmed through biomonitoring that there is a direct relationship between chemical body burden (e.g., blood lead levels), mouthing behaviors, and contact with surfaces.[382]

Many studies have been conducted to estimate the amount of soil ingested by children. Most of the early studies attempted to quantify the amount of soil ingested by measuring the amount of dirt present on children's hands and making generalizations based on behavior. Soil intake studies have been conducted using a methodology that measures

trace elements in feces. These measurements are used to estimate the amount of soil ingested over a specified period of time.

Studies of Soil Ingestion

In light of the importance of soil ingestion for estimating human exposure to contaminated soil, several literature surveys have been undertaken to identify the typical amount of soil consumed by children and adults.[53,63,64,99,224,368] Research evaluating lead uptake by children from ingestion of contaminated soil, paint chips, dust, and plaster provides the best source of information. Walter et al.[383] estimated that a normal child typically ingests very small quantities of dust or dirt between the ages of 0 and 2 years, the largest quantities between 2 and 7 years, and nearly insignificant amounts thereafter. In the classic text by Cooper,[384] it was noted that the desire of children to eat dirt or place inedible objects in their mouths becomes established in the second year of life and disappears more or less spontaneously by the age of 4–5 years. A study by Charney et al.[385] also indicated that mouthing tends to begin at about 18 months and continues through 72 months, depending on several factors such as nutritional and economic status, as well as race. Work by Sayre et al.[386] indicated that ages 2–6 years are the important years, but that "intensive mouthing diminishes after 2 to 3 years of age."

An important distinction that is often blurred is the difference between the ingestion of very small quantities of dirt due to mouthing tendencies and the disease known as pica. Children who intentionally eat large quantities of dirt, plaster, or paint chips (1–10 g/day), and consequently are at greater risk of developing health problems, can be said to suffer from the disease known as pica. This disease is known as geophasia if the craving is for dirt alone. Geophasia, rather than pica, is generally of greatest concern in areas with contaminated soil.

Duggan and Williams[387] have summarized the literature on the amount of lead ingested through dust and dirt. In their opinion, a quantity of 50 μg of lead was the best estimate for daily ingestion of dust by children. Assuming, on the high side, an average lead concentration of 1000 ppm would indicate a soil and dust ingestion rate of 50 mg/day. Lepow et al.[388] estimated an ingestion rate equal to 100–250 mg/day (specifically, 10 mg ingested 10–25 times a day). Barltrop[369] and Barltrop et al.[389] also estimated that the potential uptake of soils and dusts by a toddler is about 100 mg/day. In a Dutch study, the amount of lead on hands ranged from 4 to 12 ng. By assuming maximum lead concentrations of 500 ng/g (concentrations were typically lower) and complete ingestion of the contents adsorbed to a child's hand on 10 separate occasions, the amount of ingested dirt would equal 240 mg. Thus, in order to eat 10,000 mg of soil per day, the rate suggested by the Centers for Disease Control and Prevention, children would have to place their hands into their mouths 410 times a day, a rate that seems improbable.[390,391]

A report by the National Research Council[390] addressing the hazards of lead suggested a soil/dust ingestion rate of 40 mg/day. Day et al.[392] measured the amount of dirt transferred from children's hands (age range from 1 to 3 years) to a sticky sweet and estimated that a daily intake of 2–20 sweets would lead to dirt intake of 10–1000 mg/day. Bryce-Smith[393] estimated 33 mg/day. In its document addressing lead in air, EPA assumed that children ate 50 mg/day of household dust, 40 mg/day of street dust, and 10 mg/day of dust derived from their parents' clothing (i.e., a total of 100 mg/day).

Kimbrough et al.[368] estimated the ingestions of soil at Times Beach, Missouri, based on unpublished observations about children's behavior and hand-to-mouth activity. A few years later, Kimbrough noted that their estimate of up to 10,000 mg/day was clearly not close to reality and her personal estimate would be nearer 50 mg/day.[224] LaGoy[394] based his soil ingestion estimates on a review of the literature, in particular using empirical data derived by Binder et al.[395] and Van Wijnen et al.[396] Similarly, Paustenbach based his estimates on a review of the literature,[229] including the mass-balance quantitative study conducted by Calabrese et al. in 1989.[397]

De Silva[398,399] adopted a different approach that may overcome some of the uncertainties inherent in the assumptions of the indirect studies mentioned in earlier text by applying a *slope factor* increase of 0.6 μg/dL in children's blood lead levels for each 1000 ppm increase in soil lead (this factor was developed by Barltrop et al.[389] following his work on blood lead levels in children from villages on old mining sites). De Silva then deduced that an increase of 0.6 μg/dL in blood indicates an extra oral intake of 3.75 μg lead/day, based on an EPA calculation that an increase of 1.0 μg lead/day in children's diets produces an increase of 0.16 in the blood lead level. With a soil lead value of 1000 ppm, 3.75 mg of soil would contain 3.75 μg of lead, suggesting that 3.75 mg/day (say 4 mg) of soil was ingested by the children. However, the slope factor used here may not be the most appropriate, since mining soil wastes typically have larger-sized particles, which tend to decrease lead bioavailability compared with soil contaminated by lead smelter activity and therefore reduce the slope factor.

A major step forward beyond estimating soil ingestion using indirect measurements was the attempt to study tracer elements found in soil with elements measured in the urine and feces of children. Several studies have been conducted thus far that have used this approach.[355,356,395,397–403] One early tracer study evaluated the amount of soil eaten by 24 hospitalized and nursery school children by analyzing the amount of aluminum, titanium, and acid-soluble residue in the feces of children aged 2–4 years.[396] They found an average of 105 mg/day of soil in the feces of nursery children, and 49 mg/day in hospitalized children. Even with the limited number of children in the study, the difference between the two groups was significant ($p < 0.01$). If the value for the hospitalized children is assumed to be the background level because these substances are taken in from nonsoil sources (e.g., diet and toothpastes), the estimated average amount of soil ingested by the nursery school children would be 56 mg/day. This value is

in the lower range of estimates in the literature and supports the use of 100 mg/day as a reasonable daily average uptake of soil by toddlers (ages 2–4 years or 1.5–3.5 years).

There have been two major studies completed by Calabrese et al.[355,356,397,400,401,404] In the first, they quantitatively evaluated six different tracer elements in the stools of 65 school children aged 2–4 years. They attempted to evaluate children from diverse socioeconomic backgrounds. This study was more definitive than prior investigations because they analyzed the children's diets, assayed for the presence of tracers in the diapers, assayed house dust and surrounding soil, and corrected for the pharmacokinetics of the tracer materials.

In the second study, soil ingestion estimates were obtained from a stratified, simple random sample of 64 children aged 1–4 years residing on a superfund site in Montana.[405] The study was conducted during the month of September for 7 consecutive days. Soil ingestion was estimated by each soil tracer via traditional methods as well as by an improved approach using five trace elements (Al, Si, Ti, Y, and Zr), called the best tracer method (BTM), which corrects for error due to misalignment of trace input and output, as well as error occurring from ingestion of tracers from nonfood, nonsoil sources, while being insensitive to the particle size of the soil/dust ingested. According to the BTM, the median soil ingestion was less than 1 mg/day while the upper 95% was 160 mg/day. No significant age- (1 year vs. 2 years vs. 3 years) or sex-related differences in soil ingestion were observed. These estimates are lower than the estimates observed in the first study, which was conducted in New England during September and October.

Based on the series of early papers by Calabrese et al.,[355,356,397–401] a few generalizations can be made. The first two studies were difficult to conduct and interpret. Only children from a single climate were studied, and it can be expected that rates vary with the amount of time spent indoors and outdoors. In addition, only a handful of children have been studied (less than 500), so it is not possible to characterize the percentage of children who might tend to ingest large quantities of soil or house dust. The relevant amount of soil or house dust ingested indoors versus outdoors is not known yet. In most cases, the contaminant concentrations in dust can be quite different when found in a carpet versus the yard.[213] This was demonstrated in a more recent study that involved evaluation of aggregate daily exposures, contributions of specific pathways of exposure, and temporal variation in exposure to chlorpyrifos from a collection of indoor air, carpet dust, exterior soil, and duplicate diet samples.[170] Chlorpyrifos concentrations in each medium and self-reported rates of time spent inside at home, time and frequency of contact with carpet, frequency of contact with soil, and weights of the duplicate diet samples were used to derive exposure to chlorpyrifos from each medium, as well as average daily aggregate exposure. While it was found that inhalation of indoor air and ingestion of solid food accounted for almost all (97.9%) exposure to chlorpyrifos on average, the authors did report significant differences in average chlorpyrifos concentrations in exterior soil and carpet dust.[170] Specifically, a chlorpyrifos concentration of 204 ng/kg was reported for exterior soil, whereas carpet dust showed a pesticide concentration of 2380 ng/kg.[170] Overall, although there is some degree of uncertainty in the results of the various studies, it appears that the best estimate of soil intake for most children resides in the area of 10–25 mg/day. It appears that perhaps 1%–5% of the children may ingest much larger amounts during certain days or weeks (e.g., 2000 mg/day), but these tendencies do not occur on a chronic basis.

The issue of how much soil and house dust children eat, as well as the percent of children who are engaged in these activities, remains an active area of research.[396–398,402–404,406,407] Work by Calabrese and Stanek[408] suggests that prior work yielded reasonable results for purposes of risk assessment. Most of the values discussed here are presented in Table 10.9. As discussed previously, another area of research impacting exposure assessments of contaminated soil, which has been and continues to be actively pursued, is the bioavailability of the contaminant in the soil matrix.[51,103,104,106,110,111,115,118–121,317,409,410]

TABLE 10.9
Recommended Values for Childhood and Adult Soil and Dust Ingestion Rates Used in Health Risk Assessments

		Soil or (Dust) Intake (mg/day)	
Author	**Age Group**	**CT**	**Upper Percentile**
U.S. EPA[63,64]	6 weeks to <1 year	30 (30)	
	1 to <6 years	50 (60)	
	3 to <6 years		200 (100)
	6 to <21 years	50 (60)	
	Adults	20 (30)	
Barltrop et al. [389]	2–6	100	
Lepow et al.[388]	2–6	100–250	
Day et al.[392]	2–6	10–1,000	
Kimbrough et al.[368]	0–9 months	0	
	9–18 months	1,000	
	1.5–3.5	10,000	
	3.5–5	1,000	
	5+	100	
Hawley[724]	0–2	Negligible	
	2–6	90	
	6–18	21	
	18–70	57	
La Goy[394]	1–6	500 (max)	
	1–6	100	
Calabrese et al.[397]	1–4	27–85	
		9–16	
Paustenbach[69]	2–4	25–50	
	Adults	2–5	
De Silva[399]	Children	~4	
Calabrese and Stanek[408]	Children	30–60	

What Is the Significance of Pica?

There appears to be some confusion in the literature over what constitutes *pica*. Pica can be defined as "the habitual ingestion of substances not normally regarded as edible," but some authors have included mouthing and sucking activities in their definitions.[411] Others appear to assume that all children with pica necessarily must be habitual soil eaters. In fact, pica behavior may be generalized to the ingestion of many different (nonfood) substances, or may be specific to one substance such as paper, soap, or earth. It is likely that repetitive pica behavior specifically for dirt, or habitual *geophagia*, rarely occurs in the general population in most industrialized countries.[412,413]

Pica should, therefore, be considered a *normal* temporary phenomenon in some children. In the general population, the prevalence of both mouthing and pica, and the range of articles ingested, has been shown to decrease with age.[369] In the 1-year-old age group, 78% of children mouthed objects and 35% ingested them; this behavior decreased at the age of 4 years, when 33% were mouthing and only 6% had pica. It is also relevant to note that in certain circumstances, pica for soil may be culturally determined (such as eating clay, high in silicon and aluminum, for its medical properties in the relief of stomach discomfort and diarrhea by some Aborigines; or the custom of eating earth during pregnancy in certain cultures).[224] For example, some women in the southern portions of the United States have a craving for and eat certain clays during pregnancy. Pica may be associated with physical disorders, including iron deficiency. However, it has been debated whether pica represents a cause or an effect of these deficiencies. Pica can also be associated with mental illness. It has also been reported that 25% of institutionalized mentally handicapped adults indulged in pica of one kind or another (including bizarre objects ranging from rags and string to rocks, insects, and feces).[412]

Calabrese and Stanek[408] have indicated that in their studies, they have observed great variability in soil ingestion by children. They have noted, for example, that some children are highly variable in their soil ingestion activities, displaying little propensity for soil ingestion on one day while ingesting copious amounts the next day. While there has not been any concerted focus on the soil pica child, the available data indicate that some children ingest over 50 g of soil on particular days. They note that while it is true that some children will ingest large amounts of soil, it is far from certain whether soil pica is a behavior that only a small subgroup displays over a limited number of years (e.g., 1–6) or whether most children, on occasion, display this behavior or some combination of both behavioral patterns. Clearly, additional work is needed to understand this topic.

Soil Ingestion by Adults

For most persons beyond the ages of 5–6 years, the daily uptake of dirt due to intentional ingestion is generally thought to be quite low. With the exception of some lower-income persons who eat clays due to tradition or mineral deficiency, adults will not usually intentionally ingest dirt or soil. However, there are two other important ways in which adults eat dirt—incidental hand-to-mouth contact and through dust on vegetables. It has been shown that most soil ingested from crops comes from leafy vegetables. Interestingly, investigations at nuclear weapons trials have shown that particles exceeding 45 μm are seldom retained on leaves. Furthermore, superficial contamination by smaller particles is readily lost from leaves, usually by mechanical processes or rain and certainly by washing.[414] As a result, unless the soil contaminant is absorbed into the plant, superficial contamination of plants by dirt will rarely present a health hazard.[53,415]

The estimated deposition rate of dust from ambient air in rural environments is about 0.012 μg/cm^2-day, assuming that rural dust contains about 300 μg/g of lead (the substance for which these data were obtained). EPA has estimated that even at relatively high air concentrations (0.45 mg/m^3 total dust), it is unlikely that surface deposition alone can account for more than 0.6–1.5 μg lettuce/g dust (2–5 μg/g lead) on the surface of lettuce during a 21-day growing period.[53] These data suggest that daily ingestion of dirt and dust by adults due to eating vegetables is unlikely to exceed about 0–5 mg/day even if all of the 137 g of leafy and root vegetables, sweet corn, and potatoes consumed by adult males each day were replaced by family garden products.

With respect to the second route—unwashed vegetables—only a very limited amount of work has been conducted. It has been suggested that the primary route of uptake will be through accidental ingestion of dirt on the hands, which may be of special concern to smokers who tend to have more frequent hand-to-mouth contact. It is true that before the importance of this route of entry was recognized, persons who worked in lead factories between 1890 and 1920 probably received a large portion of their body burden of lead due to poor hygiene; however, such conditions are now rare in the United States and most developed countries.

Some persons have evaluated the exposure experience of agricultural workers who apply or work with pesticide dusts. Because of the frequency and degree of pesticide exposure during its manufacture or application, these data do not appear to be appropriate surrogates for estimating soil uptake from the hands of persons who live on or near sites having contaminated soil. In addition, most of the published studies on pesticides involve liquids such as the organophosphates, rather than *soil-like* particles. Exposure studies of persons who apply granular pesticides might be more useful for defining upper-bound estimates of dermal exposure than estimates based on dusty workplaces.[326,416]

At least one study has been conducted to specifically address soil uptake by adults involved in remediating waste sites.[202,406,408,417] The results suggest that the amount of soil eaten by these workers is much less than the default value of 100 mg/day suggested by EPA in a number of guidance documents or risk assessments.

Estimating the Intake of Chemicals via Food

Without question, the information necessary to accurately estimate the ingestion of xenobiotics via foods is one of the most complex of all exposure calculations. The hundreds of different possible foods and dozens of different chemicals that can be present as a pesticide residue and background concentrations of various chemicals in soil make this a formidable task. The methodology for estimating uptake via ingestion must account for the quantity of food ingested each day, the concentration of contaminant in the ingested material, and the bioavailability of the contaminant in the media. Over the past 20 years, a significant amount of work has been directed at understanding these exposure factors.[63,64]

The approach to estimating uptake via foods was first applied in the late 1940s by the FDA and had not changed appreciably through 2000.[418] However, because of the passage of the FQPA of 1996, which significantly amended the U.S. laws that regulate pesticides (e.g., Federal Insecticide, Fungicide, and Rodenticide Act [FIFRA] and the Federal Food, Drug, and Cosmetic Act), the methodology for estimating uptake of chemicals from foods has changed dramatically.[419] Specifically, the FQPA established a stringent health-based standard (*a reasonable certainty of no harm*) for pesticide residues in foods to assure protection from unacceptable pesticide exposure and to strengthen health protections from pesticide risks for sensitive populations. In addition, the FQPA required the U.S. EPA to consider the cumulative effects on human health that may result from exposure to mixtures of pesticides.[419] In response, the U.S. EPA Office of Pesticide Programs (OPP), in consultation with the FIFRA scientific advisory panel, has developed guidelines for the cumulative risk assessment of pesticides that share a common mechanism of toxicity.[175,420] The approach is conceptually similar to methods developed by the U.S. EPA for estimating exposure to mixtures of dioxins and dibenzofurans using toxicity equivalence factors to normalize the toxicity (i.e., binding to the aryl hydrocarbon receptor) of each member of the group with respect to that of a single chemical.[421] Specifically, the FQPA requires that all pesticide residues from foods be added together, in a prescribed manner based on target organ, with the goal of understanding the total daily dose of all residual pesticides in the diet.[160,175,420] Then, if necessary, the pesticide manufacturers are expected to calculate the necessary residue level that their chemical may have in a particular food so that the total dose does not exceed a fraction of the acceptable daily intake (ADI).

Ingestion of contaminated fruits and vegetables is a potential pathway of human exposure to toxic chemicals. Fruits and vegetables may become contaminated with toxic chemicals by several different pathways. Ambient air pollutants may be deposited on or absorbed by plants, or dissolved in rainfall or irrigation waters that contact the plants. Plant roots may also absorb pollutants from contaminated soil and groundwater. The addition of pesticides, soil additives, and fertilizers may also result in food contamination. Formulas are available to predict the concentration of chemicals from the soil, which have deposited from the air, and remain after treatment with a pesticide.

The primary information source on consumption rates of fruits and vegetables among the U.S. population is the U.S. Department of Agriculture's (USDA) Nationwide Food Consumption Survey (NFCS) and the USDA Continuing Survey of Food Intakes by Individuals (CSFII).[418,422–426] Data from the NFCS have been used in various studies to generate consumer-only and per-capita intake rates for individual fruits and vegetables, as well as total fruits and total vegetables. CSFII data have been analyzed by EPA to generate per-capita intake rates for various food items and food groups.[63,64,425,426]

Consumer-only intake is defined as the quantity of fruits and vegetables consumed by individuals who ate these food items during the survey period. Per-capita intake rates are generated by averaging consumer-only intakes over the entire population of users and nonusers. In general, per-capita intake rates are appropriate for use in exposure assessment for which average dose estimates for the general population are of interest, because they represent both individuals who ate the foods during the survey period and individuals who may eat the food items at some time, but did not consume them during the survey period. Total fruit intake refers to the sum of all fruits consumed in a day, including canned, dried, frozen, and fresh fruits. Likewise, total vegetable intake refers to the sum of all vegetables consumed in a day, including canned, dried, frozen, and fresh vegetables.

Intake rates may be presented on either an as-consumed or dry-weight basis. As-consumed intake rates (g/day) are based on the weight of food in the form in which it is consumed. By contrast, dry-weight intake rates are based on the weight of food consumed after the moisture content has been removed. Therefore, when calculating exposures based on ingestion, the unit of weight used to measure the contaminant concentration in the produce needs to be understood. Intake data from the individual NFCS and CSFII components are based on *as eaten* (i.e., cooked or prepared) forms of the food items or groups. Thus, no corrections are required to account for changes in portion sizes from cooking losses.[425–427]

Estimating source-specific exposures to toxic chemicals in fruits and vegetables may also require information on the amount of fruits and vegetables exposed to or protected from contamination as a result of cultivation practices, the physical nature of the food product itself (i.e., those having protective coverings that are removed before eating would be considered protected), or the amount grown beneath the soil (i.e., most root crops such as potatoes). The percentages of foods grown above and below ground will be useful when the contaminant concentrations in foods are estimated from concentrations in soil, water, and air. For example, vegetables grown below ground would more likely be contaminated by soil pollutants, but leafy aboveground vegetables would more likely be contaminated by deposition of air pollutants on plant surfaces. Some examples of various exposure factors and confidence ratings for liquids and food are presented in Table 10.10.[428]

TABLE 10.10
Selected Default Exposure Factor Recommendations Involving Food Intake for Citizens of the United States

Variable	Age or Geography Category	Mean	95th Percentile
Total Food (g/day)	3 to <6 years	1066	1746
	6 to <11 years	1118	1825
	21 to <40 years	1100	2110
Meats (g/kg-day)	3 to <6 years	3.9	8.5
	6 to <11 years	2.8	6.4
	21 to <50 years	1.8	4.1
Dairy (g/kg-day)	3 to <6 years	24	51.1
	6 to <11 years	12.9	31.8
	21 to <50 years	3.5	10.3
Breast Milk (mL/day)	Birth–<1 month	510	950
	1 to <3 months	690	980
	3 to <6 months	770	1000
	6 to <12 months	620	1000
Fruits (g/kg-day)	3 to <6 years	4.6	14.9
	6 to <11 years	2.3	8.7
	21 to <50 years	0.9	3.7
Vegetables (g/kg-day)	3 to <6 years	5.4	13.4
	6 to <11 years	3.7	10.4
	21 to <50 years	2.5	5.9
Grains (g/kg-day)	3 to <6 years	6.2	11.1
	6 to <11 years	4.4	8.2
	21 to <50 years	2.2	4.6
Finfish (g/kg-day)	3 to <6 years	0.19	1.4
	6 to <11 years	0.16	1.1
	21 to <50 years	0.15	1
Shellfish (g/kg-day)	3 to <6 years	0.05	0
	6 to <11 years	0.05	0.2
	21 to <50 years	0.08	0.5
Fish Intake—Recreational Anglers (g/day)	Adult (≥18 years)		
	Marine—Atlantic	5.6	18
	Marine—Gulf	7.2	26
	Marine—Pacific	2	6.8
	Rivers	20–70	
	Lakes	5–10	

Individual average daily intake rates calculated from NFCS and CSFII data are based on averages of reported individual intakes over 1 day or 3 consecutive days. Such short-term data are suitable for estimating mean average daily intake rates representative of both short- and long-term consumptions. However, the *distribution* of average daily intake rates generated using short-term data (e.g., 3 days) does not necessarily reflect the long-term *distribution* of average daily intake rates. The distributions generated from short- and long-term data will differ to the extent that each individual's intake varies from day to day; the distributions will be similar to the extent that individuals' intakes are constant from day to day.[63,64]

The variation in day-to-day intake rates among individuals will be greatest for food items or groups that are highly seasonal, and for items or groups that are eaten year-around but are not typically eaten every day. For these foods, the intake distribution generated from short-term data will not reflect long-term distribution. On the other hand, for broad categories of foods (e.g., vegetables), which are eaten on a daily basis throughout the year with minimal seasonality, the short-term distribution may be a reasonable approximation of the true long-term distribution, although it will show somewhat more variability.

The EPA's OPP uses three primary models to evaluate cumulative risk for chemicals that share a common mechanism of toxicity and involve concurrent exposure by all relevant pathways and routes of exposure. These models include (1) Dietary Exposure Evaluation Model (DEEM)/Calendex, (2) Lifeline (assesses exposure, risk, and benefits to elements of people's diets and living environment), and (3) Cumulative and Aggregate Risk Evaluation System (CARES). Each of these models uses food and drinking water consumption data from USDA's CSFII (1994–1996, 1998) and user-entered

residue data to estimate dietary exposure using probabilistic techniques. OPP sets tolerances for specific pesticides on raw agricultural commodities based on estimates of dietary risk. These estimates are calculated using pesticide residue data for the food item of concern and relevant consumption data.

The USDA has also conducted a study entitled *Food and Nutrient Intakes of Individuals in One Day in the U.S.*[422,428,429] USDA calculated mean intake rates for total fruits and total vegetables using NFCS data from 1977 to 1978 and 1987 to 1988, and CSFII data from 1994 to 1995.[422,428,429] Mean per-capita total intake rates are based on intake data for 1 day from the 1977–1978 and 1987–1988 USDA and NFCS, respectively. Data from both surveys are presented in the *Exposure Factors Handbook* to demonstrate that although the 1987–1988 survey had fewer respondents, the mean per-capita intake rates for all individuals agree with the earlier survey. Also, slightly different age classifications were used in the two surveys, providing a wider range of age categories from which exposure assessors may select appropriate intake rates. The age groups used in this dataset are the same as those used in the 1987–1988 NFCS. Information for per-capita intake rates and consumer-only intake rates for various ages of individuals is also available. Intake rates for consumers only were calculated by dividing the per-capita consumption rate by the fraction of the population using vegetables or fruits in a day.[63,64] The advantages of using these data are that they provide intake estimates for all fruits, all vegetables, or all fats combined. Again, these estimates are based on 1-day dietary data that may not reflect usual consumption patterns.[63,64]

Children's exposure from food ingestion may differ from that of adults because of differences in the type and amounts of food eaten and intake per unit body weight.[64] Recent information on consumption rates of foods among children is available from the USDA's NFCS and the USDA's CSFII. Data from the 1989–1991 and 1994–1996 CSFIIs have been used in various studies to generate children's per-capita intake rates for both individual foods and the major food groups. The Supplemental Children's Survey to the 1994–1996 CSFII (1998) was conducted in response to the FQPA of 1996,[425,426] which required the USDA to provide data from a larger sample of children for use by the EPA in estimating exposure to pesticide residues in the diets of children. The 1998 survey adds intake data from 5559 children from birth through 9 years of age to the intake data collected from 4253 children of the same age who participated in the 1994–1996 CSFII.[425,426]

Intake of Fish and Shellfish

Contaminated finfish and shellfish are potential sources of human exposure to toxic chemicals. Pollutants are not only carried in surface waters but also may be stored and accumulated in sediments as a result of complex physical and chemical processes. Consequently, various aquatic species can be exposed to pollutants and may become sources of contaminated food.[63,64]

Accurately estimating exposure to various chemicals in a population that consumes fish from a polluted water body requires an estimation of caught-fish intake rates by fishermen and their families. Commercially caught fish are marketed widely, making the prediction of an individual's consumption from a particular commercial source difficult. Because the catch of recreational and subsistence fishermen is generally not diluted in this way, these individuals and their families represent the population that is most vulnerable to exposure by intake of contaminated fish from a specific location.[63,64]

Over the years, fish consumption survey data have been collected using a number of different approaches that need to be considered when interpreting the survey results.[430] In general, surveys are either *creel* studies in which fishermen are interviewed while fishing or broader population surveys using mailed questionnaires or phone interviews. Both data types can be useful for exposure assessment purposes, but somewhat different applications and interpretations are needed. In fact, creel study results have often been misinterpreted because of inadequate knowledge of survey principles.[63,64,431,432]

The typical survey seeks to draw inferences about a larger population from a smaller sample of that population. The larger population from which the survey sample is taken and to which the survey results are generalized denotes the target population of the survey. In order to generalize from the sample to the target population, the probability of being sampled must be known for each member of the target population. This probability is reflected in weights assigned to each survey respondent, with weights being inversely proportional to sampling probability. When all members of the target population have the same probability of being sampled, all weights can be set to one and essentially ignored.[433,434]

In a mail or phone study of licensed anglers, the target population generally involves all licensed anglers in a particular area, and in these studies, the sampling probability is essentially equal for all target population members. In a creel study, the target population is anyone who fishes at the locations being studied; generally in a creel study, the probability of being sampled is not the same for all members of the target population. For instance, if the survey is conducted for 1 day at a site, then it will include all persons who fish there daily, but only about one-seventh of the people who fish there weekly, one-thirtieth of the people who fish there monthly, etc. In this example, the probability of being sampled (or inverse weight) is seen to be proportional to the frequency of fishing. However, if the survey involves interviewers who revisit the same site on multiple days, and persons who are only interviewed once for the survey, then the probability of being in the survey is not proportional to frequency; in fact, it increases less proportionally with greater frequency of fishing. If the same site is surveyed every day of the survey period with no reinterviewing, all members of the target population would have the same probability of being sampled, regardless of fishing frequency, implying that the survey weights should all equal 1.[433,435]

On the other hand, if the survey protocol calls for individuals to be interviewed each time an interviewer encounters them (i.e., without regard to whether they were previously interviewed), then the inverse weights will again be proportional to fishing frequency, no matter how many times interviewers revisit the same site. Note that when individuals can be interviewed

multiple times, the results of each interview are included as separate records in the database, and the survey weights should be inversely proportional to the expected number of times that an individual's interviews are included in the database.[63,64,433,435]

Fish and shellfish exposure assessments are among the most complicated of all assessments.[436] A significant portion of the *Exposure Factors Handbook* addresses this topic.[63,64] Recently, fairly complex methods, including Monte Carlo modeling, have been applied to resolve many of the difficulties estimating exposure of anglers and their families.[79,430]

Aggregate Exposure and FQPA

Pesticides are regulated under the FIFRA and the Federal Food, Drug and Cosmetics Act (FFDCA). In 1996, Congress passed the FQPA that amended both FIFRA and FFDCA.[419] These laws mandated EPA to register pesticides and set tolerances based on a safety determination, a reasonable certainty that use of a given pesticide or consumption of raw agricultural commodity or processed foods that contain the pesticide and its residues will cause no reasonable harm to human health or the environment. EPA evaluates risks posed by the use and usage of each pesticide to make a determination of safety. Based on this determination, EPA regulates pesticides to ensure that use of the chemical is not unsafe.

In the past, EPA evaluated safety of pesticides based on a single chemical, single exposure pathway scenario. However, FQPA requires that the Agency consider aggregate exposure in its decision-making process. Section 408(a)(4)(b)(2)(ii) of FFDCA specifies with respect to a tolerance that there must be a determination "that there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." Section (b)(2)(C)(ii)(I) states that "there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residues …." *Aggregate dose* is defined as the amount of a single substance available for interaction with metabolic processes or biologically significant receptors from multiple routes of exposure. *Aggregate risk* is defined as the likelihood of the occurrence of an adverse health effect resulting from all routes of exposure to a single substance. Conversely, *cumulative risk* is defined as the likelihood of the occurrence of an adverse health effect resulting from all routes of exposure to a group of substances sharing a common mechanism of toxicity.

As shown in Figures 10.4 and 10.5, the most basic concept underlying all aggregate exposure assessments is that exposure occurs to an individual. The integrity of the data concerning this exposed individual must be maintained throughout the aggregate exposure assessment. In other words, each of the individual *subassessments* must be linked back to the same person.[175] Because exposures are based on that received by a single individual, aggregate exposure assessments must agree in time, place, and demographic characteristics. Time–microenvironment–activity (TMA) studies have become an integral part of exposure assessment and risk management and play a key role in explaining exposure variation.[437] The individual's temporal, spatial, and demographic characteristics are then used to develop a distribution of total exposure to (many) individuals in a population of interest.[175]

Each of these parameters has imbedded attributes that must be matched to create a reasonable assessment. Some of these imbedded attributes include

- Time (duration, daily, seasonally)
- Place (location and type of home, urbanization, watersheds, region)
- Demographics (age, gender, reproductive status, ethnicity, personal preference)

Exposures to pesticides do not occur as single events, but rather as a series of sequential or simultaneous events that are linked in time and place. By performing aggregate assessment (single chemical, multiple pathway/routes), exposure and risk assessments are expected to move closer to describing the pattern of exposure actually encountered by people in the real world.[175] Developing realistic aggregate exposure and risk assessments requires that the appropriate temporal, spatial, demographic exposure factors be correctly assigned. Examples of some of these factors include sex- and age-specific body weights, regional-specific drinking water concentrations of the pesticide being considered, seasonally based pesticide residues in food, and frequency of residential pest control representative of housing type. Exposure factor information, such as that presented in the *CSFII* (1992) and *Exposure Factors Handbook*,[63,64] takes into account seasonal-, age-, and ethnic-related differences. Both documents are critical resources for aggregate exposure and risk assessments. Once an aggregate exposure and risk assessment is completed for one individual, population and subpopulation distributions of exposures and risk may be constructed by probabilistic techniques.[61]

An aggregate exposure and risk assessment is distinct from a cumulative risk assessment. Cumulative risk is defined as "the measure or estimate of distributions of exposures (doses) for a set of chemicals that act by a common mechanism of toxicity."[10,438,439] Cumulative risk assessment evaluates risks from multiple chemicals via all routes and pathways of exposure. The cumulative risk assessment considers the combined toxicological effect of a group of chemicals with a common mechanism of toxicity. The definition of a common mechanism of toxicity is defined as

> Two or more pesticide chemicals that produce an adverse effect(s) to human health by the same, or essentially the same, sequence of major biochemical events. The underlying basis of the toxicity is the same, or essentially the same, for each chemical.[10]

Specific guidance concerning conducting a cumulative aggregate risk assessment has been developed.[61,160,420]

When studying environmental health risks from chemical and nonchemical stressors, multiple levels of analyses are often considered to understand the causal pathway from combined exposures to adverse health outcomes.[439] These levels involve understanding the macro level (e.g., government policies,

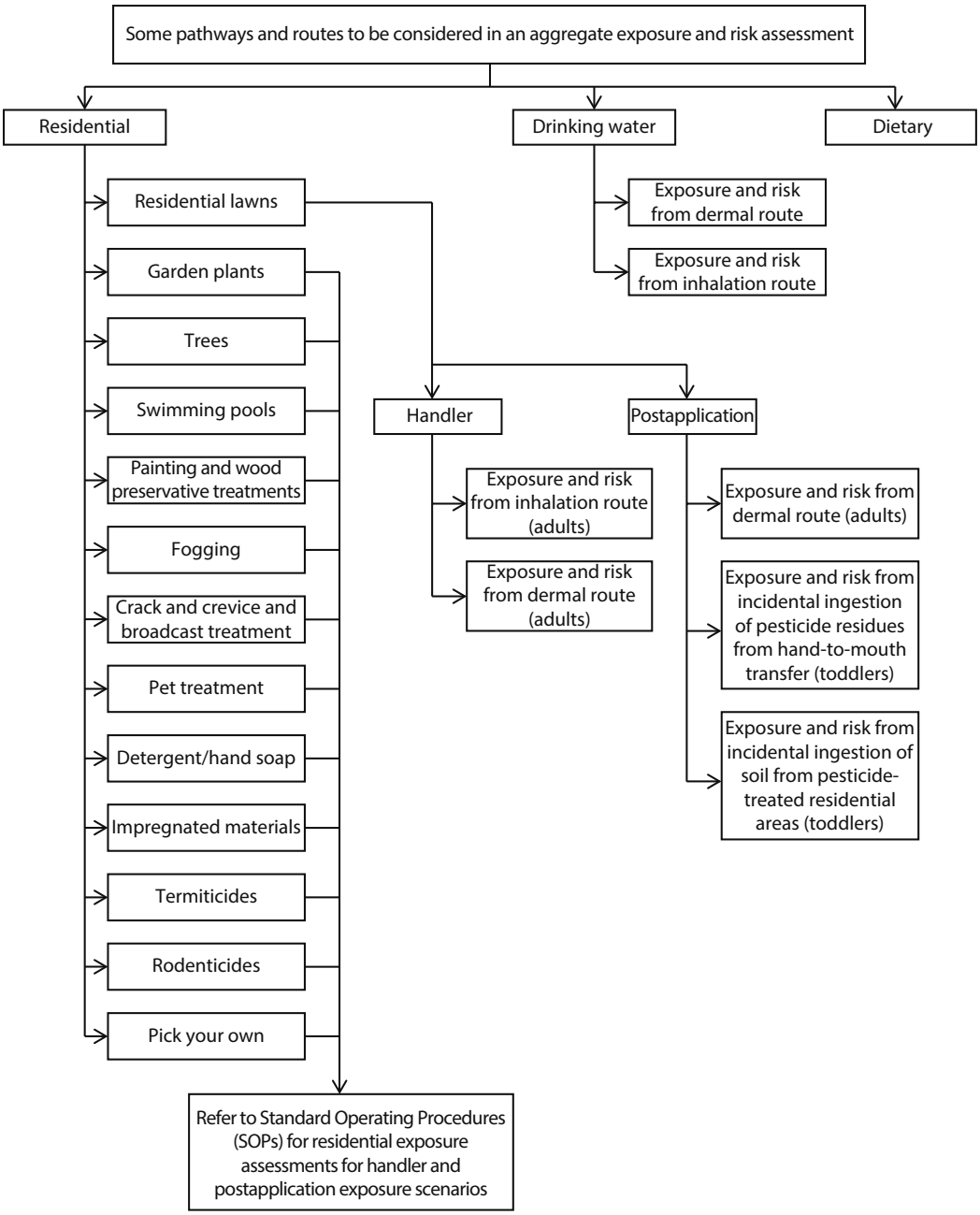

FIGURE 10.5 Factors to consider in an aggregate exposure assessment. (From U.S. Environmental Protection Agency, General principles for performing aggregate exposure and risk assessment, U.S. Environmental Protection Agency (USEPA), Office of Pesticide Programs, Washington, DC, November 28, 2001.)

regulation, market forces), the meso level (e.g., neighborhood pollution levels, social environments, community resources), and the micro level (e.g., personal exposures, body burden, health status, psychological factors, activities and behaviors, socioeconomics, physical and demographic characteristics).[439] These conceptual approaches have been applied in recent models, such as the Cumulative Environmental Hazard Inequality Index (CEHII), the World Health Organization (WHO) Urban Health Equity Assessment and Response Tool (Urban Heart), and EPA's Community-Focused Exposure and Risk Screening Tool (C-FERST).[439] In addition, the U.S. EPA Office of Research and Development along with the National Exposure Research Laboratory (NERL) in their *Cumulative Communities Research Program* is developing and applying tools to enhance cumulative risk assessments to address key questions about (1) identifying and prioritizing key chemical stressors within a given community, (2) developing estimates of exposure from multiple stressors for epidemiologic studies, and (3) tracking effectiveness of risk reduction strategies.[440] A recent paper by Barzyk et al.[438] summarizes over 70 tools available to aid in gathering information and assessing environmental issues related to community-based cumulative risk assessments. In addition, Young and colleagues have compared the use of four different probabilistic models to two deterministic models to

estimate aggregate residential exposure to pesticides within different application scenarios.[441]

Understanding aggregate exposures is a critical component of exposure and risk assessment of chemicals under the new European Union (EU) legislation of chemical management, called Registration, Evaluation, and Authorisation of Chemicals (REACH). In brief, REACH requires companies that manufacture or import more than 1 ton of a substance per year to register the substance at the new EU Chemicals Agency. The aim of REACH is to control and demonstrate the safe use of chemicals for consumers, workers, and the environment. Core to the REACH process is the development of exposure scenarios that relate to how a substance is manufactured or used during its life cycle and how the manufacturer or importer controls or recommends to downstream users to control exposures of humans and the environment.[442,443] Under this new legislation, REACH requires aggregation of exposure from all relevant identified sources, which no doubt will necessitate the use of probabilistic approaches and comprehensive databases on populations' habits, practices, and behaviors.[444]

Breast Milk

Breast milk is a potential source of exposure to toxic substances for nursing infants. Lipid-soluble chemical compounds accumulate in body fat and may be transferred to breast-fed infants in the lipid portion of breast milk. Because nursing infants obtain most (if not all) of their dietary intake from breast milk, they are especially vulnerable to exposures to these compounds. In fact, the peak body burdens of certain chemicals (such as dioxin) can reach their lifetime peak (μg/kg) on the last day of nursing at age 12–24 months. Estimating the magnitude of the potential dose to infants from breast milk requires information on the quantity of breast milk consumed per day and the duration (months) over which breast-feeding occurs.

Several studies have generated data on the ingestion of breast milk.[445–447] Typically, breast milk intake has been measured over a 24 h period by weighing the infant before and after each feeding without changing its clothing (test weighing). The sum of the difference between the measured weights over the 24 h period is assumed to be equivalent to the amount of breast milk consumed daily. Intakes measured, using this procedure, are often corrected for evaporative water losses (insensible water losses) between infant weighings.[64,448] Neville et al. (1988) evaluated the validity of the test-weight approach among bottle-fed infants by comparing the weight of milk taken from bottles with the difference between the before and after feeding weights of infants.[446] Once corrected for insensible water loss, test-weight data were not significantly different from bottle weights. Conversions between weight and volume of breast milk consumed are made using the density of human milk (approximately 1.03 g/mL).[64,448] Techniques for measuring breast milk intake using stable isotopes have been developed; however, little data based on this new technique have been published.[64,448]

Factors associated with breast milk intake include the frequency of breast-feeding sessions per day, the duration of breast-feeding per event, the duration of breast-feeding during childhood, and the magnitude and nature of the population that breast-feeds.[64] Infant birth weight and nursing frequency have been shown to influence the rate of intake. Infants who are larger at birth and/or nurse more frequently have been shown to have higher intake rates.[64,448] Also, breast milk production among nursing mothers has been reported to be somewhat higher than the amount actually consumed by the infant.[428,449] A portion of EPA's *Exposure Factors Handbook* and *Child-Specific Exposure Factors Handbook* addresses this topic, and a few published papers have offered some novel approaches.[63,64] Some examples of breast milk intake rates are presented in Table 10.11.

TABLE 10.11
Values for Daily Intakes of Breast Milk

Age (Months)	Number of Infants Surveyed	Intake (mL/day) Mean	Intake (mL/day) Range of Means	Upper Percentile[a]	References
1	77	702	600–747	1007[b]	Pao et al. (1980),[725] Butte et al. (1984),[726] Neville et al. (1988),[446] Dewey and Lönnerdal (1983)[727]
3	140	759	702–833	1025[b]	Pao et al. (1980),[725] Butte et al. (1984),[726] Neville et al. (1988),[446] Dewey and Lönnerdal (1983),[727] Dewey et al. (1991b)[728]
6	85	765	682–896	1059[b]	Pao et al. (1980),[725] Neville et al. (1988),[446] Dewey and Lönnerdal (1983),[727] Dewey et al. (1991b)[728]
9	62	622	600–627	1038	Neville et al. (1988),[446] Dewey et al. (1991b)[728]
12	51	427	391–435	900	Neville et al. (1988),[446] Dewey et al. (1991b)[728]
12 TWA	688			Range 900–1059 (middle of the range 980)	
1–6 TWA	742			1033.0	

[a] Upper percentile is reported (mean plus two standard deviations) except as noted.
[b] Middle of the range; TWA, time-weighted average.

Information on the fat content of breast milk is also needed for estimating dose from breast milk residue concentrations that have been indexed to lipid content.[63,64,447,450] Environmental chemicals with high lipid solubility are likely to be found in breast milk. These chemicals include polyhalogenated compounds, organochlorine insecticides, and polybrominated diphenylethers. For many long-lived environmental chemicals in lactating women, breast milk may be a major route of elimination.[451] These fat-soluble chemicals are incorporated into the milk as it is synthesized and they must be measured in accordance with the fat content of the milk to allow for meaningful comparisons within an individual and among populations.[452]

The lipid content of breast milk varies according to the length of time that an infant nurses, and it increases from the beginning to the end of a single nursing session. The lipid portion accounts for approximately 4% of human breast milk (39 ± 4.0 g/L).[63,64,450] Most studies in evaluating chemical exposures through breast milk have not accounted for the fact that women's stores of lipophilic chemicals in adipose tissue and breast milk are depleted over the duration of lactation.[453] There are a number of factors that affect contaminant levels in breast milk, including the health of the mother during pregnancy and during the lactation period, the presence and levels of other xenobiotics that may alter metabolism, change in body mass index (BMI) during pregnancy and lactation, diet, other factors that may mobilize fat, parity and length of previous lactation, the number of children being breast-fed at one time, maternal age, and maternal BMI.[452] In addition, variation in the time of breast milk sampling (including time postpartum and time of day), the age of the mother, and the number of previously breast-fed children can also influence measured levels.[453]

While the study of prescription drugs has provided a basis for understanding the governing principles behind transfer of chemicals through breast milk,[454] the research of environmental chemical exposure through breast milk has increased dramatically over the last few decades. One of the earliest reports of the measurement of an environmental chemical in breast milk was by Laug and colleagues in 1951, who reported that the breast milk from 32 women from the general population of Washington, District of Columbia contained DDT (1,1,1-trichloro-1, 2-bis(4-chlorophenyl)ethane) at an average concentration of 0.13 ppm.[455] Since then, a number of studies have evaluated trends of exposure to environmental chemicals through breast milk. Studies have measured chlordane, dieldrin/aldrin, heptachlor, hexachlorobenzene, hexachlorocyclohexane, dioxins and furans, polychlorinated biphenyls, polybrominated diphenyl ethers, and metals in breast milk. The data that exist suggest that bans and restrictions in recent decades on the use of many of the persistent organic pollutants have led to a dramatic decline in the levels of many of these chemicals in breast milk.[456]

ESTIMATING UPTAKE VIA INHALATION

Estimating intake via inhalation depends on only a few exposure factors, for example, inhalation rate, airborne chemical concentration, bioavailability, and, if it is a particle, particle size. In general, uncertainty in estimates of intake via inhalation is among the smallest of all exposure calculations. Inhalation rates are known to vary directly with the amount of physical activity of the persons being evaluated. Airborne chemical concentrations are obtained through either direct measurement or modeling. The form of the chemicals in the air will be a gas (includes vapors) or particles (dusts or fumes). In general, it is assumed that virtually all of the vapors or gases will be absorbed if inhaled.[43,74,255,457] This may not be the case for volatile chemicals, if the concentration in the blood is approaching steady state. In those cases, a significant fraction of the inhaled vapors will be present in the exhaled breath and, therefore, not absorbed.[129] In addition, it is usually assumed that if particles enter the lower respiratory tract, they will eventually be absorbed unless the chemical is highly insoluble. In general, it is assumed that particles less than 150 μm are inhalable, but virtually all particles greater than 10 μm (by weight) will be captured in the upper respiratory tract (nose and throat) and then ingested. It has often been assumed that particles less than 10 μm will be captured in the lower respiratory tract and nearly 100% of these will eventually be absorbed.

Exposure Science in Air Pollution Research

The field of exposure assessment in studying the effects of ambient particulate air pollution has shown some exciting new developments in the last 10–20 years. Tools such as forensic chemical mapping of sources, source apportionment, and tracer analysis for outdoor–indoor contributions of personal exposures have been coupled with health outcome data. Over the last decade, over 100 studies of more than 35 different cities have investigated the acute effects of ambient particulate matter (PM) showing increased hospital admissions and deaths from cardiopulmonary disease (e.g., asthma, chronic obstructive pulmonary disease, arrhythmia, heart attack).[458–460] The effects appear to best correlate with $PM_{2.5}$, with an increased mortality of 0.5%–1.5% for every incremental concentration increase of 5 $\mu g/m^3$.[459] While the mechanisms by which ambient PM causes increased morbidity and mortality due to respiratory and cardiovascular disease are not well understood, investigators have proposed that the ultrafine fraction of ambient PM may be responsible for, or at least contribute to, these effects. The study of these very-fine-sized particles (e.g., $PM_{2.5}$, PM with a median mass aerodynamic diameter less than 2.5 μm) as well as those much smaller (ultrafine or nanosized particles, $PM_{0.1}$, PM with a median mass aerodynamic less than 0.1 μm) has probably been among the most exciting areas of research in the environmental sciences over the past 20 years.[461–464]

The great majority of the epidemiologic literature with respect to the effects of ambient air particulate pollution on morbidity and mortality from cardiopulmonary disease has focused on the combined fine and ultrafine fraction of airborne PM ($PM_{2.5}$). Only recently have human population studies begun to separately characterize the fine and ultrafine fraction of particulate pollution episodes.[465–469] Although

changes in ST-segment depressions, blood pressure, and heart rate variability as in the case of panel-based population studies, or increased hospital admissions for cardiovascular or respiratory disease or increased stroke mortality, have been specifically associated with increases in nanosized particulate pollution, there have also been correlations reported for other constituents (e.g., nitrogen dioxide, carbon monoxide) that often accompany PM from combustion sources. In a study of eight Canadian cities to examine the association between acute exposure to ambient air pollutants and mortality, it was observed that the PM mass only explained 28% of the total health effect of the mixture, with the remaining effects accounted for by the gases.[470] Thus, differentiating the effects of nanosized particulate pollution from other copollutants will continue to pose a challenge. An increasingly greater number of studies, however, are comparing the effects of more traditionally characterized particle size fractions (PM_{10}, $PM_{2.5}$) to that within the ultrafine or nanosized range. The few studies that have made these comparisons, however, have reported varying correlations with the nanosized particles showing greater associations in some cases (e.g., heart rate variability, blood cells, platelet activation markers [CD40 ligand]) or a complete lack of association (e.g., shortness of breath, chest pain) with health effects as compared to the fine-sized particulate fraction.[471–473]

A number of studies have focused on various techniques for source apportionment of ambient PM with the hope that if PM toxicity could be determined by source types, then the regulation of PM may be more effectively implemented. While few studies have conducted such analyses, there is suggestive evidence that PM from certain combustion sources (i.e., secondary aerosols and traffic) and not other sources (e.g., soil) is associated with mortality.[474] These studies are highly complex and it can be difficult to discern trends associated with health effects due to variability of chemical speciation caused by regional, spatial, seasonal, and temporal changes, as well as overlapping chemistries of different emission sources.[474–479]

Within the last 5 years, there has also been greater recognition that exposures within the home, workplace, or other microenvironments have a greater contribution to the total personal exposure to PM than what is represented by outdoor stationary PM monitoring.[480,481] A recent study to characterize indoor sources of ultrafine PM found that outdoor ultrafine PM had difficulty penetrating the home and, based on sample measurements, estimated that for a typical suburban nonsmoker lifestyle, indoor sources provide about 47% and outdoor sources about 36% of the total daily ultrafine PM exposure and in-vehicle exposures add the remainder (17%).[480] Interestingly, cooking on gas or electric stoves and electric toaster ovens was the major source of ultrafine PM in the home with other common sources being cigarettes, vented gas clothes dryers, air popcorn poppers, candles, and electric mixers, toasters, hair dryers, curling irons, and steam irons.[480]

To better understand the health impacts of ambient PM in light of personal exposures to PM in microenvironments, researchers have developed various modeling efforts, sought to identify tracer elements to track ambient PM, and conducted large personal sampling regimens to develop infiltration rates and attenuation factors for ambient PM entering the home and contributing to person exposures.[482–487] In the conceptual framework, linking air pollution exposure to disease requires identification of individuals with increased exposures and tools to determine how and whether the exposures increased the likelihood of disease. Factors that complicate identifying this association include time–activity allocations and mobility (e.g., commuting), microenvironmental and building infiltration factors, age-specific factors (children, working parents, immobile elderly), and spatial and temporal differences in sources and concentrations of air pollutants.[488] Recent data have shown mixed results on the association between peak personal exposures to $PM_{2.5}$ from indoor versus outdoor sources and changes in heart rate variability.[489,490] Differentiating the health impacts of ambient air pollution versus microenvironmental personal exposures will certainly continue to be an active area of research given recent questions about the applicability of air pollutions measurements from a single location and the mobility of the population, as well as the contributions of consumer products and other sources to chemical exposures in the indoor environment.[488,491]

Various Inhalation Rates

A significant amount of research has been conducted to correlate various inhalation rates with different tasks and body weights. Most studies on this subject have been summarized in the most recent *EPA Exposure Factors Handbook*.[63] Data are available for dozens of different levels of physical activity and the distributions for several populations are presented.

A number of equations have been proposed for predicting the inhalation rate based on body weight.[63] The *Exposure Factors Handbook* and other sources provide a number of tables that relate physical activity with inhalation rate (see Table 10.12).

Bioavailability of Airborne Chemicals

Because the mass of chemicals inhaled is usually quite small, and because most particles less than 10 μm in diameter are thought to be fairly easily absorbed, it is generally

TABLE 10.12
Daily Inhalation Rates Estimated from Daily Activities

	Inhalation Rate (IR)		
Subject	Resting (m^3/h)	Light Activity (m^3/h)	Daily Inhalation Rate (DIR) (m^3/day)
Adult man	0.45	1.2	22.8
Adult woman	0.36	1.14	21.1
Child (10 years)	0.29	0.78	14.8
Infant (1 year)	0.09	0.25	3.76
Newborn	0.03	0.09	0.78

assumed that particles are 100% bioavailable after they are trapped in the lower lung. Likewise, it is generally assumed that most vapors and gases are completely absorbed (100% bioavailable) if they reach the lower respiratory tract. Both are conservative assumptions that should be reassessed on a case-by-case basis.

ROLE OF UNCERTAINTY ANALYSIS

Exposure assessment uses a wide array of information sources and techniques. Even when actual exposure-related measurements exist, assumptions or inferences will still be required. Most likely, data will not be available for all aspects of the exposure assessment and these data may be of questionable or unknown quality. In these situations, the exposure assessor will have to rely on a combination of professional judgment, inferences based on analogy with similar chemicals and conditions, and estimation techniques. The net result is that the exposure assessment will be based on a number of assumptions with varying degrees of uncertainty.[43]

The decision analysis literature has focused on the importance of explicitly incorporating and quantifying scientific uncertainty in risk assessments.[435,436] Reasons for addressing uncertainties in exposure assessments include the following[43]:

- Uncertainty information from different sources and of different quality must be combined.
- A decision must be made about whether and how to expend resources to acquire additional information (e.g., production, use, and emissions data; environmental fate information; monitoring data; population data) to reduce the uncertainty.
- So much empirical evidence exists that biases may occur, resulting in so-called best estimates that are not very accurate. Even when all that is needed is a best-estimate answer, the quality of the answer may be improved by incorporating a frank discussion of uncertainty into the analysis.
- Exposure assessment is an iterative process. The search for an adequate and robust methodology to handle the problem at hand may proceed more effectively, and to a more certain conclusion, if the associated uncertainty is explicitly included and it can be used as a guide in the process of refinement.
- A decision is rarely made on the basis of a single piece of analysis. Furthermore, it is rare for there to be one discrete decision; a process of multiple decisions spread over time is the more common occurrence. Chemicals of concern may go through several levels of risk assessment before a final decision is made. During this process, decisions may be made based on exposure considerations. An exposure analysis that attempts to characterize the associated uncertainty allows the user or decision-maker to do a better evaluation in the context of the other factors being considered.
- Exposure assessors have a responsibility to present not just numbers, but also a clear and explicit explanation of the implications and limitations of their analyses. Uncertainty characterization helps to achieve this.

Essentially, constructing scientifically sound exposure assessments and analyzing uncertainty go hand in hand. The reward for analyzing uncertainties is knowing that the results have integrity or that significant gaps exist in available information that can make decision-making a tenuous process.

Variability versus Uncertainty

While some authors treat variability as a specific component of uncertainty, EPA and others advise risk assessors (and, by analogy, the exposure assessor) to distinguish between variability and uncertainty.[12,492] Specifically, uncertainty represents a lack of knowledge about factors affecting exposure or risk, whereas variability arises from true heterogeneity across people, places, or time. In other words, uncertainty can lead to inaccurate or biased estimates, whereas variability can affect the precision of the estimates and the degree to which they can be generalized.

Variability and uncertainty can complement or confound one another. National Research Council[12] has drawn an instructive analogy based on estimating the distance between the earth and the moon. Prior to fairly recent technological developments, it was difficult to accurately measure this distance, resulting in measurement uncertainty. Because the moon's orbit is elliptical, the distance is a variable quantity. If only a few measurements were taken without knowledge of the elliptical pattern, then either of the following incorrect conclusions might be reached:

- The measurements were faulty, thereby ascribing to uncertainty what was actually caused by variability.
- The moon's orbit was random, thereby not allowing uncertainty to shed light on seemingly unexplainable differences that are in fact variable and predictable.

A more fundamental error in the earlier situation might be to incorrectly estimate the true distance and assume that a few observations were sufficient. This latter pitfall—treating a highly variable quantity as if it were invariant or only uncertain—is most relevant to the exposure or risk assessor.[43]

Now consider a situation that relates to exposure, such as estimating the ADD by one exposure route—ingestion of contaminated drinking water. Suppose that it is possible to measure an individual's daily water consumption (and concentration of the contaminant) exactly, thereby eliminating uncertainty in the measured daily dose. The daily dose still has an inherent day-to-day variability, however, because of changes in the individual's daily water intake or concentration of the contaminant in the water.[43]

Clearly, it is impractical to measure the individual's dose every day. For this reason, the exposure assessor may estimate

the ADD based on a finite number of measurements, in an attempt to *average out* the day-to-day variability. The individual has a true (but unknown) ADD, which has now been estimated based on a sample of measurements. Because the individual's true average is unknown, it is uncertain how close the estimate is to the true value. Thus, the variability across daily doses has been translated into uncertainty in the ADD. Although the individual's true ADD has no variability, the estimate of the ADD has some uncertainty.[43]

The preceding discussion pertains to the ADD for one person. Now consider a distribution of ADDs across individuals in a defined population (e.g., the general U.S. population). In this case, variability refers to the range and distribution of ADDs across individuals in the population. By comparison, uncertainty refers to the exposure assessor's state of knowledge about that distribution, or about parameters describing the distribution (e.g., mean, standard deviation, general shape, various percentiles).[43]

As noted by the National Research Council,[12] the realms of variability and uncertainty have fundamentally different ramifications for science and judgment. For example, uncertainty may force decision-makers to judge how probable it is that exposures have been overestimated or underestimated for every member of the exposed population, whereas variability forces them to cope with the certainty that different individuals are subject to exposures both above and below any of the exposure levels chosen as a reference point.[43]

Types of Variability

Variability in exposure is related to an individual's location, activity, and behavior or preferences at a particular point in time, as well as pollutant emission rates and physical/chemical processes that affect concentrations in various media (e.g., air, soil, food, and water). The variations in pollutant-specific emissions or processes, and in individual locations, activities, or behaviors are not necessarily independent of one another. For example, both personal activities and pollutant concentrations at a specific location might vary in response to weather conditions, or between weekdays and weekends.[43]

At a more fundamental level, three types of variability can be distinguished:

1. Variability across locations (spatial variability)
2. Variability over time (temporal variability)
3. Variability among individuals (interindividual variability)

Spatial variability can occur both at regional (macroscale) and local (microscale) levels. For example, fish intake rates can vary depending on the region of the country. Higher consumption may occur among populations located near large bodies of water such as the Great Lakes or coastal areas. As another example, outdoor pollutant levels can be affected at the regional level by industrial activities and at the local level by activities of individuals. In general, higher exposures tend to be associated with closer proximity to the pollutant source, whether it is an industrial plant or related to a personal activity such as showering or gardening. In the context of exposure to airborne pollutants, the concept of a *microenvironment* has been introduced to denote a specific locality (e.g., a residential lot or a room in a specific building) where the airborne concentration can be treated as homogeneous (i.e., invariant) at a particular point in time.

Temporal variability refers to variations over time, whether long or short term. Seasonal fluctuations in weather, pesticide applications, use of woodburning appliances, and fraction of time spent outdoors are examples of longer-term variability. Examples of shorter-term variability are differences in industrial or personal activities on weekdays versus weekends or at different times of the day.

Interindividual variability can be either of two types: (1) human characteristics such as age or body weight and (2) human behaviors such as location and activity patterns. Each of these variabilities, in turn, may be related to several underlying phenomena that vary. For example, the natural variability in human weight is due to a combination of genetic, nutritional, and other lifestyle or environmental factors. Variability arising from independent factors that combine multiplicatively generally will lead to an approximately lognormal distribution across the population or across spatial/temporal dimensions.[31,43,493]

Monte Carlo Analysis

Among the most significant advances in exposure assessment of the past 20 years is the application of Monte Carlo or other probabilistic analyses to environmental health issues.[25,83,88,494] Monte Carlo analysis has existed as an engineering analytical tool for many years, but the development of specialized computer software (e.g., Crystal Ball [Decisioneering, Boulder, Colorado], @RISK [Palisades Corp., Newfield, New York]) has allowed its application to new areas. As discussed previously, one criticism of many exposure assessments has been a reliance on overly conservative assumptions about exposure, as well as the problem of how to properly account for the highly exposed (but usually small) populations that do exist.[75,445] The Monte Carlo technique offers an approach to addressing this issue.

The probabilistic or Monte Carlo model accounts for the uncertainty in select parameters evaluating the range and probability of plausible exposure levels. Instead of specifying input parameters as single values, this model allows for consideration of the probability distributions. The Monte Carlo statistical simulation is a statistical model in which the input parameters to an equation are varied simultaneously. The values are chosen from the parameter distributions, with the frequency of a particular value being equal to the relative frequency of the parameter in the distribution. The simulation involves the following three steps:

1. The probability distribution of each equation parameter (input parameter) is characterized, and the distribution is specified for the Monte Carlo simulation. If the data cannot be fit to a distribution, the

data are *bootstrapped* into the simulation, meaning that the input values are randomly selected from the actual dataset without a specified distribution.
2. For each iteration of the simulation, one value is randomly selected from each parameter distribution, and the equation is run. Many iterations are performed, such that the random selections for each parameter approximate the distribution of the parameter. Five thousand iterations are typically performed for each dose equation.
3. Each iteration of the equation is evaluated and saved; hence, a probability distribution of the equation output (possible doses) is generated.

This technique generates distributions that describe the uncertainty associated with the risk estimate (resultant doses). The predicted dose for every 50th percentile to the 95th percentile of the exposed population and the true mean are calculated. Using these models, the assessor is not forced to rely solely on a single exposure parameter or the repeated use of conservative assumptions to identify the plausible dose and risk estimates. Instead, the full range of possible values and their likelihood of occurrence is incorporated into the analysis to produce the range and probability of expected exposure levels.[25,37,80–82,144,495]

The methodology is illustrated in the following examples. The first example is to understand the time needed to go shopping. Time spent shopping each month (minutes) is estimated by the product of two parameters: the number of trips per month and the total time spent in the store (minutes). Total time spent in the store is the sum of time spent shopping and time spent waiting in line. Using Monte Carlo techniques, a distribution of likely values is associated with each of these parameters. These distributions depend on the detail of information available to characterize each parameter. For example, the distribution compares all of the information, such as those days when the line at the checkout counter is short, as well as those when the line is long. It is noteworthy that each parameter has a different distribution: lognormal, Gaussian, and square. Total time spent shopping is then calculated repeatedly by combining parameter values that are randomly selected from these distributions. The result is a distribution of likely time spent shopping each month. Using this technique, information concerning each parameter is carried along to the final estimate.

The second example, which directly applies to toxicologists, is to build a distribution that describes the various soil ingestion rates for children. As shown in Figure 10.6, the three pertinent distributions are the basis for constructing the overall exposure distribution. Most of the variables used in an exposure assessment actually exist as ranges, rather than single point values. For instance, the common assumption that adult body weight is 70 kg will be replaced in a Monte Carlo analysis by the appropriate distribution (i.e., normal)

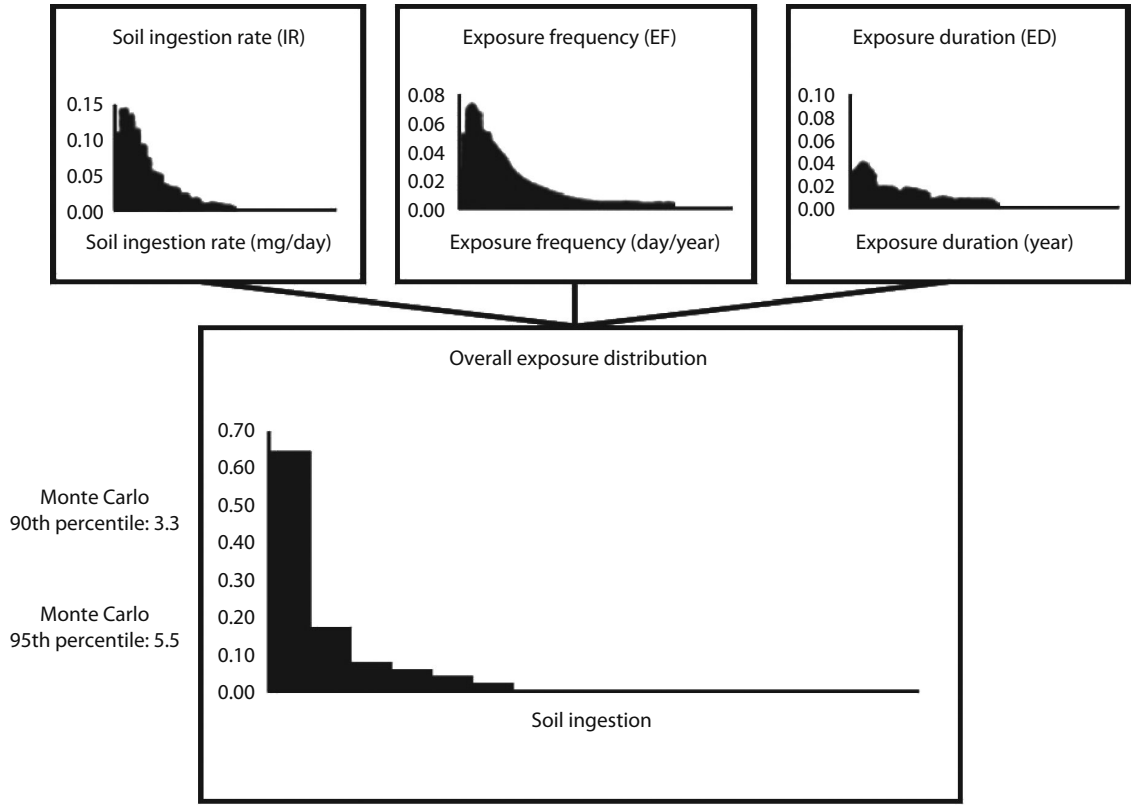

FIGURE 10.6 Example of how probability density functions (distributions) for three different related exposure factors are combined to form a distribution for the amount of soil ingested by a population of children. The Monte Carlo technique allows the risk assessor to account for the variability in many exposure parameters within a population and then produce a distribution that characterizes the entire population.

TABLE 10.13
U.S. EPA Guiding Principles for Monte Carlo Analysis

1. Conduct preliminary sensitivity analyses to identify important contributors to the assessment endpoint and its variability and uncertainty.
2. Based on the results of the sensitivity analyses, include probabilistic assessments only for the important pathways and parameters.
3. Use the entire database of information when selecting input distributions.
4. When using surrogate data, identify sources of uncertainty, and whenever possible, validate the use of these data by collecting site-/case-specific data.
5. If empirical data are collected for use in the assessment, use collection methods that improve the representativeness and quality of these data (especially at the tails of the distribution).
6. Identify when expert judgment, rather than hard data, is used in the assessment.
7. Separate uncertainty and variability during the analysis.
8. Use appropriate methods to address uncertainly and variability, e.g., 2D Monte Carlo.
9. Discuss the numerical stability of estimates at the tails of the distribution.
10. Identify which sources of uncertainty are addressed by the assessment and which are not.
11. Provide a detailed description of all models used.
12. Provide a detailed description of the input distributions, including a distinction between variability and uncertainty in these distributions, and a graphical representation of the probability density and cumulative distribution functions.
13. Provide a graphical representation of the probability density and cumulative distribution functions of each output distribution.
14. Consider the potential covariance between important parameters. If the covariance cannot be determined, evaluate the impact of a range of potential covariances on the output distributions.
15. Present point estimates and identify where they fall on the exposure distribution. If there are large differences between point estimates and Monte Carlo estimates, explain if the differences are due to changes in the data or models used.
16. Present results in a tiered approach.

of body weights (including maximum, minimum, mean, and standard deviation). Using this approach, virtually every exposure variable, whether physiological, behavioral, environmental, or chronological, can be replaced with a probability distribution.[37,77,78,80–82,144,267,434,495–504] Since no population (or individual) is exposed to a single concentration; breathes, eats, or drinks at a single rate; or is exposed for the same length of time, it is not appropriate to assess them as such. To be protective, high values are employed, resulting in the problems of compounding conservatisms mentioned previously.[66,67,75,505,506]

The probabilistic analysis addresses the main deficiencies of the point estimate approach because it imparts more information to risk managers and the public, and uses all of the available data.[507,508] The range of values (i.e., the distribution) for all the variables used in an exposure assessment is determined (e.g., normal, lognormal, uniform, triangular) and combined into a *distribution of distributions*. Because of the extrapolations involved and the assumptions made, the area of single greatest uncertainty in risk assessment is associated with the dose–response evaluations.

It should also be clear that, in addition to exposure variables, data forming the basis of the toxicological criteria (carcinogenic potency factors [CPFs] and reference doses [RfDs]) are also amenable to Monte Carlo–style analysis in which a robust database exists.[509–521] As with exposure variables, the advantage to this approach is that it allows all data to be used (and weighted appropriately, where necessary), thus avoiding reliance on a single experiment or endpoint.

Probabilistic analyses have in recent years been recognized in regulatory guidance,[63,83,88] and EPA's Risk Assessment Forum has published a document of principles for conducting Monte Carlo analyses (Table 10.13).[511] EPA and a number of states (and other countries) have published a comprehensive guidance document on how to conduct Monte Carlo assessments.[83,88]

Like traditional exposure analysis, one challenge to performing a Monte Carlo analysis properly is having appropriate distributions for use in the analysis. Numerous studies on individual variables have been published in the risk assessment literature,[71,76,83,319,403,406,507,522–526] and the impact on the distributions employed on the outcome has also been discussed.[31,527–531] It should be noted that these techniques can be combined with other advanced risk assessment methods (i.e., PBPK modeling) to further reduce uncertainty in risk estimates.[84,532] Two-dimensional Monte Carlo analyses and probabilistic approaches, in general, have been developed that take into account both variability and uncertainty and have been utilized as a method to quantify uncertainty in sensitivity analyses.[86,533–537] Information appropriate to probabilistic analyses can often be found in published papers in fields quite distant from the environmental sciences.

Case Study Using Monte Carlo Technique

An example might be useful.[524] Assume that persons are likely to be exposed to contaminated drinking water at the maximum contaminate level (MCL). Concern has

been raised that these regulatory limits are not sufficiently protective and that certain federal and state regulatory programs (i.e., Resource Conservation and Recovery Act) are justified in requiring groundwater remediation to levels below that of drinking water standards. In order to test this supposition, it is necessary to evaluate the possible incremental cancer risk of exposure via tap-water ingestion, dermal contact with water while showering, inhalation of indoor vapors, and ingestion of produce irrigated with groundwater, using a probabilistic approach. PDFs for each exposure variable (e.g., water ingestion, skin surface area, fraction of exposed skin, showering time, inhalation rate, air exchange and water use rates, exposure time) are then identified and used in the appropriate exposure equation to calculate dose and risk. A commercially available software package (i.e., @RISK) could be used to conduct the Monte Carlo analysis.[504]

Some have suggested that the Latin Hypercube (LHC) approach offers some advantages to traditional approaches for identifying the correct number of iterations. Often, one can reach convergence sooner with LHC than the Monte Carlo option in @RISK/Crystal Ball. In addition, LHC is more reproducible (to the hundredth decimal place). The Monte Carlo option needs more iterations to reach convergence.

The results of such an analysis are presented in Table 10.14.[524] The risk associated with exposure to water at the current MCL for four different contaminants, as well as the 50th and 95th percentiles of exposure as determined by the probabilistic analysis, is shown. At the 50th percentile level (*the best estimate*), the risk ranges from 6×10^{-7} (tetrachloroethylene) to 9×10^{-6} (chloroform), while at the 95th percentile (*the upper-bound risk*), these risks range from 4×10^{-6} (tetrachloroethylene) to 1.5×10^{-4} (chloroform). These values can be compared to the point estimate risks calculated for the MCLs, which range from 7×10^{-6} (tetrachloroethylene) to 5.4×10^{-5} (vinyl chloride). For the 50th percentile (average) person, all calculated risks are within the range of *acceptable* risks adopted by regulatory authorities for Superfund sites (1×10^{-4} to 1×10^{-7}). For the 95th percentile person (upper bound), the risks are still mostly below the 1×10^{-4} benchmark risk level generally used to separate acceptable from unacceptable risks. For tetrachloroethylene, these results are 30 (50th percentile) to 3 (risk at the MCL) times below the reasonable maximum exposure risk of 2×10^{-5} developed by combining the 95th percentile values for each exposure variable using standard EPA risk assessment methodologies. This point estimate is greater than the 99th percentile of risk and is consistent with statements regarding the conservatism of the reasonable maximum exposure (RME) approach. These results suggest that chemical residues in drinking water at the MCLs will be health protective and that remedial goals based on de minimis requirements (1×10^{-6}) might be unnecessarily low.[524]

TABLE 10.14
Risks Calculated for Exposure to Four Halogenated Solvents in Water Using Probabilistic Analysis at the MCL and for the 50th and 95th Percentile Exposures

Chemical	50th Percentile Risk	95th Percentile Risk	MCL Risk
Tetrachloroethylene	0	0.000005	0.000007
Chloroform	0.000009	0.00014	0.000017
Bromoform	0.000002	0.000016	0.000023
Vinyl chloride	0.000005	0.000029	0.000054

In terms of estimates for the reasonably maximally exposed (RME) individual, which often serve as the basis for regulatory decisions, several observations on the utility of probabilistic assessment can be made. First, exposure assessments that incorporate two to three direct exposure pathways usually show that the 95th percentile probabilistic estimates are three to five times below the traditional RME estimates. Second, for multipathway assessments that contain several indirect exposure pathways, the 95th percentile probabilistic estimates can be as much as an order of magnitude below the RME estimates. Third, when the number of distributions used in the exposure assessment is 10 or more, the difference between the 50th- and 95th-percentile estimates may be between 5 and 10. Finally, in such assessments, the difference between the RME estimates and the 95th percentile probabilistic estimates can be as high as 100. In the probabilistic approach to estimating exposure and risk, the complete range of potential risks can be illustrated along with the likelihood estimates and estimates of uncertainty associated with such risks. While the availability and confidence of distributions for exposure variables differ, risk assessors ought to take advantage of this and similar approaches in their risk assessments to advance and improve the process. In addition, since the highest degree of uncertainty in risk assessment tends to be the CPFs, attention ought to be directed to applying probabilistic analysis to the development of toxicity criteria in a similar manner.[514,521]

Sensitivity Analysis

In addition to establishing exposure and risk distributions, probabilistic analysis can also identify variables with the greatest impact on the estimates and illuminate uncertainties associated with exposure variables through sensitivity analysis.[538–543] Sensitivity analysis is the study of how the uncertainty in the output of a model (numerical or otherwise) can be apportioned to different sources of uncertainty in the model input. The sensitivity analysis is hence considered by some as a prerequisite for model building in any setting and in any field where models are used.[537] Sensitivity analysis can help in identifying critical control points, prioritizing additional data collection, and verifying and validating a model.[534] This provides some insight into the confidence that resides in exposure and risk estimates, and has two important results. First, it identifies the inputs that would benefit most from additional research to reduce uncertainty and improve risk estimates. Second, assuming that a thorough assessment

has been conducted, it is possible to phrase the results in more accessible terms, such as

> The risk assessment of PCBs in smallmouth bass is based on a large amount of high-quality reliable data, and we have high confidence in the risk estimates derived. The analysis has determined that 90 percent of the increased cancer risk could be eliminated through a ban on carp and catfish, but there is no appreciable reduction in risk from extending such a ban to bass and trout.[520]

Such a description provides all stakeholders with considerably more information than a simple point estimate of risk based on a traditional exposure and risk assessment.[142]

If the most *sensitive* exposure variables are based on limited or uncertain data, confidence in these estimates will be poor. Robust datasets, on the other hand, lead to increased confidence in the resulting estimates. In the example mentioned in the earlier text involving smallmouth bass, sensitivity is defined as the ratio of the relative change in risk produced by a unit relative to change in the exposure variables used. A Gaussian approximation (the product of the normalized sensitivity and the standard deviation of the distribution) of intake was used to allow both sensitivity and uncertainty to be gauged. In this case, the true mean of each distribution was chosen as the baseline point value, and the differential value for each variable was calculated by increasing this value by 10%. For each variable, the differential value was substituted, the risks recalculated, and the baseline value replaced.[524] Sensitivity was calculated using the following formula:

$$\text{Sensitivity} = \frac{|\text{Risk}_{\text{baseline}} - \text{Risk}_{10\%}|}{|\text{X}_{\text{baseline}} - \text{X}_{10\%}|} \times [\sigma]$$

where

$X_{baseline}$ and $X_{10\%}$ are the baseline and differential values for the variable X, respectively

σ is the standard deviation for the distribution of variable X

The sensitivity of each variable relative to one another is assessed by summing the unitless sensitivity values and determining the relative percent that each variable contributes to the total.

Table 10.15 identifies the most important variables in the probabilistic analysis for tetrachloroethylene. In this case, the most sensitive exposure variables in household exposure to tap water are exposure time in shower and exposure duration. Relatively small changes in these variables will result in relatively large changes in the risk estimates. Since these estimates are based on actual time-use studies and census information, this suggests a high level of confidence can be placed on this estimate, particularly if site-specific data are being used. If the critical variables (in terms of sensitivity) were not based on robust data, this would suggest that the risk assessment could be improved by additional research on these exposure variables. It is interesting that the form of the distribution chosen for the variables is less important than the validity of the data.[72] When the empirical distribution of the tap-water ingestion rate was substituted with a lognormal distribution,[544,545] the resultant change in the risk estimates was less than 1%.[504]

TABLE 10.15
Results of Sensitivity Analysis for Tetrachloroethylene Exposure in Household Water

Exposure Variable	Sensitivity (Unitless)	Percentage Rank (%)
Shower exposure time	0.000004	55.0
Exposure duration	0.000001	20.0
Plant–soil partition factor	0	8.4
Water ingestion rate	0	4.6
Surface area of exposed skin	0	4.4
Body weight	0	3.8
Dermal permeability constant	0	1.8
Skin fraction contacting water	0	1.5

In this case, the value of the sensitivity analysis is that it allows input variables to be ranked in order of importance and confidence in the output to be established to a higher degree than previously possible. As pointed out by EPA,

> [W]here possible, exposure assessors should report variability in exposures as numerical distributions and should characterize uncertainty as probability distributions. They need to identify clearly where they are using point estimates for "bounding" potential exposure variables or estimates; these point estimates should not be misconstrued to represent, for example, the upper 95th percentile when information on the actual distribution is lacking.[520]

As noted by EPA, such explicit presentation of the data reduces the temptation to use the exposure assessment process for veiling policy judgments.[546]

EVOLVING RESEARCH IN EXPOSURE ASSESSMENT

The field of exposure assessment will continue to benefit from ongoing research efforts. The following are some fruitful areas of ongoing research.

Bioavailability

Areas of applied research that will improve the practice of exposure assessment include bioavailability, speciation, chemical fate, and the role of biological monitoring. For over 20 years, consideration of the bioavailability of a chemical in a various media has become an increasingly important aspect of the exposure assessment process.[51,102,118,120,121,547] Alexander (1995) has shown that a variety of organic chemicals in soil lose the ability to interact with biological receptors over time,[548] despite the fact that the chemical concentration

in soil remains largely the same. The alteration in bioavailability extends across the various routes of exposure as well.[106–108,111–113,115,119,122,125,314–316,547,549,550] Inorganic compounds, even those posing a potentially significant degrees of hazard (i.e., cyanide), react similarly.[551–554] These losses in hazard potential are presumably due to irreversible chemical interactions with soil constituents. Table 10.16 indicates that the bioavailability of lead added to soil is immediately halved and that it is further reduced over time.[555] This would suggest that an assumption of 100% bioavailability of this compound (and many others) from soil is erroneous. It is also clear that the environmental media in which the compound occurs will influence its uptake into the body.[549] EPA recognized this fact when it developed an RfD for manganese and acknowledged that the bioavailability and resultant dose of manganese can drastically differ depending on whether the chemical exists in solid matrices (e.g., food, soil) or water.[10] One simple method to improve bioavailability estimates is to conduct extractions under more biologically relevant conditions.

Bench-scale extraction experiments in simulated gastric fluids or sweat can be used to inexpensively and accurately measure how readily environmental residues can be released from the media in which they occur.[102,124,556] As with inhalation or ingestion of vapors or solutions, both the release and absorption rates of agents from an environmental matrix (i.e., soil) across biological membranes need to be incorporated into the risk assessment when such data are available, and generated when absent. This need is particularly of issue for assessing dermal exposure. The problem for materials in aqueous solutions is less problematic than from solid matrices.[91] For liquids, permeability constants expressed in terms of agent weight per unit area per time (mg/cm^2/min) have been developed for a number of agents, and in vivo and in vitro techniques or mathematical models exist to develop similar flux rates if needed.[260,261,264,288,290,298,316,550,557–560] From soil, however, the typical approach in many risk assessments has been to assume a constant percent absorbed from soil adhered to skin as a default. For volatiles, an absorption rate of 25% has been used. For semivolatiles and inorganics, absorption rates of 10% and 1% have been used, respectively.

Some experimental data for absorption are available for a few agents (e.g., PCBs, DDT, dioxin, benzo[a]pyrene), suggesting that the simple assumption of a constant percentage absorbed may overestimate or underestimate the dose depending on the agent, co-contaminants, soil type, exposure duration, and similar considerations.[125,314,316,410,550] The impact of this default approach results in an instantaneous dermal dose being assumed, regardless of whether the soil remains in contact with the skin for 1 min or 1 day. This assumption, together with the questionable route-to-route adjustment of toxicity criteria from oral to dermal previously discussed, results in the dermal absorption of agents from soil, which arguably should present a minor exposure and risk in most cases, being a major driver in the risk assessment of soil-bound contaminants.

TABLE 10.16
Effect of Matrix and Aging on the Bioavailability of Lead from Soil

Treatment			
Lead Acetate (ppm Diet)	Soil Lead (ppm)	Tibial Lead (Standard Deviation)	Relative Lead Absorption
—	—	0.3 (0.3)	—
—	11.3	0	—
50	—	247 (10)	100
50	11.3	130 (30)	53
—	706	40 (6)	16
—	9,95	108 (26)	44
—	1,080	37 (7.3)	15
—	1,260	53.6 (7)	22
—	10,420	173 (22)	70

Chemical Fate

Risk assessors ought to incorporate information on the fate of chemicals in the environment in their exposure estimates, whenever possible.[552] Many organic compounds tend to degrade over time, and may disappear from exposed surfaces relatively quickly or otherwise change.[69,561] As suggested earlier, inorganic compounds may also undergo changes in the environment over time that affect their fates.[554,555] Influencing factors include degradation by sunlight, soil and water microbes, evaporation, and chemical interactions. The resultant changes can dramatically alter the outcome of exposure assessments.[51,551] For instance, most criticism of incinerators has focused on the inhalation risk of dioxin emitted from the stacks. As it turns out, the environmental half-life of dioxin (as a vapor) is only 90 min because of photolytic degradation. By contrast, the half-life of dioxin in soil or fly ash is 50–500 years. The focus of concern is often not the main risk issue when environmental fate is considered because levels and availability change over time.[7] Incorporation of half-life data into risk assessments can have substantial benefits for improving understanding of the potential exposures and risks associated with a specific situation.[2,562] In a similar manner, the risk from persistent contaminants (i.e., DDT) in fish has usually been assessed using results from the analysis of raw fish fillets in combination with assumptions about the size and number of fish meals. The effects of cleaning and cooking on these residues are not typically considered, but have been shown to be reduced substantially in many cases (i.e., 50% or greater).[563,564] Since many of these risk assessments form the basis of fish advisories or bans with potentially significant economic repercussions, there is obviously an important reason to make these exposure estimates as accurate as possible. In addition, since there are known health benefits to fish consumption, making recommendations against eating fish based on theoretical risk needs to be rigorously defended.[565]

Biomarkers and Biomonitoring

There is general agreement among the scientific community that diseases that contribute the greatest public health burden to society result from complex interaction between genetic and environmental factors, such as chemical pollutants, nutrition, lifestyle, infectious agents, and stress.[176,566–568] The field of epidemiology is a critical field for understanding these interactions. The cornerstone of exposure assessment in epidemiologic studies is the development of the exposure metric, the estimate of exposure for each individual of the study population.[176]

The past two decades have witnessed a dramatic increase in the level of research activity, derivation of theoretical constructs, and development of practical applications for the direct measurement of biological events or responses that result from human exposure to xenobiotics.[211,569] These measurements, conveniently grouped under the descriptor *biological markers* or *biomarkers*, reflect molecular and/or cellular alterations that occur along the temporal and mechanistic pathways connecting ambient exposure to a toxicant and eventual disease. As such, an almost limitless array of biomarkers is theoretically available for assessment, and only a minute fraction of these has been recognized and investigated to date.[569–571]

The term biomarker is a general term for specific measurements of an interaction between a biological system and an environmental agent.[166,572] Biomarkers can be broadly grouped into several categories: biomarkers of internal exposure, biomarkers of early biological effects, susceptibility biomarkers, genomic biomarkers, and biomarkers of health risk.[573] The International Programme on Chemical Safety defines three classes of biomarkers: biomarker of exposure, effect, and susceptibility:[572]

1. Biomarker of exposure is defined as an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism.
2. Biomarker of effect is a measurable biochemical, physiological, behavioral, or other alteration within an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment or disease.
3. Biomarker of susceptibility is an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance.

Some events that can technically be classified as biomarkers of chemical exposure (e.g., hematological changes following high levels of exposure to lead or benzene, acetylcholinesterase inhibition by organophosphates) have been measured for decades. However, the recent surge of interest in this field has been driven by technical advances in analytical chemistry and molecular genetic techniques and by the recognition that *classical* toxicology and epidemiology may not be able to alone resolve critical questions regarding causation of environmentally induced disease.[211]

Epidemiology relies on inference of associations between exposure and response variables. Typically, the measurements of response in epidemiologic studies reflect late-stage endpoints of morbidity, mortality, body weight decrease, tumor development, and tissue pathology.[176,574,575] Defining risk at a late stage in the disease process provides little opportunity to intervene and redirect the outcome. It is clearly more desirable to identify early changes in biologic processes that can serve as predictive markers of exposure, of early effect, or of susceptibility.[176,576]

Important early applications of biomarkers to characterize environmental and occupational exposures have also been explored by several groups in the United States and abroad. Biomarkers of internal dose reflect the absorbed fraction of a xenobiotic, that is, the amount of material that has successfully crossed physiological barriers to enter the organism.[577] Consequently, the magnitude of the biomarker accounts for bioavailability and is influenced by numerous parameters such as route of exposure, physiological characteristics of the receptor, and chemical characteristics of the xenobiotic. In general, simple measurement of xenobiotic levels in biological media (blood, tissue, urine) can provide data on internal dose, and this is called biomonitoring.[578–580] As employed in most studies, biomonitoring indicates the presence of the substance or marker in the body at a single point in time, corresponding to when the specimen was taken, but such data alone provide no information on the source, pathway, the magnitude, the frequency or the duration of exposure.[581,582] Biomonitoring of exposure involves the measurement of the concentration of a chemical in a given biologic matrix during or after absorption, distribution, metabolism, and elimination (ADME), and its concentration level depends on the amount of the chemical that has been absorbed into the body, the pharmacokinetics (ADME) of the chemical, and the exposure scenario, including the time sequence of exposure and time since last exposure.[583,584] Ideally, in order to link the dose with adverse health outcomes, measurements of the BED, the dose at the target site that causes an adverse health effect, are preferred.[582,583]

Biomarkers reflect internal dose (in terms of proximity to downstream events in the sequence) and could include the measurement of a metabolite in selected biological media, particularly if such metabolite is active or critical to the toxic effects seen.[211] Ideally, a biomarker should be biologically relevant, sensitive, and specific (i.e., valid) and should be readily accessible, inexpensive, and technically feasible. Analytical, metabolic, and source specificities are important aspects to consider when identifying an appropriate biomarker, where the analytical specificity refers to the capability of the analytical method to exclusively measure the chemical (parent or metabolite) of interest, metabolic specificity means that the chemical measured is derived exclusively from the parent chemical of interest, and source specificity indicates the source of the chemical in the body.[585] This combination of requirements is rarely achieved, and some trade-off

TABLE 10.17
Biomarkers Examined for Selected Occupational and Environmental Chemicals

	Biomarker		
Chemical	**Exposure**	**Effect**	**Susceptibility**
PAH	DNA adducts[a]	*hprt* mutation	GST-M1
	Hb adducts	*gpa* mutation	NAT-2
	Serum albumin (SA) adducts	*fes* oncogene activation	CYP1A1
	Urinary 1-HP[a]	*ras* p21 level	CYP2A2
	Sister chromatid exchange (SCE)	DNA single-strand breaks	
	SCE (high-frequency cells)	Chromosomal aberrations	
		Micronuclei	
1,3-Butadiene	Hb adducts[a]	*hprt* mutation	
	SCE	Chromosomal aberrations	
	Urinary metabolites	Micronuclei	
		ras oncogene activation	
Acrylamide	Hb adducts[a]		
	Urinary metabolites		

[a] Biomarkers for which cumulative data indicate the best correlation with ambient exposure.

is inevitable in order to obtain useful biomarker data in a timely manner. A few promising examples are presented in Table 10.17. The validation process for a biomarker involves determining the relationship between the biological parameter measured and both upstream and downstream events in the continuum, that is, the dose–response curve must be characterized.[211]

For the majority of chemicals of interest, occupational or environmental sources may not be the only source of exposure. The identification of the exposure depends on the concentration difference in the *exposed* individual or group, in comparison to the general population.[585] Over the last decade, the U.S. Centers for Disease Control and Prevention (CDC) has expanded its biomonitoring efforts in order to better characterize potential trends in chemical exposures to the general population.[166] In its recent National Health and Nutrition Examination Survey (NHANES) reflecting biomonitoring data between 2007 and 2010, the CDC applied a new sampling methodology, in which all Hispanics, not just Mexican Americans, were evaluated and race/ethnicity domains were combined (12–15 and 16–19 years of age) or split (40–49 and 50–59 years of age) to increase the number of participants aged 40+ and decrease 12- to 19-year-olds from previous cycles. The CDC released the National Report on Human Exposure to Environmental Chemicals, which provided a cumulative analysis (containing all the results from previous reports) and new data, including that for 75 new environmental chemicals for years 2003–2004.[586] The Fourth National Report on Human Exposure to Environmental Chemicals represents exposure information and separate analyses by age, sex, and race/ethnicity on 212 environmental chemicals in people who had blood and urine samples since 1999.[586] In addition, the most recent Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables,[587] provides new data since the release of the fourth report, including updated tables for 119 chemicals in the fourth report[586] and tables for 34 new chemicals. The national exposure information identifies which chemicals are within Americans in measurable quantities; determines whether exposure levels are higher among population subgroups; determines how many Americans have levels of chemicals above recognized health threshold levels; establishes reference ranges that define general population exposure so unusual exposures can be recognized; assesses the effectiveness of public health efforts to reduce population exposure to selected chemicals; and tracks over time trends in U.S. population exposure.[588]

These data have been proven to be very useful in understanding national trends of chemical exposure, as well as the effectiveness of laws intended to restrict environmental emissions. Biomonitoring of lead and persistent organic pollutants are probably the two best examples of how biomonitoring can confirm the reduction of human exposures as a result of restrictions of environmental emissions. Blood lead measurements in the population were important in identifying lead in gasoline as a significant source of human lead exposure and documenting the reduction in blood lead levels in the population as a result of removing lead from gasoline and other products in the United States.[588] In the United States, the mean lead blood concentration in children during the consecutive phases of NHANES II, III, and IV in 1976–1980, 1988–1991, and 1991–1994 were 150, 36, and 27 μg/L, respectively.[166,589] Serum cotinine levels in the early 1990s found more widespread exposure to environmental tobacco smoke (ETS) in the United States than previously thought and additional measurements in 1999 and 2000 documented major declines in exposure to ETS as a result of public health

actions in the 1990s.[588] The results of biological monitoring for evaluating the background contamination or the trends regarding contamination have also been used to compare internal exposure to organochlorine compounds or the trends in the concentration of dioxins in breast milk.[166,590–592]

With the increased analytical sensitivity to detect chemicals in various biological media and the increasing use of biomonitoring data in an integrated approach for epidemiologic studies, there is a substantial need to design and interpret such data that would support environmental health decision- and policy-making and provide informative risk communication to the public. Improved methods to provide quantitative links between biomonitoring information and the potential for adverse health risk would certainly assist with human health risk evaluations. Several organizations have offered guidance to enhance the application of biomonitoring data in these kinds of assessments.[593] The National Research Council (NRC) committee on Human Biomonitoring for Environmental Toxicants in the report *Human Biomonitoring for Environmental Chemicals* provides guidance on designing and conducting studies as well as research to enable interpretation and reporting of biomonitoring results to the individuals measured and the public.[594] The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) published a report, *Guidance for the Interpretation of Biomonitoring Data*, which includes a framework for interpreting human biomonitoring data. In addition, the International Life Science Institute's Health and Sciences Institute (ILSI/HESI) biomonitoring committee seeks to identify and refine effective biomonitoring tools and data to characterize exposures to chemicals.[593] More recently, the International Council of Chemical Associations' Long-Range Research Initiative (ICCA-LRI) sponsored a workshop, *Twenty-First Century Approaches to Toxicity Testing, Biomonitoring, and Risk Assessment*, and identified research needs to close the gap between interpretation and understanding of emerging biological data and rapid technology advancements.[595] As noted by the National Academy of Sciences (NAS), a critical prerequisite for meaningful use of data from new technologies will be to distinguish between the changes representing perturbations in cells or tissues that may resolve themselves through normal homeostatic mechanisms and those changes that represent a true activation of toxicity pathways that lead to disease.[596] Biomonitoring assay methods have incredible sensitivity to detect chemicals and metabolites in biological media in the parts per billion and even parts per trillion. An important question that arises with these enhanced detection limits are whether and how the measurable levels potentially link to predictions of adverse health outcomes and the relevance for characterizing true population exposures.[595]

Biomonitoring in Environmental Science and Health Risk Assessment

Biomonitoring data can be used to identify early indicators of a biological effect for assessing health risk and contributors of exposures, which can support rational health risk management planning. Biomonitoring has been used as a tool to validate other exposure assessment techniques. For example, in a review of 14 different concurrent or consecutive passive dosimetry-biomonitoring studies of agricultural workers, it was shown that the total absorbed dose estimated using passive dosimetry, human kinetics, and dermal absorption data for eight different active pesticide ingredients was similar to measurements of human urinary biomonitoring data.[597] In addition, biomonitoring data have been used to examine the validity of parental questionnaire responses of child touching and mouthing behaviors during outdoor play in comparison to data derived from video assessments.[382] Through biomonitoring of blood lead levels, it was confirmed that hand-to-mouth activities as determined by video monitoring were directly correlated to child blood lead levels and parental questionnaire responses did not accurately reflect children's oral behaviors, play habits, or play environment.[382] It has been suggested that biological monitoring for exposure assessment may also be a useful tool following chemical incidents.[598] Such application of biological monitoring has proved successful following chemical incidents involving mercury, methylmercury, PCBs, and dioxins,[599–605] as well as other relatively long lifespan biomarkers including sulfur mustard,[606] ethylene oxide,[607] dichlorvos,[608] and acrylonitrile.[609]

More recently, a novel screening tool, termed biomonitoring equivalents (BEs), has been proposed as a means to assist in the interpretation of biomonitoring data for public health risk assessment.[610–612] BE is the concentration or range of concentrations of a biomarker of exposure consistent with existing exposure guidance values and is analogous to biological exposure indices (BEIs) developed by the American Conference of Governmental Industrial Hygienists (ACGIH). More specifically, BEs are the concentrations of a chemical in the body that are equivalent to guidance values (e.g., RfDs, minimum risk levels [MRLs], tolerable daily intakes [TDIs], and their underlying toxicological points of departure) and are calculated using chemical pharmacokinetic properties that dictate the relationship between external and internal exposures.[610–612] The BE approach has also been suggested as an opportunity to use chemical-specific information and the relationship between the biomarker and critical dose metric to refine uses of default uncertainty assumptions in the risk assessment process.[610] The first application of the BE approach involved 2,4-dichlorophenoxyacetic (2,4-D) in which pharmacokinetic data and models were applied to existing health-based exposure guidance values from the U.S. EPA to estimate corresponding BE values for 2,4-D in plasma and urine.[613,614] Although these approaches can provide useful insights relating biomarker levels to regulatory requirements under the simplified assumption of chronic, steady-state exposures, these techniques are not applicable to reconstructing real-world exposure scenarios, because such scenarios often involve nonsteady, transient exposures with variable frequencies, durations, and intensities.

Recent Applications of Biomonitoring in Exposure Science

Research over the last decade has expanded the use of biomarkers in applying human biological monitoring data of exposure to individual disease and susceptibility information.[211,615,616] The advancement in analytical methods has allowed researchers to measure markers in a variety of biological specimens, including serum, cord blood, urine, feces, hair, bone, teeth, breast milk, saliva, and exhaled breath.[583,590,617–620] In addition, recent technical advances have also allowed researchers to use biomarkers to address questions about the effectiveness of personal protection equipment and engineering controls in preventing exposures in the workplace,[618] contribution of different exposure pathways on biomarker patterns,[574] effect of genetic polymorphisms on biomarkers of mutagenicity,[616,621] impact of personal activities on individual exposure,[164,590] and correlation of biomarkers of exposure and disease risk.[620,622]

Research efforts, such as the NHEXAS,[623] the CTEPP,[624] and other U.S. EPA programs, have focused on developing databases of exposures of human populations to a wide range of pollutants in air, water, food, soil, and indoor/residential environments, over a wide range of space and time scales. For example, the University of California, Berkeley, CHAMACOS collected biomarkers in farming communities for pesticides and other important pollutants from mothers and their newborn children from conception through early childhood.[163] Data from NHEXAS have been used to understand lead, phenanthrene, naphthol, and chlorpyrifos environmental concentrations in outdoor air, soil, indoor air, dust, dermal, water, beverages, and diet solids in relation to urine and blood concentrations.[164] In particular, these data were used to evaluate how personal activities and lifestyle factors (e.g., seasonal differences, ventilation [window, central air/heat], home environment [paint, cement, carpet, fireplace], pesticide use, garden care, smoking, gas grill use, vacuuming method, schedule [work at home], and personal hygiene) may contribute to exposure to the specific chemicals within the home environment.[164] Georgopoulos and colleagues summarized and assessed the feasibility of applying exposure reconstruction approaches to some of the most comprehensive national databases that include complementary exposure and biomarker data.[625]

The measurement of chemicals and biomarkers has revolutionalized the field of exposure assessment. Current challenges with the increasing analytical sensitivity of detecting chemicals in various biological media and the more widespread application of these tools in population-based studies involve the interpretation of these data for minimizing exposure and health risks, as well as effectively communicating the risk trade-offs to the general public. The University of Michigan Dioxin Exposure Study (UMDES) is one study or series of studies that will likely serve as a model for future research integrating questions about complex environmental exposure pathways by which chemicals in soils, sediments, fish and game, homegrown produce, and household dust lead to increased body burdens.[279–282,626–628] This study was undertaken in response to concerns that discharge of dioxin-like compounds from the Dow Chemical Company facilities in Midland, Michigan resulted in contaminated soils in the Tittabawassee floodplain and areas of the city of Midland, and potentially led to an increase in residents' body burdens of polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs).[281] Environmental contamination was believed to result from an aerosol plume from historic incinerators that deposited PCDDs on surface soils downwind of the plant and through releases into the Tittabawassee River downstream of the plant from chloralkali operations during World War I. In addition to measuring soil, household dust, and serum dioxin concentrations, other parameters potentially influencing exposures and dioxin body burdens were assessed, including demographic factors (i.e., age, BMI, breast-feeding history, race, smoking), residence factors (i.e., location, use, time), work history, water activities, fish and meat consumption, and hunting activities. A principal focus of the study was to evaluate activities that involve contact with contaminated soils, river sediments, and household dust. These included living on contaminated soil, living with contaminated household dust, and pursuing activities in the contaminated water bodies and floodplain (boating, swimming, picnicking, hiking). Little evidence was found that these activities were associated with increased serum dioxins.[279] The study also demonstrated that direct environmental exposures cannot be properly assessed without careful control of age, sex, BMI, smoking, and breast-feeding; these demographic factors largely accounted for the variation in serum dioxin concentrations with little apparent contributions from environmental exposures.[279] Despite this, small but statistically significant differences in serum dioxin concentrations were detected in populations living in the Tittabawassee River floodplain and within the city of Midland.[280]

While the researchers cautioned that the study results of the UMDES could not be extended to assess the potential health risks to children living in areas of dioxin-contaminated soil, recent data on the pharmacokinetic characteristics of dioxins in children from the Seveso, Yucheng, and Yusho experiences offered some insights on this topic.[604,629–632] Using the internal dose model of dietary and soil PCDD/F exposures in young children developed by Kerger et al.,[629] the possible impact of oral and dermal uptakes from the contaminated soils and house dust on the serum lipid toxicity equivalents (TEQ) concentrations of children was evaluated.[632] Consistent with the reported findings of the UMDES for soil/dust and serum lipid TEQ in adults,[279] the internal dose model predicts that child residents exposed to 10–1000 pg/g TEQ using the 50th percentile uptake factor are not likely to show measurably increased TEQ body burdens from soil/dust ingestion and dermal contact.[279] This evaluation also supported the well-established contention that background dietary concentrations of PCDD/Fs are far more substantial contributors to child TEQ body burdens compared to soil and house dust ingestion.[53,213,633]

Biomonitoring Data for Forward and Inverse Analyses

Biomonitoring data have been applied in a *forward* mode of analysis, whereby biological doses and effects are assessed using exposure measurements and, more recently, in an *inverse* mode of analysis, in which biomarkers are used to estimate or reconstruct external exposures. The forward analysis utilizes direct application of environmental exposure, toxicokinetic and toxicodynamic models either empirical or mechanistic (i.e., physically and biological based), where as the reconstruction analysis requires application of numerical model inversion techniques in conjunction with toxicokinetic and/or toxicodynamic models.[625]

PBPK models are broadly applicable as tools for relating dose biomarkers to measures of population exposure and health risk.[168] This approach was evaluated in the EPA dioxin reassessment, in which PBPK models were used to evaluate the reasonableness of their earlier estimated cumulative dietary intake of dioxin compounds.[634,635] Wallace and Pellizzari[637] and Wallace[636] assessed the utility of using exhaled breath for estimating exposure and body burden for VOC based on PBPK models. Chinnery and Gleason[638] and McKone[559] used PBPK models of chloroform applied to breathe samples reported by Jo et al.[351] to determine the relative contribution of inhalation and dermal exposure routes for adults showering with water containing residual chloroform from disinfection.[350] More recently, measurements of serum lipid 2,3,7,8-tetrachlorodibenzodioxin (TCDD) concentrations in adults and/or children from Seveso, Italy; Vienna, Austria; and Yucheng and Yusho, China have been used to model the distribution and elimination of dioxins.[604,629,631,639–641]

Several factors can influence the feasibility of reconstructing exposures from biomarkers. These factors include the specificity and sensitivity of the biomarker in relation to the exposure event(s); biochemical properties and biological factors (e.g., genetic polymorphisms) that impact the absorption, distribution, metabolism, and elimination; and various exposure characteristics (e.g., frequency, magnitude, duration, pathway), as well as supplemental exposure information (e.g., behavior, activities, macro- and microenvironment source locations, personal monitoring). Inherent in the use of biomonitoring data in these frameworks is the assumption that the relative magnitude and variation of the biomarker in the biological media are representative of the external chemical exposure. With this general assumption, reverse dosimetry methods have been used to estimate daily intakes of chemicals (i.e., phthalates, bisphenol-A, pesticides) to the corresponding biomarker concentrations in population surveys.[642–644]

While all of these factors are considered, the applicability and adequacy of the forward model (e.g., toxicokinetic/toxicodynamic model) and the efficacy of the computational inversion technique for reconstruction are the two most important factors for use of biomonitoring data for exposure reconstruction.[625] Reverse dosimetry or exposure reconstruction from biomonitoring data was recently used to demonstrate the application of PBPK modeling in estimating population exposures to trihalomethanes from biomonitoring results.[645] However, even with detailed information from national studies, such as the NHEXAS and the NHANES, it is not necessarily enough for detailed reconstruction of exposures under various conditions. For example, information about the last urinary void time associated with urinary biomarker samples can significantly improve interpretation of urinary biomarker data.[625]

Many compounds have short half-lives relative to the intervals between exposures and significant intraindividual, and within-day biomarker variability can exist due to fast chemical absorption and elimination. Reverse dosimetry may not be appropriate if the time frame of exposure characterization is not appropriately matched to the health outcomes of interest. For example, if the health outcome of interest is a chronic disease, a single measurement of a transient biomarker is not likely to provide accurate characterization of an individual's exposure leading up to the disease.[646] However, pilot studies to examine serial biomarker performance in relation to exposure patterns can help determine biomarker variability and timing to inform exposure characterization strategies and sample size requirements.[647] Incorporating such information in future biomonitoring study designs would be beneficial for these types of reverse analysis.

Other Emerging Applications of Biomarkers

Biomarkers are an important component of the emerging discipline of molecular epidemiology, which seeks to expand the capabilities and overcomes the limitations of classical epidemiology by incorporating biological measurements collected in exposed humans.[211,648] Biomarkers have also recently been suggested as an important potential tool in the emerging science of characterizing the *exposome*, which represents the totality of exposures received by a person during life and offers the potential to investigate environmental causes of chronic diseases.[649] Early efforts at utilizing biomarkers to make quantitative estimates of exposure and to predict human cancer risk were made by Ehrenberg and Osterman-Golkar.[650] Using ethylene oxide as a model xenobiotic, these investigators explored the use of macromolecular reaction products (i.e., Hb adducts) as internal dosimeters. By employing Hb adduction data, they predicted the level of ambient ethylene oxide that would correspond to a tumorigenic dose of γ-radiation, which they termed the *rad-equivalent dose*. Seminal work in the area of biomarkers as applied to the molecular epidemiology of cancer was performed by Perera and Weinstein,[651] who proposed the use of such techniques to identify environmental contributors to human cancer incidence.

Persistent and nonpersistent chemicals can react with biomolecules such as DNA, Hb, or fatty acids to form biomolecular adducts. By using these adducts as a surrogate for exposure, a greater length of time (dependent on the life of the adducts) can pass after exposure before measurements are collected.[583] Biomarkers with half-lives of 7 days or longer exhibit physiologic dampening of fluctuations in external

contaminant levels and can offer advantages when compared to short-lived biomarkers or exposures assessed by air monitoring.[652] Ehrenberg and Osterman-Golkar first proposed using Hb adducts to monitor the internal dose of alkenes and epoxides such as ethylene oxide over two decades ago.[650] This methodology has since evolved into a widely used and highly sensitive technique for quantitating N-terminal Hb adducts of a variety of xenobiotic metabolites in human blood. Hb adducts have been employed as internal exposure biomarkers for aromatic amines, nitrosamines, PAHs, and other compounds.[211]

Toxicogenomics-based methods have recently been used in laboratory settings to develop biomarkers of exposure, early biologic response, and susceptibility.[176] Toxicogenomics is a broad field that seeks to define, on a global basis, the levels, activities, regulation and interaction of genes, mRNA transcripts (transcriptomics), proteins (proteomics), and metabolites (metabolomics) in a biologic sample or system.[176] The approaches have been used for classifying exposures to a variety of chemicals and drugs, for example, hydrazine, 2-bromoethanamine, lead, acetate, cadmium, and acetaminophen based on mechanism of action and dose; they have been used for classifying health outcomes for cardiovascular disease and cancer based on disease status and severity.[176,653–666] The primary basis of classification and discovery in these studies is the molecular signature. Once the discriminating elements of the molecular signature are identified, biologic function can be inferred by mapping components to known biologic pathways and verifying functionality in follow-up studies.[176] However, some of the greatest challenges with this technology is that background levels of expression and variability for mRNA transcripts, proteins, and metabolites in human tissues are currently not known, but must be defined if toxicogenomic methods are to be used to assess personal exposures in epidemiologic studies. Expression levels are expected to vary widely because of differences in diet, lifestyle, health status, and genetic predisposition.[176] Despite the enormous promise of toxicogenomics for advancing our understanding of the relationship between environmental exposure and disease, the challenge has been, and will continue to be, the development of genetic and biologic markers that are predictive of adverse health outcomes in both experimental and human studies.[176]

One such area of active research with respect to biological markers for environmental contributions to disease is that of epigenetics.[667–671] Epigenetics refers to any change in gene expression that is stable between cell divisions but does not involve changes in the DNA sequence of the organism. Mechanisms involved with epigenetic changes include methylation of cytosine residues in the DNA, remodeling of chromatin structure, regulatory processes mediated by small RNA molecules, and other gene silencing pathways.[670] However, one of the most significant challenges with this area of research is understanding the *normal* epigenome in regard to the distinction between adaptive and adverse epigenetic changes. Without detailed characterization of the epigenome, including stable and variable methylation sites, it is not possible to evaluate whether an epigenetic alteration is an adverse effect from exposure to an exogenous agent or is part of the normal epigenetic variability.[668] As described in a workshop by the National Academy of Sciences' Standing Committee on Use of Emerging Science for Environmental Health Decisions *Use of Emerging Science and Technologies to Explore Epigenetic Mechanisms Underlying the Developmental Basis for Disease*, epigenetic testing is not yet sufficiently validated for the regulatory process because (1) no single test is ideal for epigenetic effects, (2) normal methylation patterns and long-term effects are not well understood, and (3) standardized tiered screening scheme to prioritize chemicals is lacking.[668]

Statistical and Analytical Issues

Despite the use of precise and reproducible analytical methods, we often do not have enough data of chemical concentrations to estimate exposure with great certainty. Because of resource availability, over the past 20 years, it has often been the case that a single round of analytical results or samples collected for other purposes serves as input and the surrogate for long-term or lifetime exposure.[672] As noted previously, chemical concentrations vary over both time and space, which makes the task of dose estimation all the more difficult.[563] For instance, using the (estimated) average dose to predict the typical lifetime dose may seriously overestimate or underestimate the actual dose. In addition, the average dose may be less important in the biological scheme of things than peak exposures or exposures at specific times (i.e., developmental effects) and ought to be considered as such in the evaluation of exposure.[174] Techniques do exist for estimating long-term exposure from short-term data,[673–675] but the reliability of these estimates is uncertain. Similarly, a variety of mathematical or bench-scale models exist that have been used to estimate exposure in the absence of measurements or long-term monitoring data.[676] As has been noted on several occasions, "all models are wrong, but some are useful," and risk assessors should carefully evaluate mesoscale and microscale models, as well as model outputs, for relevance and accuracy. Often, field measurements can serve as useful and relatively inexpensive *reality checks* on model results.

Equally important in exposure assessment are the statistics used to analyze field data. Environmental data are most often lognormally distributed. Under such conditions, a geometric average is generally assumed to be a better measure of the central tendency of data than the arithmetic mean.[677] Despite this, the arithmetic mean (and the 95% upper confidence limit of the arithmetic mean) is typically used to identify environmental concentrations for use in exposure assessment. Since the advances in analytical chemistry have improved our ability to measure trace amounts of chemicals in different media and identify potential sources in some situations, less reliance should be placed on the use of mathematical models to predict the distribution of chemical and physical agents in the environment, and actual field data should be collected.

Another important issue in exposure assessment is how the analytical limit of detection (LOD) is handled in calculations. An agent reported as a nondetect may be treated as a numerical zero, or occurring at the LOD or some fraction of the LOD, typically one-half of the LOD or the LOD divided by the square root of two, for purposes of calculating statistics. The manner in which censured data are assessed may affect the outcome of the risk assessment process.[678–685] For instance, analysis of highly contaminated samples or samples containing interfering substances may result in high LODs. Under such conditions and in the absence of additional analysis, assuming that nondetects are present at one-half the LOD could result in the exposure assessment and subsequent risk assessment being driven by compounds that are not truly present in the environmental media. When such an approach is used on a site that may be only 2%–10% contaminated (based on surface area), the predicted severity of the level of contamination will be much higher than what actually exists.[677] In these cases, it is often appropriate to insert a value lower than one-half the LOD when conducting exposure assessment calculations.

The practical result of these decisions can be illustrated by considering the following 11 data points resulting from analysis of field samples: Nondetect (ND), ND, ND, ND, ND, 5, 6, 6, 8, 55, and 500 ppm. The results are lognormally distributed as expected. The detection limit is 0.05 ppm, and nondetects are assumed to be present at one-half the detection limit (0.025 ppm). Using these assumptions, the arithmetic mean of the dataset is 52.7 ppm, while the geometric mean is 1.3 ppm. The practical consequence of choosing one descriptor over the other may be to misidentify or mischaracterize the dose and ultimately the risk, and will influence regulatory decisions involving remediation and regulation.

CLOSING THOUGHTS

The field of exposure assessment has evolved significantly over the past 20 years. We have learned a great deal about where people are exposed to xenobiotics and the relative degree of exposure. Not that long ago, most of our concerns were about industrial chemicals in our water, ambient air, and the soil. Today, we know that indoor exposure to particles, vapors, and gases in the home (influenced by smoking) often represents the predominant source of exposure for most persons. Aggregate exposure assessment and biomonitoring have changed the field of exposure assessment tremendously and are moving research to look at more complex and less obvious sources and pathways of exposure. A greater portion of our work in the future will undoubtedly focus on better understanding the individual contribution that environmental and indoor sources have to personal exposures,[176,220,222] differential exposures among susceptible populations (e.g., children, elderly, disease compromised),[686] and biological markers that identify biologic events early in the exposure-disease continuum.

Of the four portions of a risk assessment, exposure assessment has made the biggest improvement in quality over the 30-year history of health risk assessment. Often, exposure assessments will contain less uncertainty than other steps in a risk assessment, especially the dose–response portion. Admittedly, there are a large number of factors to consider when estimating exposure and it is a complicated procedure to understand the transport and distribution of a chemical that has been released into the environment. Nonetheless, the available data indicate that scientists can do an adequate job of quantifying the concentration of the chemicals in the various media and the resulting uptake by exposed persons if they account for all the factors that should be considered.

There are at least 11 significant lessons we have learned about conducting exposure assessments in recent years. Had we not had to learn through experience, avoiding these lessons could potentially have saved the United States hundreds of millions of dollars and thousands of person-years of work.

First, experience has shown that in our attempts to be prudent, we placed too much emphasis on the so-called maximally exposed individual (MEI).[68,70,71,76] Often, the results of those analyses were misinterpreted by the public and/or misrepresented by some scientists or lawyers. Often, as a result, poor decisions were made by risk decision-makers.

Second, as we have learned how to accurately characterize the risks of exposure for about 95% of the population, more emphasis has been placed on evaluating the various special groups (e.g., Eskimos, subsistence fishermen, dairy farmers)[79,359,451,590,687–690] and potentially susceptible populations (e.g., children, fetuses, elderly).[162,165,169,370,374,376–379,583,686,691–702] Although the risk for these populations needs to be understood, the typical levels of exposure for the majority of the population should be the initial focus of the assessment. Risk managers need to understand the size of the exposed populations and the risks.

The third lesson is a variation of the second—do not allow the repeated use of conservative assumptions to dictate the results of the assessment. In recent years, many investigators have addressed this issue and have demonstrated its importance. Monte Carlo techniques can generally be successful in addressing this problem.

Fourth, we have learned that risk managers and the public want to understand the statistical confidence in our estimates of risk. Sensitivity analyses can yield important information about the critical exposure variables.[534–537,678,679,684,703] Furthermore, most risk assessments can benefit from analyses of both variability and uncertainty. Without these, risk managers are not fully informed.

Fifth, we have improved our techniques for statistically handling samples that have no detectable amount of a contaminant. Frequently, regulatory agencies have used the LOD of the analysis or one-half the LOD in the exposure calculations

relying on the premise that the contaminant might be present at that level. We learned that when such an approach is used (without reflection) on a site that may only be 2%–10% contaminated (based on surface area), the impact of a few samples on the results could lead us to improper conclusions about the level of risk to persons who live there or nearby.

Sixth, we have gained a significant degree of confidence in our ability to estimate historical exposures, so-called dose-reconstruction or retrospective risk assessments. Over the past 20 years, these assessments have been used in epidemiology studies to understand the likely exposure to workers and/or those in the community nearly 40–50 years ago based on estimated chemical usage and emission data, measured data, and models.[29,30,37,204,209,210,237,704–709]

Seventh, we now understand the need to quantitatively account for indirect pathways of exposure. For example, the uptake of a contaminant in water by humans due to ingestion is obvious (and direct), but the uptake of the same contaminant by garden vegetables due to watering or uptake via the inhalation of volatile contaminants from the water while showering are indirect pathways that had not always been evaluated in assessments. Perhaps the most important indirect route of exposure, which had not been considered before 1986 when regulating airborne nonvolatile chemicals, is the ingestion of particulate emissions that have deposited onto soil and plants and are subsequently eaten by grazing animals.[60,212]

Eighth, we have learned that children and their exposure patterns are unlike those of adults.[162,165,169,370,374,376–379,583,686,691–702] As some have said, in more ways than one, children are not miniature adults! Their intake of certain foods, percentage of time outdoors, proximity to carpets, and inhalation rates per body weight are all different.

The ninth lesson learned is to use biological monitoring to validate or confirm the predicted degree of human exposure. Over the past 20 years, analytical chemists have increased their ability to detect very small quantities of dozens of chemicals in blood, urine, hair, feces, breath, and fat.[164,166,168,452,453,573,581,583,585,588,590,615–622,639,652,690,710–714] For many chemicals, these data represent a direct indicator of recent exposure and, in some cases (such as PCBs and dioxins), chronic exposure. Validation of our exposure assessments should be one of the major areas of study during the next few decades (both through biomonitoring and molecular epidemiology).

Tenth, it has become clear that in most cases, the most significant risks due to exposure to chemicals occur in the workplace. Even though great strides have been made in industrial hygiene over the past 50 years, the doses to which persons can legally be exposed are much greater (often by a factor of 100) than those to which most persons not in those occupations will ever be exposed.

Eleventh, and perhaps most important, we have learned that (for most persons) exposures to chemicals and bacteria in the home pose a greater risk than to those in the ambient air or through the ingestion of water. Many fine studies conducted in the 1970s through the current day continue to show that in-home exposures to most chemicals are 2–20 times greater than that present in the ambient environment.[22,217,219,222,283,357,363,366,457,504,715–723]

We have come a long way in a short time. Several professional societies including the International Society of Exposure Science (ISES), Society for Risk Analysis (SRA), American Industrial Hygiene Association (AIHA), Air and Waste Management Association (AWMA), American Chemical Society (ACS), Society of Toxicology (SOT), International Society for Regulatory Toxicology and Pharmacology (ISRTP), and others have all placed an emphasis on improving the practice of exposure assessment. All indications are that the information we have gained has significantly improved the quality of recent risk assessments and it can be expected that due to better exposure assessments, future decisions by risk managers will be much better informed.

QUESTIONS

10.1 Describe ways in which biomonitoring data are being used to understand exposure and environmental health risk.

10.2 In case study, several pesticides were detected in soil surrounding a residence. Describe the various media and pathways in which a family may be exposed to these pesticides, as well as the different factors that would need to be considered to conduct a quantitative exposure assessment of this particular family. Include topics related to interpretation of environmental and biological sampling, behavioral and susceptibility factors, and pesticide contamination within various media besides soil.

10.3 Name five emerging areas involved with different aspects of exposure science.

10.4 What factors influence the uptake of chemicals from soil particles through the skin, and what are some of the important criteria for assessing experimental dermal absorption studies?

10.5 What methods have researchers used to quantify soil ingestion in children?

10.6 Name at least four different national databases that are available to identify activity and behavioral patterns and/or biomonitoring data for different demographics within the general population.

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11 Epidemiology for Toxicologists

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CONTENTS

INTRODUCTION

It is not by accident that a chapter on epidemiology has been included in a text on toxicology (Figure 11.1).

An important role of toxicology is to provide a basis from which to infer causation of an association of exposure to a chemical agent with adverse health outcomes as may be observed in epidemiological studies. The limitations of epidemiological studies include such factors as uncertainty in exposure classification, numerous factors that may confound interpretation, and potential exposures to multiple agents. Although toxicological studies can be carried out in a

"And it was so typically brilliant of you to have invited an epidemiologist."

FIGURE 11.1 And it was so typically brilliant of you to have invited an epidemiologist. (From William Hamilton, The New Yorker Collection, www.cartoonbank.com.)

controlled manner that limits potential confounding and lessens uncertainty regarding exposure, limitations do exist and include use of surrogate species or in vitro test systems, limited statistical power, and, typically, potentially higher exposures than those occurring in the human populations studied in epidemiological investigations. In evaluating the potential for adverse effects of xenobiotics and natural toxins on human health, there has been a long historical interaction between the fields of epidemiology and toxicology [1]. Often, associations of adverse human health outcomes with exposures to xenobiotic or natural toxins have been demonstrated to be causal based on the results of toxicological investigation. Conversely, observations of effects in toxicological studies have led to identification of those effects in exposed human populations based on epidemiological investigations. Just as toxicological data have traditionally held a principal role in the human health risk assessment process, the value and potential supremacy of epidemiological data have gained increasing recognition. Therefore, it is important if not necessary for toxicologists to have an understanding of the manner in which epidemiological studies are undertaken and interpreted and how the data from epidemiological investigations may be used in the processes of hazard evaluation and risk assessment.

Although it cannot be overemphasized that an understanding of basic epidemiological principles has always been of value to the practicing toxicologist, the consideration and use of epidemiological data in a regulatory context has gained significant ground in recent years. For example, two recent regulations, the 2007 European Union Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) and the U.S. Occupational Safety and Health Administration (OSHA) 2012 updated final rule for hazard communication standard (HCS), both emphasize the use of human data in establishing effect levels and in classifying chemicals for hazard communication, respectively.

REACH Guidance on Epidemiology Data

REACH guidance on information requirements and chemical safety assessment identifies human data as a valuable source of hazard information [44]. The guidance cites the advantages of human data as being the direct application to the human species; obtained, in most cases, from relevant exposure conditions; and often derived from a more heterogeneous population when compared to toxicological data. Epidemiological studies are noted as a major source of human data for hazard assessment under REACH. Despite the potential uncertainties inherent in evaluation of epidemiological data, such human data are recognized under REACH as the most relevant in assessing potential health hazard and risk to human populations. However, it is emphasized that any interpretation of epidemiological data be accomplished in consultation with experts in the methodology used in epidemiological studies as well as experts in the clinical aspects of the data under consideration. The REACH guidance illustrates the processes used for integrating human data into the derivation of critical derived no effect level (DNEL) and derived minimal effect level (DMEL) values.

Ensuring the quality of human data used in human health hazard assessment is necessary under REACH. REACH regulation relates specifically to evaluation of human information. Six adequacy criteria are provided for assessing

adequacy of human data. These are the following: (1) the proper selection and characterization of the exposed and control groups, (2) adequate characterization of exposure, (3) sufficient length of follow-up for disease occurrence, (4) valid method for observing an effect, (5) proper consideration of bias and confounding factors, and (6) a reasonable statistical reliability to justify the conclusion.

OSHA Guidance on Epidemiology Data

In 2012, OSHA modified the HCS to comply with the United Nations Economic Commission on Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (http://www.unece.org/trans/danger/publi/ghs/ghs_rev04/04files_e.html). The GHS states that "In addition to animal data and valid in vitro testing, human experience, epidemiological data, and clinical testing provide important information that should be considered in application of the GHS." In the OSHA HCS, it is stated that "epidemiological data and experience on the effects of chemicals on humans (e.g., occupational data, data from accident databases) shall be taken into account in the evaluation of human health hazards of a chemical." Further, the final rule states that

> Where evidence is available from both humans and animals and there is a conflict between the findings, the quality and reliability of the evidence from both sources shall be evaluated in order to resolve the question of classification. Reliable, good quality human data shall generally have precedence over other data. However, even well-designed and conducted epidemiological studies may lack a sufficient number of subjects to detect relatively rare but still significant effects, or to assess potentially confounding factors. Therefore, positive results from well-conducted animal studies are not necessarily negated by the lack of positive human experience but require an assessment of the robustness, quality and statistical power of both the human and animal data.

The OSHA HCS recognizes that epidemiological data are often key in evaluating the potential human relevance of effects observed in nonhuman studies:

> Route of exposure, mechanistic information, and metabolism studies are pertinent to determining the relevance of an effect in humans. When such information raises doubt about relevance in humans, a lower classification may be warranted. When there is scientific evidence demonstrating that the mechanism or mode of action is not relevant to humans, the chemical should not be classified.

Search for Scientific *Truth* Regarding Human Disease Etiology

The search for scientific *truth* regarding the causes of human disease (also referred to as causal inference) is a laborious multistep process, a winnowing of a large number of postulated hypotheses down to the few that can be supported with data derived from testing and observation. Success depends on the replication of results, coherence of evidence from many different fields, and, ultimately, an understanding of the underlying biological mechanisms of action. In evaluating the potential human health effects of chemical exposures, three major sources of scientific information are used by the courts, various government agencies, and the larger scientific community: experimental laboratory research, controlled clinical investigations, and observational epidemiology studies. These three are not mutually exclusive in method or thought; nonetheless, each makes a unique contribution toward understanding the etiologies of human disease and each has certain inherent limitations. Ultimately, the determination of causation depends on the demonstration of a meaningful elevated risk for the disease among those with the *exposure* and a biological explanation for the excess. The former can only be obtained via epidemiology studies; the latter usually comes from an interplay of information derived from experimental laboratory research and controlled clinical investigations.

Toxicology has become stylized to the extent that the format of research results can be both predictable and quantitative. This made it convenient for information derived from toxicology studies to be used in quantitative risk assessments and related regulations. This convenience facilitated a major concern regarding extrapolation beyond the data to make inferences about health effects for levels of exposure that were not administered (i.e., the much lower exposures observed in humans). The 2007 U.S. National Research Council report (*Toxicity Testing in the 21st Century: A Vision and a Strategy*) proposed relying on less animal studies and more in vitro methods using human cells in a high-throughput context in order to identify biological *toxicity* pathways that would be sufficiently perturbed to lead to adverse health outcomes [96]. Risk assessments would then shift from high-dose observations to identifying the doses below which an exposure would not likely result in pathology [6]. Furthermore, in vitro methods do not take into account detoxification processes that may occur in vivo. Therefore, epidemiology studies of human exposure (low) levels would become much more relevant in the risk assessment process.

Clinical investigators also administer measured doses according to a predetermined schedule, to humans, thereby eliminating the need to extrapolate between species. Other technical biases, at least in theory, are minimized by randomly assigning potential study participants to either the exposed group or the unexposed controls; however, humans are not passive participants in health research. At the very least, they must consent to be studied. Some do not, and key characteristics of those who do and those who do not may be markedly different, quite possibly to the extent of compromising the utility of the initial randomization. Furthermore, specific subgroups (e.g., only men or only those who are patients of a single clinician) may be eligible for inclusion in the research. For all of these reasons, *controlled* clinical trials are, at best, quasi-experimental research. This means that care must be exercised in extrapolating their findings too broadly.

The two major strengths of observational epidemiology research are that one, it studies humans and, two, it deals

with the effects of real exposures—actual levels, durations, and patterns of exposure to individual agents and to mixtures. If epidemiology studies are well done, they furnish results that reasonably can be extended to larger populations. Unfortunately, epidemiologists often handle exposure as a qualitative variable (either as a yes/no or some variation of high, medium, and low). This can limit the utility of the research results for those who require quantitative information. Even when afforded an opportunity to analyze a continuous exposure variable (e.g., serum concentrations of a low-level environmental contaminant), whose measurement is not inexpensive, epidemiologists are prone to analyze the data in qualitative (categorical) terms with cut points that may just be a few nanograms per milliliter (ng/mL). Occasionally, both qualitative and quantitative estimates are provided. In addition, because the research is observational in the sense that the investigators simply observe natural experiments and do not exercise control over the key variables, epidemiologists routinely must grapple with a number of technical biases that are largely transparent to those in the other two fields. If these biases (in particular, selection, information bias [misclassification], and confounding) are not adequately addressed during the study design or data analysis, the study results may be unduly imprecise and important associations missed (i.e., false negative) or conversely the biases result in overestimates of risk (false positive). Alternatively, the results may be relatively precise but precisely inaccurate, thereby also leading to interpretations that are incorrect. Some of these problems can be exacerbated if the epidemiologist utilizes secondary sources of data (data originally gathered for purposes other than the specific research project, possibly even for reasons unrelated to research), especially if the methods for the original data collection process were poorly documented.

One other point differentiates epidemiology from toxicology and clinical medicine research. To call oneself an epidemiologist does not require any specific set of educational requirements, testing, or state or national licensure. Avenues exist to obtain formal training through doctoral and postdoctoral graduate degree programs in epidemiology, but this does not prevent someone with minimum training to declare himself or herself an epidemiologist. Toxicologists have various board certification processes such as the American Board of Toxicology (Diplomate, American Board of Toxicology) or the American College of Veterinary Toxicology (board certification) that certify a measure of competency. Medical professionals (e.g., MD, DVM, DDS) have their respective examination processes for state licenses and board certification requirements. Beyond graduation with a degree in epidemiology (MPH, MS, PhD), the closest *approval* process within the U.S. epidemiology community is voluntary membership in the American College of Epidemiology (ACE) that assigns a committee to review membership applicants based on their curriculum vitae to determine whether they can be credentialed as *members* or *fellows*. Only a handful of epidemiologists ever took the ACE examination that was discontinued in the late 1980s. A selective American Epidemiology Society exists for senior level epidemiologists. Other societies may have different requirements but not require examination. Epidemiologists have different strengths based on their experiences. Some enter the field with strong statistical skills; others have extensive training in human biology. Others arrive from the social and behavioral sciences. The formal graduate degree programs in epidemiology require a proficiency in both biology and statistics, but individuals with any level of education can gather a dataset, analyze it, report the results, and call their effort epidemiology research. The rule of thumb for the consumer of epidemiology results therefore, especially if a report appears in other than the peer-reviewed literature, is *caveat emptor*. Furthermore, peer review should not necessarily be viewed as a process that leads to epidemiologic research excellence either. Nor should a journal's impact factor [70]. The ultimate peer reviewer is the reader of an epidemiology paper. *Caveat emptor* indeed.

Historically, epidemiology has been defined as the study of the distribution and determinants of disease in humans [85]. Although commonly used, this definition is incomplete. Epidemiologists certainly search for the factors associated with human disease but they also attempt to identify both interventions that likely will benefit those who are at risk for getting the condition (perhaps because of unique patterns of exposure to combinations of putative agents or a genetic predisposition for reacting adversely to such exposures) and treatments that will help control or cure any significant pathology once it occurs. They also, implicitly or explicitly, try to determine which agents do not cause a specific disease, which interventions will not be successful, and which treatments are not effective.

As with toxicology and clinical research, epidemiology practice is an amalgam of subject-specific knowledge and methods. And just as clinical specialties and areas of expertise in toxicology have evolved with the growing complexities of each of those two fields, epidemiology is divided into a number of overlapping subgroups: occupational, environmental, reproductive, cardiovascular, cancer, infectious disease, molecular, genetic, nutritional, medical device, clinical, etc. Some of these are subdivided still further; for example, AIDS is a subcategory of viral, which, in turn, is a subset of infectious disease. Although certain knowledge and techniques may be unique to a subgroup, many concepts are common across the disciplines. As practiced today, epidemiology research sometimes involves multidisciplinary teams (e.g., epidemiologists, statisticians, physicians, geneticists, industrial hygienists, sociologists) as research studies have increased in population sizes and scientific complexities. Very large multi-institutional studies are not uncommon.

Primary Objective of Chapter

The primary objective of this chapter is to introduce those concepts that span epidemiology so toxicologists might become better consumers of the epidemiology literature. The topics include measures of disease frequency, measures of risk and association, study methods, and issues. All of

these are interrelated so the order of presentation is somewhat arbitrary, but the first three set the stage for the last one, in particular the key issues impacting validity—selection, information bias (misclassification), and confounding. Each of these in turn is presented to show how it can bias the measures of association. The intent is to make the readers more sensitive to possible biases so they might determine for themselves how well the investigators recognized a potential problem and addressed it during study design, data collection, and data analysis—and during data interpretation.

Several of the major points are illustrated with examples from the epidemiology literature or, to a lesser extent, with toxicology or clinical references. In part, this was done to emphasize the point that all science—experimental, quasi-experimental, and observational—is based on assumptions that may not be correct. Some of these assumptions are relatively innocuous. Conversely, some are so important that, if violated, the process is severely biased and any policies based on that science also are likely flawed. Those are the two poles of a continuum, but validity is not a dichotomous variable like pregnancy. Evaluating epidemiology research very rarely leads to *black or white* conclusions but rather varying shades of gray. The challenge for the consumer of scientific reports is to determine which data provide useful information, which data do not, and which data lie somewhere in between—intriguing enough to warrant additional research but not strong enough to merit intervention. This winnowing equates to first identifying potential problems that may have compromised the validity of the research; second, determining the probability to which the problems occurred; and, third, estimating the impact, if any, they had upon the results.

This chapter is by design a very limited overview. For those who wish to have a more detailed presentation of epidemiology, the list of references includes a number of published textbooks [31,55,115,116,124]. Note that, throughout this chapter, key *terms of art* are highlighted. Most can be found in Last's *Dictionary of Epidemiology*, an invaluable resource to any technical library [84]. Additionally, found in Appendix A at the end of this chapter is the Strengthening the Reporting of Observational Epidemiology Research (STROBE) statement, which provides an outline that can be used for critiquing epidemiology reports [134,136]. Found in Appendix B are five examples from the scientific literature that illustrate, in more detail, some of the epidemiologic concepts presented in this chapter. Three of these examples are based on the authors' epidemiology experience.

MEASURES OF DISEASE FREQUENCY

Data, information, and knowledge are related but not equivalent terms. Data are gathered by the investigator during the course of an investigation. Information is analyzed data. Knowledge is meaningful information that can be used to predict or solve problems. By way of analogy, data are bricks, information the wall, and knowledge the building. Just as good bricks can be put together poorly to build an unstable wall, so can data be valid but aggregated in a fashion that produces useless information. Pseudoknowledge may be a function of either bad data or useless information, just as a structurally unsound building may result from poor-quality bricks or an unstable wall. When information is biased (with error), the term information bias will be used in this chapter. Misclassification occurs as a result of the biased information.

Prevalence and Incidence

For the epidemiologist, the two general types of data are *prevalence* and *incidence*. Prevalence is what is observed at a single point, a snapshot of what is prevalent, what exists, at a specific point in chronological or biological time. For example, the number of toxicologists currently employed by the federal regulatory agencies is prevalence data. Incidence is the number of incidents, of new events, that develop over time. Because it represents a delta (a change), incidence data have to be gathered at two or more points in time. In a sense, if prevalence is a snapshot, incidence is a movie. The number of toxicologists hired by the federal agencies in any given year is an example of incidence data. They did not work for the agencies at the start of the year (observation point one), but they did work for the government later in the year (observation points two or more).

Parenthetically, when epidemiologists speak of prevalence data, they are usually referring to *point prevalence*, but they may mean *period prevalence*. Period prevalence is a combination of what exists at the beginning as well as what occurs during a specified period. The number of toxicologists who were employed by the federal agencies at any time during a given year is period prevalence data. It includes those who were working at the beginning of the year (point prevalence data) and those who were hired during the year (incidence data). Period prevalence may or may not be the same as the number of those who were employed at the end of the year (more point prevalence data) because some toxicologists may have left government employment during the period of observation.

Whether data are period prevalence or incidence can sometimes be difficult to discern because both refer to events occurring during a span of time. The key is whether the data represent a combination of existing and new events (period prevalence) or just new events (incidence). Unless otherwise noted, the term *prevalence* is used in this chapter as a synonym for *point prevalence*.

The difference between prevalence and incidence data is important for at least three reasons. One, incidence data can be used to evaluate cause and effect; prevalence data usually cannot, at least not without additional assumptions. However, unlike incidence data, prevalence is used to measure the disease burden in a population. Two, because prevalence data can be gathered at a single point in time, it is much easier to obtain; therefore, many reports in the medical literature are based on prevalence data. Three, the medical literature can incorrectly use the two terms interchangeably; as a consequence, reports that use valid prevalence data to develop nonsense information about cause and effect appear in even the most prestigious journals.

Although the two terms are different, they are related [64]. Under steady-state conditions (i.e., incidence rates and disease duration are stable over time), prevalence (P) is a function of both the incidence (I) and the duration (D) of the disease where $P/(1 - P) = I \times D_{average}$. The quantity $P/1 - P$ is the prevalence odds. For small prevalence where $1 - P$ approximates 1 (e.g., $P < 0.1$), the equation could be rewritten as $P = I \times D_{average}$. For any prevalence proportion, $P = I \times D_{average}/1 + I \times D_{average}$.

What this means is that a chemical may not cause a disease, may not increase the incidence of the disease, but it may still be associated with a higher prevalence of the condition. Whether that is good news or bad depends on the circumstances; for example, the incidence of diabetes may be quite stable in a population, but if that population is given access to a chemical called insulin, then the prevalence of the condition likely will increase dramatically. It will increase because the insulin extends the duration of the disease by allowing more of the afflicted to live longer.

Conversely, the prevalence of minor birth defects (prevalence because the events are measured at a single point in biological time: birth) could be lower among live children born to women exposed to some agent, not because the agent prevents the development of minor defects in utero but because the agent causes major malformations, including some among those fetuses who happen to have minor defects. If the major malformations lead to early spontaneous abortions, the *incidence* of minor defects might be quite stable, but the duration in utero of those with both types of congenital defects would be shortened and fewer newborns with minor problems would be observed at birth. The *prevalence* of minor defects among live births would be lower.

Figures 11.2 and 11.3 illustrate these points. In both figures, a group of six patients (A through F) is observed for 2 years. In Figure 11.2, the condition is time limited; it spontaneously resolves, it is cured through some treatment, or the patient dies. The point prevalence at the initial baseline observation (year 0) is one (patient A). Two additional cases subsequently occur (patients C and F) and all three resolve before year's end. At the end of year 1, the point prevalence is zero, the incidence is two, and the period prevalence is three. During the following year, two more cases develop (patients B and D) and one patient dies (patient D) of an unrelated cause before the end of the year. At year's end, therefore, the point prevalence is zero, the incidence is two, and the period prevalence is also two.

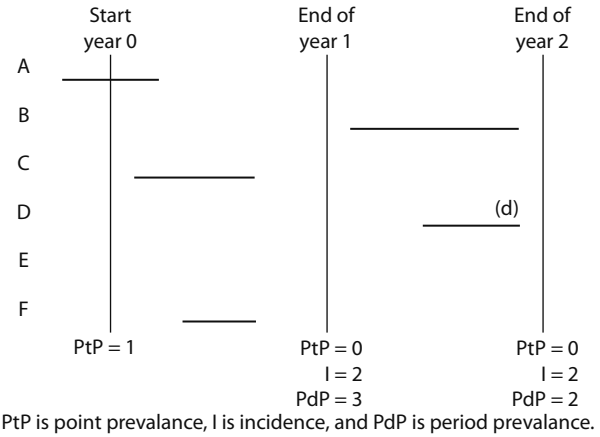


FIGURE 11.2 Prevalence versus incidence (time-limited condition).

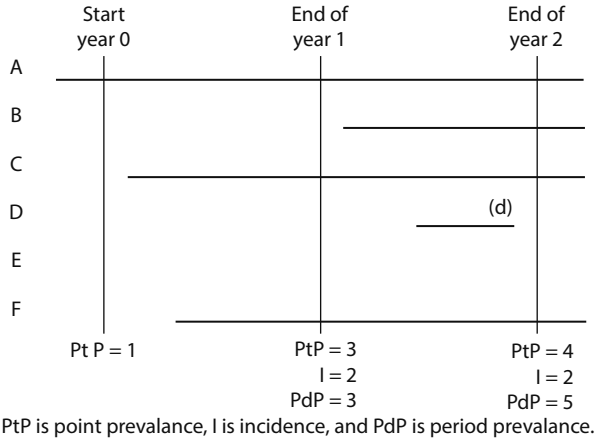


FIGURE 11.3 Prevalence versus incidence (condition chronic).

In Figure 11.3, the condition is chronic, perhaps because, like diabetes, it has been extended through treatment. Note that the incidence is exactly the same as in the previous example. It is two in each year; however, the extended duration has impacted both measures of prevalence. The point prevalence at the time of the three observations (baseline, year 1, year 2) is, respectively, one (patient A), three (patients A, C, and F), and four (patients A, B, C, and F). The period prevalence for the first year is three. For the second, it is five even though patient D died before year's end. Each of these three measures of disease provides valuable information, but using either type of prevalence data for interpretations about cause and effect depends on assumptions about *incidence time* (i.e., when the health event actually occurred) and disease duration that are often untestable or incorrect.

Rates

Technically, incidence and prevalence refer to numerator data; however, in both the epidemiology and medical literature, these terms often are used interchangeably with, respectively, *incidence rate* and *prevalence rate*. An incidence rate is the number of new events of a disease in a defined population that occur during some specified period of time. A prevalence rate is the number of cases of disease observed in a defined population at a point in time. In both, the numerator is a subset of the denominator. Obviously, errors of count in either the numerator or the denominator can impact the accuracy of a rate; nonetheless, in some technical reports, the former may not be a subset of the latter, and the description as to how either was compiled may be less than clear.

Rather than presenting a rate as the actual numerator (the exact number of new events observed) in comparison to the actual denominator (the precise count of the group under study)

at or during the period of observation, for convenience, a rate is usually given as the number of cases per 100 or per 1,000 or per 10,000. For example, if the study group had 486 persons and 5 new events occurred during a 12-month period of observation, then the incidence rate might be presented 1.0 per 100 per year (5 divided by 486 times 100) or, alternatively, as 10.3 per 1000 per year.

As opposed to toxicology, in epidemiology the study groups can be either *fixed* or *open* (sometimes referred to as *dynamic*). In a fixed study group, those included are defined at the start and followed over time. If no losses occur during the period of study, the group may be called a *closed population*. In this situation, when the population size is fixed, the rate becomes the proportion of people who become cases among those in the population at the start of the interval. This is referred to as an *incidence proportion* and it is dimensionless. In an open study group, individuals may be added or lost during the time of study. Just the events that occur and just the time that passes during the period each individual was under observation are counted. This so-called person-time experience assumes that observing 10 people for 1 year is the same as observing 1 person for 10 years. In some situations, the assumption is appropriate; in others, it may not be. The determination of which is which depends on the underlying biological model. Occupational epidemiologic studies are almost always open as each employee followed has his or her own hire date, termination date (quit, retired, lost to follow-up), and perhaps end-of-follow-up date (lost to follow-up, deceased, followed to end of study).

MEASURES OF EFFECT AND ASSOCIATION

To the general population, the concept of risk can seem straightforward. It is interpreted as a probability. *Risk* is defined as the probability that a disease develops in a person within a specified time interval. However, it can be easily misunderstood since it may be used without a clear time reference. To say a man over 65 years of age has an 8% risk of dying from cardiovascular disease does not place such risk in a time period. Is it within the next week, month, year, or remaining lifetime? The following measures of risk and association provide epidemiologic perspective.

Absolute Risk

It is an immutable fact of life that we are all going to get ill at some time and ultimately we are all going to die. On a personal level, the questions for each of us are by what disease and when? Epidemiologists are also interested in those questions, but they are particularly interested in whether the disease occurs more frequently or more severely in association with some type of exposure. In other words, when it comes to identifying the causes of disease, what is at issue is whether the *absolute risk* for a specific disease among the exposed is greater than the absolute risk of that same disease in the unexposed.

Relative Risk

Epidemiologists speak of the *counterfactual ideal*, which is comparing the same exposed person with his unexposed counterfactual experience [65]. That is, compare the experience of a person with exposure to the same exact experience during the same time period but without the exposure. Of course, this is not possible as time cannot be duplicated but it implies the person has the same experience twice, once with the exposure and once without the exposure. Likewise, this counterfactual ideal experience could be considered with a population.

Although the counterfactual experience is not possible to obtain whether on an individual or population basis, epidemiologists attempt to mirror it by comparing a population with exposure to a different population without exposure assuming the two populations have comparable risk for disease except for the exposure. *Absolute effect measures* are differences in occurrence measures and are not dimensionless. The *effect* of exposure can be measured as the risk difference (RD) (*incidence proportion* exposed minus *incidence proportion* unexposed) or the *incidence rate difference* (*incidence rate* exposed minus *incidence rate* unexposed).

Relative effect measures are ratios of occurrence measures and are dimensionless. A *relative measure of effect* for risk is determined by the RD divided by the risk in the unexposed:

$$\text{Relative effect} = \frac{\text{RD}}{R_{\text{unexposed}}} = \frac{R_{\text{exposed}} - R_{\text{unexposed}}}{R_{\text{unexposed}}} = \text{Risk ratio} - 1$$

$$\text{where Risk ratio} = \frac{R_{\text{exposed}}}{R_{\text{unexposed}}}$$

Epidemiologists ignore subtracting the 1 (since this is a baseline value) resulting in the *risk ratio* (RR) having the numerical range from zero to infinity.

In a similar manner, incidence rate data can result in a *rate ratio*:

$$\text{Relative effect} = \frac{\text{ID}}{I_{\text{unexposed}}} = \frac{I_{\text{exposed}} - I_{\text{unexposed}}}{I_{\text{unexposed}}} = \text{Risk ratio} - 1$$

$$\text{where Risk ratio} = \frac{I_{\text{exposed}}}{I_{\text{unexposed}}}$$

It is important to remember that risks are not directly comparable to rates because of different units. The risk ratio and rate ratio are often called *relative risks*. An risk ratio is proportionate to a rate ratio based on the time (T) at risk:

$$\text{Risk ratio} = \frac{R_{\text{exposed}}}{R_{\text{unexposed}}} = \left(\frac{I_{\text{exposed}}}{I_{\text{unexposed}}}\right)\left(\frac{T_{\text{exposed}}}{T_{\text{unexposed}}}\right)$$

A risk ratio and a *rate ratio* (*relative risks*) are key measures of association between exposure and disease. If the relative risk is appreciably greater than 1 among those with a particular

exposure, it is possible that the agent may be causing the disease. Maybe. Conversely, if the relative risk is below 1, the agent may be protecting against the disease. Maybe. And, if the relative risk approximates 1, there may be no meaningful association between the two variables. Once again, may be. *Maybe* is an important caveat in all three situations because how well the *apparent relative risk* (the number derived as the result of a particular investigation) corresponds to the *true relative risk* (the actual underlying biological truth) depends not only on the statistical stability of the estimate of relative risk but also on how well the potential technical biases of selection, information bias (misclassification), and confounding were controlled in the study design, during data collection, and by data analyses.

Recall now that a measure of effect was based on the underlying counterfactual principle of what happens to one population under two entirely distinct conditions for which only one can occur. A measure of association refers to what happens in two distinct populations. It is possible that the two populations may be the same population studied under two different time periods. For example, measure the incidence of a specific cancer in a community before and after application of an aerial insecticide.

Standardized Mortality Ratio

In occupational cohort mortality studies, the measure of association may be provided as a *standardized mortality ratio* (SMR). Toxicologists often encounter occupational epidemiology study results in their review of specific chemical hazards; thus, an extended description is provided herein. The SMR is the ratio of the number of deaths observed in the study group to the number that would have been expected to occur in the absence of exposure if the exposure did not affect the distribution of person-time. Occasionally, the SMR is presented as a ratio of *observed to expected* (O/E) deaths and may be more easily understood in communication meetings to workers or the public when expressed as an SMR. The interpretation of an SMR parallels that of a relative risk. Historically, the O/E ratio was multiplied by 100 where an SMR = 100 was equivalent to observed deaths equal expected deaths. Over the past 20+ years the multiplication by 100 has gained disfavor such that an SMR of 1.0 is analogous to a relative risk of 1.0. Because the observed number of deaths occurs in discrete increments and the number of expected deaths is a continuous variable (i.e., the expected deaths might be a biologically impossible number such as 1.27365…), by convention, many epidemiologists will not calculate an SMR if the number of observed deaths is less than 2 [31]. They may simply provide the two numbers (the observed and the expected) or just offer a confidence interval. Sometimes, they will do neither and merely indicate that the numbers were too small to be meaningful.

The expected number of deaths in an occupational population is obtained by multiplying the stratum-specific mortality rates in the reference population times the corresponding number of person-years in the study population and then summing across all strata. At a minimum, these stratum-specific rates will be age, sex, race, and calendar-year specific. For many occupational studies, the mortality experience of a national white male population is used as a reference, even if a small number of those in the occupational cohort are of a different race or ethnic group. The assumption is that the calculations of expected deaths will be adequate. Sex-, race-, and disease-specific mortality rates are available for most general populations. Essentially, the mortality rates of the general population are assumed to be the desired counterfactual rates. However, there will be other important differences between the exposed population and the general population besides their basic demographic distributions. Using an SMR approach also means that the investigator is assuming that no one in the reference population was exposed to the same degree as the study population. If the exposure is relatively rare among those in the reference population, the assumption is probably reasonable because the mortality experience of those few who were exposed would have had very minimum impact on the population statistics. On the other hand, if the exposure is relatively common (e.g., something like chlorinated drinking water), then the assumption may be unreasonable and another type of study would have to be done to obtain valid information. The occupational study population may be different than the reference population with other habits including smoking, dietary preferences, access to medical care, socioeconomic status, etc.

Another important caveat of an SMR analysis is if subgroups of the occupationally exposed group have different distributions such as age or calendar-year. This comparison is often done to assess a dose–response trend. Because each subgroup's SMR is weighted by the person-time experience, there can be residual confounding if such person-time is substantively different. When the subgroups have little differences in person-time across the strata, then residual confounding is less likely.

Choice of the referent population usually encompasses both national and regional (e.g., state, multicounty) mortality rates. It is conceivable that SMR associations may be quite different based on the referent populations used. There are many examples in the literature when this occurrence happens. For example, Olsen et al. reported the lung cancer mortality experience of a large population of male chemical workers in Freeport, Texas [100]. Using the U.S. mortality rates, the SMR was 1.14 (95% CI 1.04–1.25). Using Texas mortality rates, the SMR was 1.13 (95% CI 1.03–1.23). However, using the regional five county mortality rates, the SMR was 0.92 (95% CI 0.83–1.00). This example illustrates the point that regional rates are generally preferred in the interpretation of occupational data.

Instead of using mortality data, similar calculations can be performed for a *standardized incidence ratio* (*SIR*). Instead of death being the outcome of interest, it is illness, oftentimes cancer, but the calculations and the resultant interpretations are basically the same. So, too, are the underlying assumptions. SIRs are often calculated when the observed cancer incidence of the cohort is determined through record linkage

with regional cancer registries and expected numbers are calculated using cancer incidence rates from the reference population, which is that covered by the regional cancer registry. Currently in the United States, every state has a functional statewide cancer registry system although their longevity differs greatly between states.

Proportional Mortality Ratio

On increasingly rare occasions, it may not be possible to define a cohort, but information exists on those in the group who have died. Company and union records have been used to conduct such analyses. The *proportional mortality* (or *morbidity*) *ratio* (PMR) is a measure of the relative importance of an individual category of disease *among those with any disease* to the corresponding proportions among a reference population. As such, both numbers in the ratio are numerator data. Although it is a convenient measure to obtain, it must be used with caution in etiologic research because it compares proportions and not rates. It makes the assumption that a higher proportion of a particular disease is the same as an increased frequency of that disease. Because a PMR calculation works like a teeter-totter, that assumption may be invalid. Although a higher proportion of a disease may be due to an increased incidence, it also may simply be a function of a lower frequency (and therefore a lower proportion) of some other condition, disease B. For example, a higher PMR for cancer among an occupational group with a certain exposure may mean that more of those with the exposure were developing (and dying) from cancer than those in the reference population, but it is also consistent with the interpretation that those with the exposure were *not* dying more often from cancer; they were just dying less often from noncancer events. In other words, in a PMR analysis, an apparently *adverse* finding may be spurious (e.g., solely a function of the *healthy worker effect*) [89].

Attributable Fraction

As previously defined, the relative measure of effect was defined as the RD between the exposed and unexposed groups divided by the risk in the unexposed group. The attributable fraction is defined as the RD between the exposed and unexposed groups divided by the risk in the exposed group [65]:

$$\text{Attributable fraction} = \frac{\left(R_{\text{exposed}} - R_{\text{unexposed}}\right)}{R_{\text{exposed}}}$$

The attributable fraction can be understood as the proportion of disease in an exposed group that may be caused by the exposure if there were no biases in the determination of the risk. For example, if the risk in the exposed group was 0.15 and the unexposed 0.12, then the attributable fraction = 0.20, which is 20% of the risk among the exposed. A second calculation is required when applied to the target population from which the exposed group originated. The overall attributable fraction needs to take into account the proportion of all cases in the source population that is exposed. For example, if the proportion of cases in the population that is exposed is 8%, then the overall attributable fraction for the population is the attributable fraction among the exposed (0.20) multiplied by the proportion of exposed cases in the population (0.08). This equals 0.016, or 1.6% of all cases in the population are attributable to the exposure. In other words, among all cases in the population, 1.6% are attributable to the exposure. A major caveat to the interpretation of an attributable fraction is the fact that although the calculation is straightforward, the term *attributable* is dependent upon knowing causation is certain. In most instances, this is not the situation and thus any calculation needs to clarify this point. To further confuse the picture, different terms are used in the epidemiology literature. The aforementioned attributable fraction has also been called an attributable risk, attributable risk percent, and attributable proportion. Attributable risk has also been defined as the RD or the rate difference. It is always best for the definition to be clearly defined and understood. Because of this confusion, Rothman et al. recommend the term attributable risk not be used [65].

With two incidence rates, the rate difference also provides a measure of the excess burden of disease that an exposed population might expect to experience as a result of the exposure if there is a causal relationship. It represents the amount of the disease that would have never occurred if the exposure had been prevented. Note that the two measures—the rate ratio and the rate difference—provide very different information. The higher the *relative risk*, that is, above one, the greater the likelihood that a true cause-and-effect relationship exists, but a high *relative risk* for a very rare disease among a few individuals with a unique exposure may be of de minimis concern from a public health perspective, whereas a lower *relative risk* for a relatively common condition might equate to an enormous number of cases. By way of example, it is generally accepted that excess exposures to vinyl chloride monomer cause angiosarcoma of the liver. The *relative risk* for this association is quite high, but the total number of excess cases, worldwide, approximates 100. By way of contrast, the *relative risk* for heart disease among cigarette smokers is only about 1.5, but the rate difference equates to a large number of cases—many, many orders of magnitude more than 100. This is because both the disease and the exposure are relatively common. From a public health perspective, it is much more important to control the excess risk of disease related to smoking than it is the risk associated with vinyl chloride monomer. Yet, for the purpose of establishing a cause for the disease, it took many fewer epidemiology studies to establish an etiologic association between vinyl chloride monomer and liver angiosarcomas than it did for cigarettes and cardiovascular problems.

Rate ratios and rate differences are derived from research in which two groups are defined based on exposure status, and the disease patterns of each are followed forward in time. On occasion, it is easier to get groups based on whether they do or do not have a specific disease and then collect data on previous exposures. For example, it may be more convenient to identify all those who developed lung cancer during some

period, possibly via the use of data from a tumor registry, and identify a comparable group of healthy individuals from the general population. Of course, how this control selection is done is not an easy undertaking if biased selection is to be avoided. Gathering data on previous exposures from those in each group (or from their next of kin) would allow the calculation of an *odds ratio* (OR), the odds of having been exposed to a particular agent given one had the disease versus the odds of having been exposed to that same agent among the healthy controls. If a study is done properly, the OR will approximate the *relative risk*; for example, if the *relative risk* for getting lung cancer among cigarette smokers is 10, the OR of having been a cigarette smoker among those with lung cancer also will be about 10. For simplicity, the rest of the text will focus predominately on two measures of association: the relative risk and the OR.

METHODS

Cohort

Over the years, epidemiologists have developed a variety of methods to evaluate cause and effect. The most intuitively obvious, and the most analogous to the approach used in toxicology, is the *cohort* study. A cohort is simply a group with some common characteristic (e.g., gender, ethnic background, health behavior, or exposure to a particular chemical or medicine). In a cohort study, the incidence disease experience of two cohorts are compared, one with an exposure and one without. Construction and follow-up of cohorts, however, is anything but simplistic. Ideally, multiple cohorts, each with a different level of exposure, are identified with different levels of exposure that are assessed either quantitatively or qualitatively(e.g., low, medium, high). Irrespective of the number of groups, conceptually, exposure status is determined first, and health data—on subsequent mortality, morbidity, clinical measurements, etc.—are then gathered forward in time. Depending upon the time period spent in the cohort, an individual's exposure experience may vary considerably and only some of this exposure may have relevance to the disease in question. The induction period is defined as period of time from causal action until disease initiation. How much exposure is required to initiate the causal action? Is the risk of exposure effects best described by an average intensity exposure, cumulative exposure, or peak exposure? What is the amount of time necessary for any of these exposures to initiate the disease process? Oftentimes, the investigator does not know the induction period. Exposures that occur during the induction period may not be relevant to the initiation of the disease. Epidemiologists often lag exposures to minimize recent exposures that occurred near the time of diagnosis of a chronic disease since the disease process had already begun prior to the exposure. Apportioning person-time to the correct exposure category is paramount. For example, if a person worked 40 years at a manufacturing plant and began his exposure to a specific chemical in year 11, then considering his first 10 years of employment related to this exposure would be invalid. Occupational cohort definitions generally require a minimum time spent employed before entry into the cohort (e.g., 1 month, 6 months, 1 year). Counting this period as eligible time at risk is also invalid since all cohort members had to survive through this time period to enter the cohort. This is referred to as immortal person-time and should be excluded from incidence analyses. Inclusion of immortal time would bias estimated disease rates downward.

Cohort studies can be defined as to when the exposure information is collected in relation to the disease in question. If the exposure status is determined in the present and the health data are then gathered into the future, the term *prospective cohort study* has been used. Prospective cohort studies, for all of their advantages, may not be the method of choice in preliminary investigations of the causes of disease, especially if the disease has a long latency. As opposed to toxicologists who dose animals of species with relatively short life spans (a standard chronic feeding study of mice takes 2 years), epidemiologists examine a long-lived species, humans; therefore, if they only did prospective cohort studies of chronic disease, they likely would complete very few projects during their professional careers. To overcome this problem, epidemiologists will often use historical records—personnel files, medical archives, industrial hygiene reports, etc.—to define their exposed and unexposed study groups at some arbitrary date in the past. They will then gather health data on each individual in the study groups from that point up to the present. These are sometimes called *retrospective cohort studies*. Irrespective of whether the starting point for a cohort study is at the present or in the past, the results are based on incidence data presented as relative risks (and, if appropriate, RDs).

Figure 11.4 illustrates how this is done. Two groups of healthy individuals are identified at a point in time. One group is selected because they have (or had) a known or presumptive exposure to a specific agent; the second because they don't have (and ideally never had) the exposure. The health experience of those in each group is then compiled in an equivalent fashion over some defined period of time. This health experience is converted into incidence rates and the rates compared by means of a relative risk. It is possible to calculate relative risks for all health events combined (e.g., total causes of mortality) or for any number of distinct outcomes (e.g., just deaths due to angiosarcoma of the liver). When the cohort approach is used in exploratory data analysis, it can be considered an exposure in search of a disease, a hypothesis-generating exercise. If it targets just one or a limited number of specific associations of a priori concern, it is akin to hypothesis testing. Many epidemiology studies are a combination of both, and it may be difficult for the reader to discern which associations were of concern at the beginning of the research and which were simply serendipitous findings [7].

In the example, each group at the start of the study had 10,000 individuals; therefore, the marginals for the 2×2 table are both 10,000. During the period of study, X individuals in the exposed group were observed to have developed the disease (cell a), while the remainder ($10{,}000 - X$) did not (cell b). The

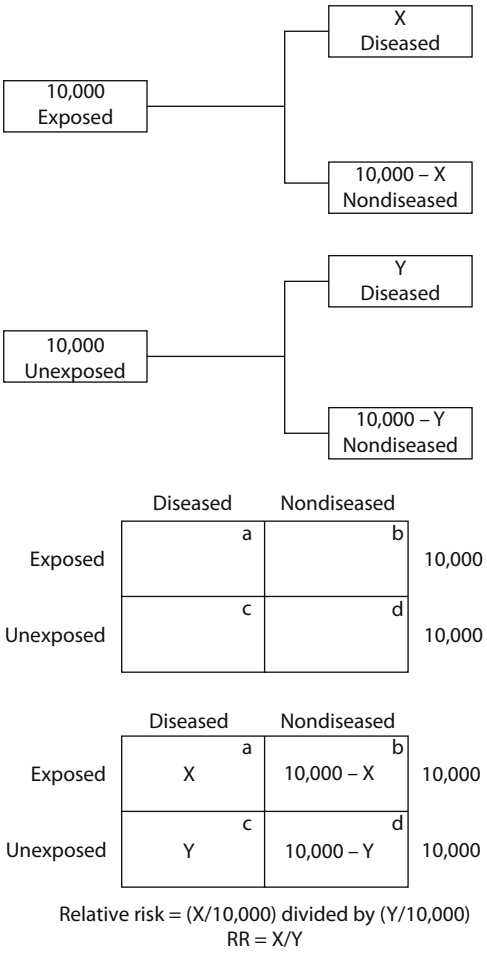


FIGURE 11.4 Illustration of the cohort method.

incidence rate for the exposed is *X* divided by 10,000 (10,000 being the total of those in cells *a* and *b*). Among the unexposed, *Y* developed the same disease (cell *c*) and 10,000 − *Y* did not (cell *d*). The incidence rate among the unexposed is therefore *Y* divided by 10,000. Dividing *X* over 10,000 by *Y* over 10,000 gives the relative risk. Because both groups had the same denominator, this particular *relative risk* simplifies to *X*/*Y*. In real life, that seldom happens. Furthermore, the denominator is not people but person-time (i.e., sum of all individuals' times each person's follow-up time in the study).

Hypothetically, the investigators might have found that 50 individuals among the exposed developed the disease and only 10 among the unexposed (Figure 11.5). After inputting these numbers into the table, the resultant calculations would produce an *relative risk* of 5. The exposed had five times the risk of developing the disease as did the unexposed, assuming there was no selection, misclassification, or confounding bias and the finding was not a chance occurrence.

In a cohort study, those in both groups must be free of the condition at the start of the investigation. This implies that no one in either the exposed or the unexposed group is eligible until they are first examined and determined to be disease free. In other words, the first step of any prospective incidence study is, conceptually, a *cross-sectional* or

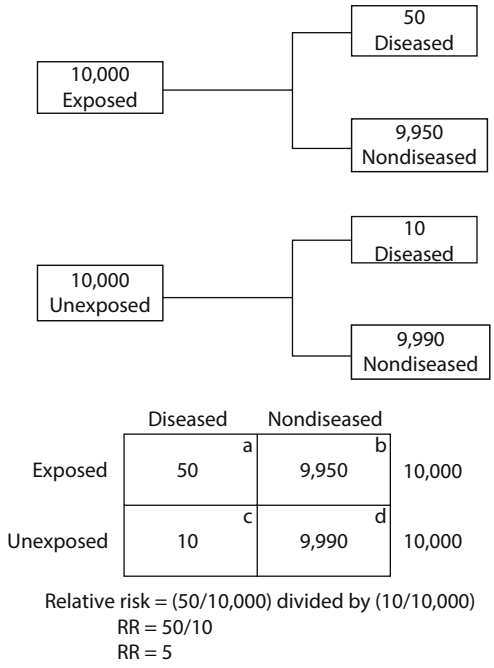


FIGURE 11.5 Illustration of the cohort method; RR = 5.

prevalence study. The data from this cross-sectional study, even though they are collected on two or more cohorts, cannot be used to make interpretations concerning etiology. They are prevalence data.

A cohort study can be a very labor-intensive process. The cohort has to be identified. Exposure histories have to be compiled and validated. Study subjects (or their next of kin) may have to be traced and contacted and data obtained on personal habits, hobbies, and a host of other variables. Medical records must then be collected and coded. Many things can complicate the process. All of this results in a study that is very expensive to conduct. Therefore, once a cohort is established, the study investigators, and their successors, try to follow the cohort for long periods of time. This is especially true when the incidence of disease is relatively infrequent. The use of disease registries (e.g., regional cancer registries) has assisted with disease identification through record linkage. However, most diseases are not found in registry systems. The amount of medical information can vary from person to person simply because of differences in healthcare-seeking behavior. The study subjects may have many different physicians, each providing a different level of care, possessing diverse diagnostic skills, and having office records with unique formats. Many states and municipalities have disparate rules governing access to government records such as death certificates. In addition, litigation and regulations may delay the process of data collection [8,10,38].

Case–Control

Cohort studies are not ideally suitable for the study of rare diseases. In the hypothetical example, 20,000 individuals were tracked to identify the 60 who actually got the disease.

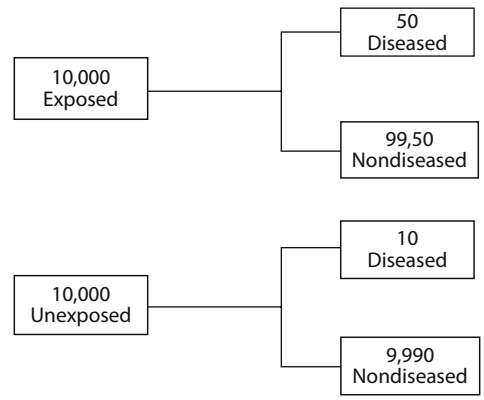
To overcome this and other inefficiencies of cohort studies, epidemiologists developed the *case–control* method. With case–control studies, the past exposures of those with some disease are compared to the past exposures of those who do not have the disease; for example, smoking histories might be compared between men who do and do not have lung cancer. Cases in the case–control approach could be the same as those included in the cohort design. Controls would be a sample from the source population from which the cases originated. If cases are a sample (e.g., 100% or a random sample) and controls are a random sample from the source population, then the case–control study is considered to be population based. This is considered the most ideal type of case–control study. Two important considerations for control selection are as follows: (1) controls need to be selected from the same source population that gave rise to the cases and (2) controls should be selected independently of their exposure status. Of course, the latter is also true for cases. Density sampling refers to the longitudinal sampling of controls based on the amount of person-time that a person is at risk of disease in the source population. In this regard, a case could be eligible to be a control up until the case's diagnosis.

If the cases originate from a medical database, for example, then the source population can be less clear and is defined as a secondary base. For example, an investigator could select cases and controls from the admission practices of a large hospital but the potential for biased selection of cases and controls becomes increasingly problematic. These are referred to as hospital- or clinic-based controls. Other types of control selections that may occur, when the source population is not defined, may include neighborhood and friend controls. It has been shown when cases are deceased, it remains preferable to use living, rather than dead, controls as the latter would not represent the exposure distribution in the general population.

Special types of case–control studies include nested case–control, case–cohort, and case–crossover studies. Briefly, a nested case–control study occurs within a cohort study, which allows for random sampling of both cases and controls. A case–cohort case–control study occurs when everyone within the cohort has an equal chance of being included in the study as a control regardless of person-time in the cohort. This design is often employed with several different types of cases using the same set of controls from the source population. A case–crossover study involves one or more pre- or postdisease time periods selected as the matched control period for the case. Since the case and control are the same individual, age, sex, and birthdates are automatically matched.

Because the study participants for a case–control investigation are first determined in the disease axis of the 2 × 2 table and data are then gathered on exposure status to fill in each of the four cells, the odds of past exposure are calculated, respectively, for the disease group and for the nondiseased control group. These odds are then compared to develop an OR. Because the magnitude of the OR in well-done case–control research closely approximates that of the underlying RR, it allows the interpretations of a case–control study to parallel those of the cohort method: An OR appreciably above 1 suggests a causal association between the disease and the exposure, an OR appreciably below 1 suggests protection, and a ratio near 1, plus or minus, suggests no association between the exposure and the disease.

If we go back to the hypothetical example, there were 60 new cases of disease in this closed population (Figure 11.6). Knowing the age, gender, race, and perhaps other key characteristics of the diseased, 60 nondiseased individuals could be randomly selected from the remainder of the 20,000. These 120 would constitute the bottom marginals of the 2 × 2 table, 60 in each column. Data could then be collected on past exposure. In this particular example, 50 of the diseased group would end up in cell *a* and 10 in cell *c*. Among the nondiseased, approximately 30 would end up in cell *b* and another 30 in cell *d*. Parenthetically, the nondiseased in each exposure category were approximately the same, 9,950 and 9,990; therefore, random sampling of 60 from the aggregate 19,940, should select equal numbers from each group: 30 and 30. With data in all four cells, the odds of exposure among



Odds ratio = odds of exposure among the diseased vs. the odds of exposure among the nondisease
Odds ratio = 50/10 divided by 30/30
OR = 5
The cross-product ratio is *ad* divided by *bc*
OR = 50 × 30 divided by 30 × 10
OR = 5

FIGURE 11.6 Illustration of the case–control method; OR = 5.

the patients would be 50–10 (5–1) and the odds of exposure among the controls would be 30–30 (1–1), giving an OR of 5. Conveniently, the calculations for case–control studies often simplify to a *cross product ratio* (*ad* divided by *bc*). In the example, 50 times 30 divided by 30 times 10 simplifies to 5.

All too frequently, it has become common practice by epidemiologists (and others) in reviewing the literature to describe ORs greater than one simply as a *positive association*—regardless of their magnitude or statistical significance. In part, this is done because the OR is dimensionless (as is the relative risk and the SMR). However, in reality, it must be remembered that the OR represents the odds for disease given a certain degree of exposure compared to the odds of disease for a lesser amount of exposure. With the advent of biomonitoring of low levels (parts per billion or lower) of environmental chemicals in humans, it is not uncommon to see ORs reported for quartile ranges of biomonitoring serum levels in the general population where such quartile concentration ranges are only a few parts per billion (ng/mL) or less. See Example 11.B.5 for such an instance related to subfecundity and perfluorochemicals. This practice ignores the actual comparison of two different odds based on an exposure level as well as the considerably much higher concentrations necessary to observe an adverse effect in toxicological studies. Thus, the sole use of the term *positive association* (or negative association) in the epidemiologic literature should be curtailed. By itself, this terminology poorly describes epidemiologic study findings and is therefore invariably misleading.

For this case–control study in Figure 11.6, an evaluation of just 120 individuals provided the same information as a study of 20,000. In the context of the time, effort, and cost, gathering data on such a limited number of study subjects can be a tremendous advantage. Paradoxically, the small size of the study can also be a problem because seemingly minor amounts of bias can have a dramatic impact on the OR.

Avoiding inadvertent bias can be more difficult in case–control studies because the health outcome has already occurred. If the investigators or the study subjects are not properly blinded, this knowledge can impact both who participates in the research and what data they provide. Social forces still may influence the results; for example, those with disease may be more inclined to participate in the research because they have a greater need to understand why they became ill. Also, the cases who selectively participate may expend greater effort toward trying to remember their past exposures and thereby provide more comprehensive or more valid data than the controls. This is called *recall bias*. It is a type of *information bias (differential misclassification)* that when present often leads to overestimates of risk.

Another methodological issue with a case–control study is that it may utilize a combination of prevalence and incidence data instead of just incidence data and thereby limit its utility for etiologic interpretations. Even with its limitations, a case–control approach can be very attractive. Because the two groups are initially defined on disease status, data on any number of exposures can be collected. As a consequence, a large number of different associations can be evaluated simultaneously and rapidly reduced to just a few that deserve further study. For that reason, an exploratory case–control study can be considered a disease in search of an exposure, the mirror image of the hypothesis-generating exercise done in a cohort mode.

A case–control study also can focus in depth on just one disease exposure association, testing a hypothesis derived from case reports or other types of research with much greater sophistication than might be feasible in a cohort study. In certain situations, it can be advantageous to use the cohort and the case–control approach in series to generate a relatively small and well-defined number of hypotheses. Such a *nested case–control study* can combine the strengths of both methods; for example, the cohort approach could be used to identify a cluster of disease within a broadly defined group, perhaps all those ever employed at a multiple-chemical-manufacturing facility, and a case–control study could then be implemented within the larger cohort not only to narrow the focus to those few agents that appear to be most important for that particular disease but also to do so with proper adjustments for confounding. This integrated approach, therefore, can achieve both efficiency and rigor.

Cohort and case–control studies are sometimes referred to as *analytic* research, in contrast to other types of epidemiology investigations that are simply *descriptive* of time, place, and person. In theory, the term *analytic* should be restricted to those studies designed to test a priori hypotheses, but in practice, it is often used more broadly to refer to any cohort or case–control research, regardless of whether it generates or tests hypotheses. That is unfortunate, because it blurs the distinction between these two important stages of discovery and the role each plays in the search for the causes of human disease.

Cross-Sectional

A study that ascertains its individual study subjects without regard to disease or exposure status is a cross-sectional study. These are relatively inexpensive endeavors compared to cohort and case–control endeavors and the study findings can be quickly ascertained and reported. The cross-sectional study examines prevalent cases since there is no longitudinal component to a cross-sectional study. Because disease and exposure information are obtained at the same time, it is not possible to examine an exposure response temporal relationship. Be aware that all too frequently investigators pay brief homage to this critical limitation in the discussion of their cross-sectional study but ignore it as they try to make etiological inferences from associations generated from other temporally challenged cross-sectional studies. Another important consideration is that in cross-sectional studies, the cases tend to be selected from individuals with longer duration of disease. Cases with shorter duration are less likely to be sampled at any point in time. This is called *length-biased sampling*. Should exposure influence the duration of the disease, then erroneous inferences are a logical consequence.

Ecologic

An ecologic study is when the unit of analysis for the exposure is generally described as an index of exposure for a group and the disease outcome is a mortality or incidence rate for that group or population [92]. The unit of analysis is not the individual. A classic example of an ecologic study relates national or regional mortality and incidence rates with per capita consumption of dietary food groups that show *western diets* are either associated or not associated with specific types of disease. Ecologic measures may be classified into three types: (1) aggregate measures of observations derived from individuals in each group, (2) environmental measures that are physical characteristics of the place in which members of each group live or work, and (3) global measures that are attributes of groups for which there is no similarity at the individual level. Ecologic studies can be an important study design used by environmental epidemiologists who may utilize geographic information system (GIS) in their analyses. The ecologic fallacy is the well-known bias that can arise from ecologic studies. An association observed between variables at a group level does not necessarily reflect the biologic effect at the individual level [92]. The lack of knowledge regarding heterogeneity of exposures and covariates within groups is also problematic. As a result, analyses at a group level may result in vastly different conclusions than analyses performed by individuals within the group.

Case Studies or Case Series

Hypotheses for analytic epidemiology may originate from toxicology studies or from epidemiology investigations, but many evolve from clinical observations and are published in the form of *case studies* or *case series*. Although based a great deal on intuition, a case study is a time-honored way for a clinician (e.g., physician, veterinarian, dentist) to develop new theories about the causes of human disease. It has been said with some justification that every human carcinogen was first identified by an astute clinician who published his or her findings in the form of a case study or case series. Nonetheless, that does not mean case studies can be used to unerringly identify new etiologic associations. Although the theories derived from case studies are not always wrong, history teaches that they are seldom right [11,40,112]. Determining which is which depends on data developed by others using experimental, quasi-experimental, and observational research. If we go back to the 2 × 2 table, we can see why.

To test a hypothesis about a new cause for human disease (to identify an elevated risk in analytic epidemiology research), data are needed in all four cells of the 2 × 2 table—data that are properly defined on both variables. Case studies tend to focus just on those in one of the four cells: cell *a*, the exposed with disease. Very little if any data are gathered by the clinician on those in each of the other three cells. Furthermore, those from whom data are gathered are a *convenience sample*. They probably are not a representative sample of any well-defined group, especially not a representative sample of the source population from which they arose—irrespective of their exposure history. They are not because physicians tend to direct their efforts toward diagnosing and treating those with medical problems.

In Figure 11.7 examples a through c, the three 2 × 2 tables represent the three possible types of association. In the first, the 50 in cell *a* translates to an RR of 5; in the second, the 30 to an RR of 1; and in the third, the 20 to an RR of 0.5. The three relative risks have very different meanings. Although it is conceivable that any clinician practicing in a community might become suspicious if a cluster of 3 or so patients came to him or her with the same rare disease and all had a similar exposure history, based on the information available, the clinician would not be able to determine whether the cluster was a subset of those in cell *a* from Figure 11.7a, b, or c. Most clusters, however provocative, yield few scientific insights [113]. Furthermore, additional case reports do not satisfy the need for replication and confirmation. Once a testable hypothesis has been formulated, additional case reports proposing the same hypothesis contribute nothing. The fact that cluster investigations offer few scientific insights does not mean, however, that some type of interaction with the concerned public should be ignored. As Bender and colleagues [11] aptly stated, an appropriate public health response to a cluster inquiry from the public should be *responsibly responsive*.

By way of example, in the silicone breast implant controversy, it was originally hypothesized that women who received this medical device were at increased risk for

Example a. Relative risk is 5

	Diseased	Nondiseased	
Exposed	50	9,950	10,000
Unexposed	10	9,990	10,000
	60	19,940	20,000

Example b. Relative risk is 1

	Diseased	Nondiseased	
Exposed	30	9,950	10,000
Unexposed	30	9,990	10,000
	60	19,940	20,000

Example c. Relative risk is 0.5

	Diseased	Nondiseased	
Exposed	20	9,950	10,000
Unexposed	40	9,990	10,000
	60	19,940	20,000

A physician sees three patients with the condition and all three were exposed to the same chemical, leading him to conclude that the disease in all three was caused by the chemical exposure. Is he correct?

FIGURE 11.7 Case studies and case series.

breast cancer. The theory was based on clinical observations, and concern was increased because of an animal toxicology study that demonstrated an Oppenheimer effect, the tumorigenic properties of foreign bodies as observed in rodents [20,52,91]. As a result of subsequent research, both experimental and observational, something between Figure 11.7b and c is now thought to most closely approximate the association between silicone breast implants and human breast cancer. It is being theorized that these medical devices or the materials from which they were constructed offer some type of protective effect against breast cancer [23,127]. The current data-based theory is in exact opposition to the hypothesis originally derived from the case reports.

Interestingly, no action has been taken on this information. Why? Probably because even though the epidemiology study results have been reasonably consistent and demonstrate coherence with the findings of the experimental animal research and the public health implications of such an association could be profound considering both the frequency and the life-threatening characteristics of the cancer, the underlying biological mechanisms of protection have not been identified.

ISSUES

The objective of an epidemiologic study is to produce a valid and precise estimate of the effect of an exposure on the occurrence of disease in the studied source population [66]. Validity refers to the accuracy of the estimate of an effect. Accuracy reflects two sources of error: random and systematic. Random error reflects the precision of the estimate of effect. A larger sample size (study population) results in a lower amount of random error and therefore higher precision. Systematic error reflects the biases incurred by the study design, study conduct, and data analyses. Systematic errors affect validity regardless of the study sample size. Fewer systematic biases generally reflect the greater the validity of the estimate of effect but the magnitude of error from different systematic biases should not be viewed as equal. Internal validity refers to that within the source population. External validity (generalizability) refers to the inferences considered outside the source population to another target population. There are three categories of violations to internal validity: selection bias, information bias, and confounding. Information bias is a function of nonrandom measurement error and is oftentimes referred to as misclassification.

Everything in the scientific literature must be read with a degree of healthy skepticism [107]. This can be difficult enough within a single field, but it is truly a daunting task when a scientist tries to evaluate the merit of work from a different discipline yet alone with epidemiology, which often encompasses multidisciplinary teams of researchers. If a toxicologist understands the basics of data, measures of disease frequency, measures of association, and methods, the epidemiology literature can be screened using the mantra of *selection*, *information bias (misclassification)*, *confounding*, *chance*, and *causation*.

The order of this mantra is important. If obvious systematic errors (biases) are related to selection, information, or confounding, it may make very little sense to spend time trying to evaluate the merit of the investigators' statistical analyses, much less to assume the findings have any biological meaning. It is no accident that the scientific literature has a highly stylized format: some variation of abstract, introduction, methods and materials, results, discussion, and conclusion. This format allows the reader to rapidly focus on the key components of the work. If the authors provide a one-sided presentation of the topic in the introduction, supply insufficient detail regarding their methods and materials, or do not critique their own work in the discussion—pointing out the potential biases of selection, information, and confounding and how they were addressed—the reader should exercise considerable caution before accepting either the results or the conclusions, even as provisional truth. A guide (see Appendix A) was written, entitled Strengthening the Reporting of Observational Epidemiology (STROBE), whose objective is to allow for better clarity of epidemiology research findings (as written by the author) and understanding of epidemiology reports (as read by the consumer) [134,136].

Selection

In epidemiology, *bias* is used to denote a deviation from the truth but not necessarily to imply that the deviation was intentional [7,132]. *Selection bias* refers to errors that are related to systematic differences between those who are and are not selected in a study. Even if the data gathered are valid for those who are examined, it may be inappropriate to use any information derived from the data for purposes of extrapolation to a larger population; for example, the results of a study of hormone replacement therapy among women cannot logically be extended to men. In epidemiology research, various types of selection bias can be introduced by the study subjects, the investigators, or even traditional medical practice and other social forces.

Self-selection occurs in both clinical research and some epidemiology studies. It is well recognized that those who participate in controlled clinical investigations, that is, those who actually sign informed consents, may not be representative of the general population; therefore, even with randomization of treatment, care must be taken before extending the study results too broadly. A similar problem occurs in observational studies in which some type of active participation, some type of action on the part of the study subjects, is required. For example, informed consent is required for any epidemiology study in which biological samples are collected. Usually, the more invasive the procedure, the more disinclined are the potential subjects to participate and the greater the potential for bias; however, in other situations, this bias may be less obvious or, paradoxically, so obvious that it is largely overlooked. As an example, how many times have you received a questionnaire in the mail and, rather

than responding, tossed it away? By doing so, you introduced a potential *participation bias* into that investigator's work, the potential for which may not be acknowledged in the final report.

In certain types of observational research, self-selection is not a problem. Projects that can be conducted without the active cooperation of the subjects often are able to achieve close to 100% follow-up; for example, occupational cohort mortality studies that utilize personnel records and industrial hygiene reports to identify the exposed and death certificates to document the cause of death can be conducted with little or no self-selection [98]. However, whether the information provided has relevancy is a different question. Using death certificates to study conditions that have high survivorship lacks scientific justification.

The same arguably holds for some studies that utilize medical records, but only if the medical records relate to the total health experience of a well-defined population. Such is the case in certain countries with socialized medicine in which all the hospital and clinic records are available for the entire citizenry. The Scandinavian countries are well known for their record linkage systems that are founded on a personal identification number at birth that is used throughout life to link to numerous computerized health care, income, work history, education, and residential databases.

Either intentionally or not, investigators can introduce selection bias when they decide who to study, especially if they make a greater effort to obtain participation among the exposed more than the unexposed, or the diseased than the healthy. Figure 11.8 is an advertisement that appeared in a Kansas paper in the late 1980s. It apparently was placed by investigators who wished to identify more subjects for a research project and thereby improve its statistical power. What they presumptively did not recognize was that by recruiting simultaneously on both health outcome (non-Hodgkin's lymphoma [NHL]) and exposure (2,4-dichlorophenoxyacetic acid [2,4-D]), they would introduce a significant selection bias into their work, one potentially so severe as to possibly invalidate any of their findings.

A spuriously elevated relative risk can be predictably found in any research in which the study subjects are selected on the joint characteristics of the condition of interest and the putative agent of concern. This is also referred to as Berkson's bias [12]. People with multiple diseases will be overrepresented in a hospital population compared to the general population according to the laws of probability. A spurious association can then be obtained because of the different probabilities of admission to a hospital for those with the disease, without the disease, and with the characteristic of interest.

WANTED
Kansas farmers suffering from non-Hodgkins lymphoma especially those exposed to herbicides such as 2,4-D. Needed to interview for research article. Please send name, address, and phone number to Box P7629 Classified Dept., The Star, 1729 Grand, K.C., MO 64108.

FIGURE 11.8 Recruitment of study subjects.

Figure 11.9 illustrates the dynamic that leads to *Berkson's bias*, a particular type of selection bias that occurs as a result of the patterns of referral, either self-referral or physician referral [57]. Although there is some merit in asserting that the 250 individuals who initially consulted a physician represent those with the more definitive illness among the 1000 in the population at risk and thus are legitimate subjects for etiologic research, it is less likely that the same thing can be said about the 5 referred to a specialist or the 1 who finally ended up at a university center. Patients seen by specialists or at tertiary referral centers include a disproportionate number whose disease is complicated, obscure, or atypical. A spuriously elevated relative risk will predictably be found in any research in which the study subjects are selected on the joint characteristics of the condition of interest and the putative agent of concern.

Even if no formal study is conducted, the specialist may develop a marked suspicion concerning the presumptive cause for the condition and then act on that presumption. Once it becomes known in the community that a physician or a referral center is interested in patients with a particular condition, especially when it occurs in conjunction with exposure to a specific agent, additional referrals or self-referrals further compromise the value of the sample for etiologic research [125]. Ironically, the more caring the physician in the sense of being more willing to provide therapy to those who have been unsuccessfully treated or refused treatment by others, the more that physician becomes a magnet for these patients.

In evaluating the literature, the reader needs to ask two questions related to selection bias: Was the sample that the investigators were attempting to study truly representative of some larger group? Were the researchers successful in getting participation from all or a large majority of those they sought to study? An individual epidemiology report probably will have little or no value if the answer to either question is *no*. The operative term in the previous sentence is *probably*. It is important to note that not every potential selection bias is real; therefore, studies with less than 100% participation should clearly not be dismissed as meaningless. The question is how does one determine whether or not a study with less than optimal participation provides relatively unbiased results? Usually, one cannot make that determination from the single study. The question can be addressed in the context of the larger body of literature. If the results of the potentially flawed study are comparable with those of other work in which selection bias is a lesser concern, the consistency suggests a cross validation of findings. On the other hand, if the results of

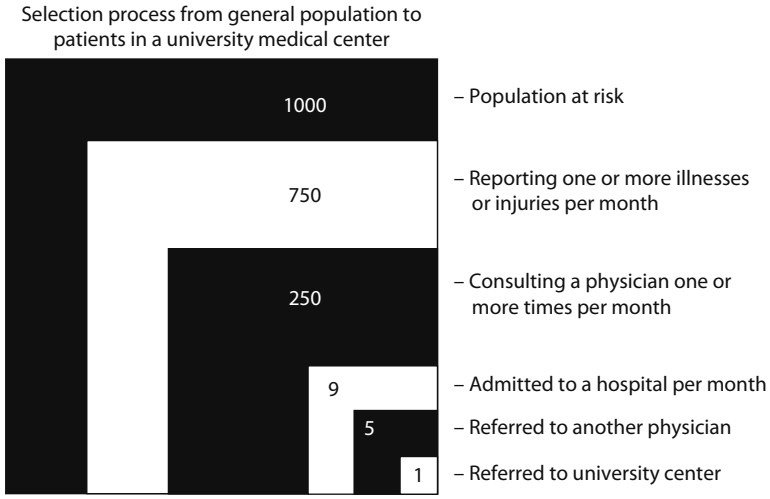


FIGURE 11.9 Berkson's bias: potential selection bias by referral. (From Gehlbach, S.H., *Interpreting the Medical Literature*, McGraw-Hill, New York, 1993. With permission.)

multiple studies are markedly different, it raises concern that the findings of one or more of the reports are biased.

Information Bias

Information bias, also known as *measurement* or *misclassification bias*, is a systematic error arising from the inaccurate measurement or inappropriate classification of subjects on the study variables—either exposure (to the putative agent or confounder) or health outcome. At some level, all measurement or classification is inaccurate. The errors may be large or small, and, in turn, depending on the use to which the data are put, these errors may be important or meaningless. As an example, in measuring blood pressure, some physicians routinely round up to the next increment of 5 (e.g., 140 mmHg systolic and 90 diastolic or 145 and 95), others round down, and still others record to the closet unit of 2. The experienced clinician tends to make these measurements consistently on the same two of the five Korotkoff sounds, but which two may vary from physician to physician [54]. These variations from the true blood pressure probably have very little importance in the clinical setting if the patient is consistently measured and treated by the same physician, but they could be very important if treatment is provided by multiple physicians. They also could be important if the clinical data were used to judge the relative efficacy of a variety of treatments as administered by different physicians.

Epidemiologists are concerned whether the misclassified information is the consequence of the disease or exposure [66]. In the most basic 2 × 2 terminology, a variable is misclassified on a dichotomous exposure (yes/no) or disease (yes/no) level. Classification error that depends on the actual values of other variables is referred to as differential misclassification. Classification error that does not depend on the actual values of other variables is called nondifferential misclassification. Misclassification can occur within exposure or disease. For example, individuals classified as exposed may not be exposed and individuals classified as nonexposed may be exposed.

Nondifferential misclassification of a dichotomous exposure will always bias an effect toward the null value (i.e., toward a relative risk = 1.0). If there are several categories of exposure, the nondifferential misclassification, however, may be biased toward or away from the null value depending on the categories to which individuals were misclassified. Differential misclassification of exposure could either increase or decrease an apparent effect. It is not readily predictable without further investigation. Nondifferential misclassification is the more prevalent condition. Be wary of epidemiologic studies whose investigators confuse the lack of knowledge of differential misclassification (due to lack of any investigation of it within the study) as reason to believe the measures of effect reported had to be biased toward the null due to the more likely presence of nondifferential misclassification. A lack of data to investigate differential misclassification is an inadequate excuse to noncritically accept as fait accompli nondifferential misclassification in any epidemiologic study.

One of the most common types of information bias is *recall bias* that occurs in case–control studies. Cases and controls may recall past exposures differently due to their disease and nondiseased conditions. Because of their disease, cases may spend considerable time pondering why they were diagnosed with their condition. Independent of any research study, the cases may question their immediate and distant past history for suspected possible (exposure) causes. In this Internet age, ready access to global search engines makes information and opinions readily available to cases and their relatives from medical professionals, patients, consumers, and advocates. Controls, on the other hand, would unlikely go through the same recall experience until questioned in a research setting. The time and energy spent on such recall by a control is likely to be considerably less than the case.

Example 11.B.2 reviews recall bias through the differential recall by type of respondents for the agricultural use of the phenoxy herbicide 2,4-D. This recall was dependent on the type of respondent for cases of NHL in a series of case–control studies conducted by the National Cancer Institute (NCI) [27,28,73,143]. Interviews conducted of the next of kin of NHL cases recalled greater 2,4-D exposure use than interviews of living NHL cases. The controls were interviewed directly as they were not deceased; thus, differential misclassification of exposure occurred likely due to recall bias by the type of respondent.

An epidemiologist might use various techniques to avoid or reduce the potential misreporting. Concealing the intent of the research from the study subjects is one, but such blinding of subjects is increasingly difficult to use in a climate of mandated informed consent and almost instantaneous dissemination of news about the latest health controversy. Another approach is to add a dummy health variable whose association with the exposure is biologically implausible; for example, a query about dental caries could be incorporated into a study evaluating the effects of exercise on angina. If a strong correlation exists between the frequency with which the study subjects reported the dummy variable and the health outcome of concern, one should suspect a misreporting problem. In such a situation, it may be necessary to validate the reports—perhaps, if feasible, by examining a subset of the respondents or via review of medical records that predate the controversy or by use of a biological marker such as saliva cotinine for cigarette smoking [83,111,137].

At a minimum, the processes by which the data were collected should be well defined. Even then, there could be problems. It is well recognized by the seasoned researcher that mechanical or electronic instruments of assessment periodically must be calibrated to ensure a consistency of measurement over time. To achieve validity, they must be calibrated to an external standard. The application of other data collection tools such as questionnaires may be less than rigorous. With survey instruments, the order in which the questions are posed can be important. Even if the questionnaire is not open-ended, the words themselves may have alternative connotations for different ethnic or racial groups. To the extent possible, epidemiology research should use tools whose strengths and limitations are well recognized or should incorporate a validation pilot into the research project.

Diagnostic bias, a type of observer bias, occurs when a physician's diagnosis is influenced by his or her knowledge of certain exposures or surrogates of such exposures. In a study of eosinophilia–myalgia syndrome, Wagner et al. [138] found up to a sixfold increase in diagnosing the condition when physicians were told the patients had ingested L-tryptophan even though use of this dietary supplement was not part of the definition for the condition.

Similar biases occur in toxicology research, both nondifferential and differential. The traditional acceptance of tumors, benign and malignant, as a surrogate for cancer is one form of misclassification. In well-conducted studies, it probably is nondifferential, but any time the methods for disease determination differ between the exposed animals and the controls, it could be differential. For example, if more histopathological slides are made or read for the exposed animals than the controls, it is more likely that small occult tumors will be found among the exposed. This is differential misclassification, one that would introduce an overestimate of risk.

Sensitivity and Specificity

Consistency (i.e., precision) of measurement, although important, does not ensure the absence of measurement bias, whatever its underlying cause. As illustrated in Figure 11.10,

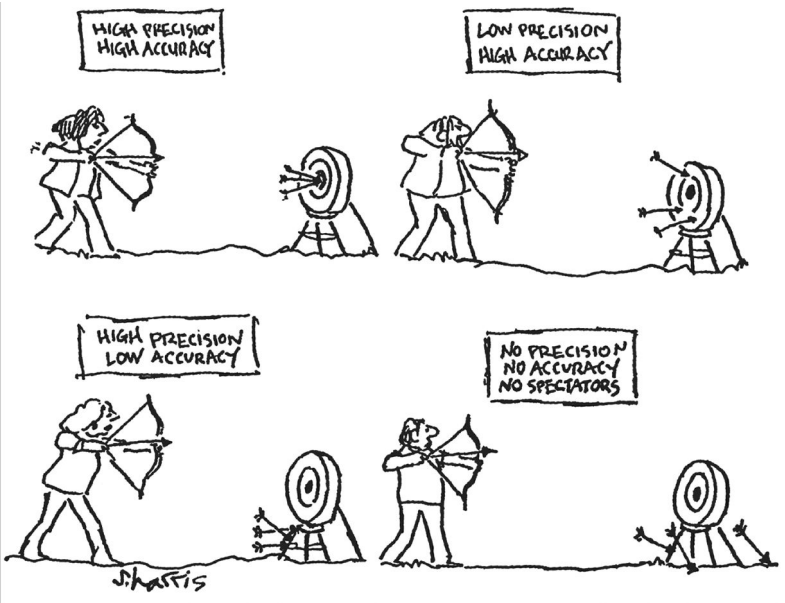


FIGURE 11.10 Precision and accuracy.

it is possible to be precise and precisely incorrect. What is more important is accuracy (i.e., the validity of the data). The key measures of validity are *sensitivity* and *specificity*. Sensitivity is a measure of how well the test identifies a true condition (disease or exposure). Specificity is a measure of how well it documents a true noncondition (the absence of disease or exposure).

It is actually more complicated than that. A number of tests used in medicine, such as blood cholesterol or antinuclear antibody status, do not clearly separate the normal individuals from those who are abnormal [124,128]. The distribution of values in each group overlap (Figure 11.11). In such situations, the operational diagnostic break point between the two can be somewhat arbitrary. It can be set to identify all the true abnormals (point a; all the true positives) but only by accepting a certain number of false positives—incorrectly labeling some normal individuals as abnormal. Or the break point could be moved to correctly identify all the true normals (point b), but only at the expense of getting more false negatives—misclassifying some of the abnormal patients. In other words, where the break point is set can impact the sensitivity and specificity of a test, and if sensitivity is improved, it may mean the specificity has been compromised and vice versa. For example, one could arbitrarily declare all chemicals human carcinogens. Such an error on the side of caution would certainly correctly label all the true carcinogens and would guarantee a sensitivity of 100%, but the specificity of such a strategy would be abysmal because the number of false positives would be huge.

Note that to determine the sensitivity and specificity of a test, its results must be compared to those of a *gold standard*, an accepted test or procedure that reliably determines the presence or absence of the condition. Why then is it necessary to have the new test? Why not just use the gold standard? The new test may be needed because the gold standard is not as useful a tool in the clinical setting. It might be more expensive, inconvenient, invasive, or dangerous.

Paradoxically, data gathered by means of a gold standard actually might have little utility for etiologic research in and by itself but still may be important for the development of tools that can be used in such investigations. For example, explantation (the surgical removal of a medical device such as a breast implant) is considered the gold standard for implant rupture; however, for both technical and ethical

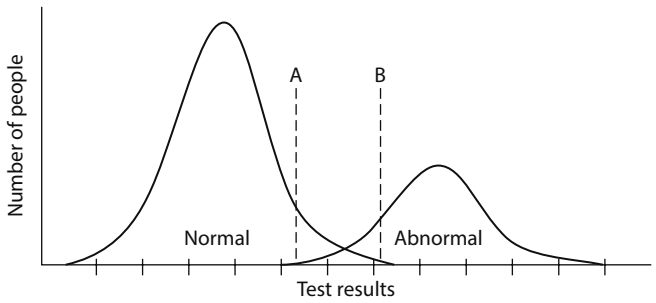


FIGURE 11.11 Sensitivity versus specificity.

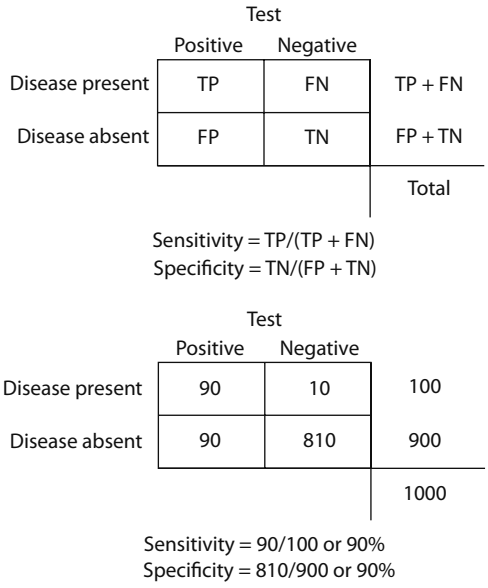


FIGURE 11.12 Sensitivity and specificity.

reasons, it can only be used to collect prevalence data. Other noninvasive techniques such as mammography, ultrasound, and magnetic resonance imaging (MRI) can collect incidence data and at lesser risk to the patient, but the relative validity of each can only be established via the gold standard of explantation.

Given that there is a suitable gold standard, Figure 11.12 shows how these measures are calculated. In the figure, TP is true positive, FN is false negative, FP is false positive, and TN is true negative. Sensitivity is obtained by dividing TP by (TP + FN) and specificity by dividing TN by (FP + TN). For this particular example, the sensitivity and specificity are both 90%, quite good for most clinical tests [112]. Sensitivity reflects how well, given that the condition is actually present, the test detects the condition. Conversely, specificity is a measure of how well, given that the condition is really absent, the test does not erroneously document its presence.

Predictive Value Positive and Predictive Value Negative

In real life, whether or not the condition is actually present is unknown before the test is performed. That is the reason for doing the test! For most investigations, what is of greatest interest is the predictive capabilities of a test: how well, given the test result is positive or negative, it respectively predicts the presence or absence of the condition. These measures, *predictive value positive* (PV+) and *predictive value negative* (PV–), can be obtained by making calculations in the vertical axis of the 2 × 2 table (Figure 11.12). In this example, the PV+ is 50% and the PV– is 98.8%. What this indicates is that among those who are diagnosed as having an illness on the basis of an abnormal test result, only 50% of them are truly diseased, but among those whose test result was in the normal range, 98.8% are actually healthy.

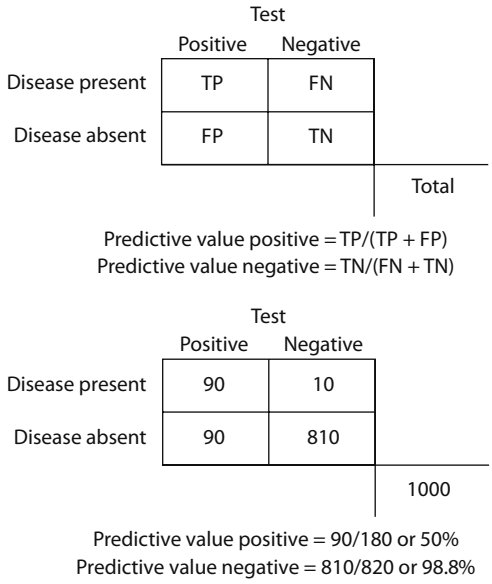


FIGURE 11.13 PV+ and PV–.

The two sets of measures are related but not equivalent. Although the sensitivity and specificity are relatively stable attributes of a test, the predictive values vary widely as a function of the background frequency of the condition being studied. Figure 11.13 illustrates this point. For a given test, the less frequent the condition, the lower the PV+ and the higher the PV–. When the disease frequency drops to 1 in a 1000, the PV+ is less than 1%. In other words, if used as a screening tool the test would label patients as abnormal incorrectly more than 99 times out of 100. This interplay between the underlying validity of a test and the relative frequency of the condition being studied not only impacts epidemiology, but it also has important implications for medical surveillance (and the government regulations that mandate, fund, or otherwise support such procedures) as well as toxicology, in particular as it impacts risk assessment.

A blue-ribbon panel of experts, for example, recommended that routine mammography screening be restricted to women over 50 or those in high-risk groups. In both, the frequency of breast cancer is orders of magnitude higher than it is in the general population of younger-aged women. This recommendation ignited a fire storm of controversy, and the panel, in part apparently due to pressure from congress, subsequently modified its recommendation to include younger women [48]. This may prove to be a mistake. Predictably, what will happen is that the medical system will be flooded with false positives [46] (Figure 11.14).

Given:
Sensitivity = 90%
Specificity = 90%

Disease frequency	PV+	PV–
1 in 10	50%	98.8%
1 in 100	8.3%	99.9%
1 in 1000	0.9%	99.99%

FIGURE 11.14 Predictive values as a function of disease frequency.

False-positive breast cancer screening tests among younger women have a number of downsides. One, a false-positive test can severely frighten patients, and many among those subsequently told the test was incorrect will retain a lingering anxiety at the very least. Two, an abnormal mammogram is often checked by means of a biopsy; this surgical procedure is associated with a certain small but predictable risk of infection, bleeding, loss of sensation, and adverse reactions to anesthesia, and for women without breast cancer, it is an unnecessary risk. Three, false positives put a strain on our medical care delivery system and misdirect limited resources. Four, procedures that produce false results cost money, a financial burden that must be borne by the individual patient in the form of direct payments and by society at large in the form of increased insurance premiums and higher taxes. Five, and arguably of greatest importance, some young women after one, two, or more false-positive reports may lose confidence in the procedure. A certain number of these will drop out of the mammography program and never reenroll. This means that they will not get the screening test later when they would benefit from it most.

The results of toxicology studies are not immune to this problem, in part because their results are routinely extrapolated to humans. If a high dose of an agent is found to cause tumors among rodents, it is current policy to assume that it will cause some form of cancer in humans at lower levels of exposure. Unfortunately, although the sensitivity of toxicology research is quite high (but not perfect), its PV+ for extrapolations between different species of rodents is low, on the order of 50% in a study of various chemicals purposefully selected because of their presumed carcinogenicity [75]. Arguably, its PV+ is even lower for humans, especially for chemicals being tested simply to satisfy a mandated protocol.

Descriptive epidemiology research, such as that done by Robinson et al. [109], focuses on a single group. Nonetheless, misclassification obviously can produce erroneous information. In analytic epidemiology, the problem is compounded because data are gathered on and compared between two or more groups. This can lead to errors that are either *nondifferential* or *differential* (Figure 11.15).

Given:
True relative risk = 1 (i.e., background incidence rate is equivalent in the exposed and the unexposed)
Differential misclassification fixed
Sensitivity and specificity among the exposed: 95% and 90%, respectively
Sensitivity and specificity among the unexposed: 90% and 95%, respectively

Background incidence rate	Apparent relative risk
10 per 100 per year	1.9
1 per 100 per year	5.5
1 per 1000 per year	9.2

FIGURE 11.15 Risk estimates in the presence of differential misclassification.

Confounding

Confounders are the extraneous factors that explain or produce all or some of the difference between the measure of association and the measure of effect that would be obtained with a counterfactual ideal [65]. In a research study, this difference may be an overestimation or underestimation of the measure of effect in disease frequency between the exposed and unexposed.

A confounder has three necessary characteristics:

1. A confounding factor must be an extraneous risk factor for the disease.
2. A confounding factor must be associated with the exposure under study in the source population.
3. A confounding factor must not be affected by the exposure or the disease. It cannot be an intermediate step in the causal path between the exposure and the disease.

Confounders can either be directly measured or a surrogate for a confounding factor. Age, for example, is a surrogate for a constellation of biological, environmental, and social factors that individually and in aggregate are associated with increased risks to certain diseases. The same can be said for race and ethnic background. Different confounding factors have different effects. None is a universal confounder.

In addition to being an alternative cause, the second necessary characteristic requires a confounding factor to be associated with the exposure under study in the source population. As with the biases related to selection and misclassification, the degree of differential distribution of the confounder between exposed and unexposed determines the direction and magnitude of the error. In addition, the relative potency of the confounder can also, to a greater or lesser extent, influence the apparent relative risk or OR. Cigarette smoking, for example, is one of the major determinants of lung cancer. An epidemiology study investigating the carcinogenic potential of a particular exposure vis-à-vis lung cancer has to take smoking into consideration as a possible confounder.

Smoking is also associated with mortality due to cardiovascular disease, but not to the degree to which it causes lung cancer. In other words, equivalent amounts of unequal distribution between the two study groups may not have the same impact on the measures of risk for different conditions because the potency, the biological activity of a confounder, varies from disease to disease. With lung cancer, smoking equates to a relative risk of perhaps 10, whereas for cardiovascular mortality, the relative risk lies closer to 1.5; for still other diseases, it has an RR that approximates 1 (no effect).

Very few diseases have only one etiology. Even a rare malignancy such as angiosarcoma of the liver has a number of alternative causes aside from vinyl chloride monomer [49]. Agents with high potency are relatively easy to discern. It is those with lesser biological activity that are more difficult to identify. An indeterminate number of the latter undoubtedly have not yet been discovered. Theoretically, because all of the causes for the various diseases are unknown, some level of confounding may occur in any epidemiology study (and any toxicology study for that matter). In addition, it is highly likely that there are synergistic and antagonistic actions between various agents, both exogenous and endogenous, further complicating the picture.

The third necessary factor of a confounder, it must not be an intermediate step between exposure and disease, is met if the confounder precedes exposure and disease. However, this may not be readily discernable.

A confounder is not the same as an *effect modifier*, although an agent, depending on the study, can be one, both, or neither. Effect measure modification produces a nonuniformity of effect across various levels of the effect modifier [65]; for example, the consequences of exposure to pathogenic organisms vary by immunization status. Under the assumption that study biases have been adequately controlled, analyzing for statistical interactions is considered a method for analyzing effect-measure modification. However, statistical interactions are not straightforward as interactions observed in additive models (linear regression) would not necessarily be apparent in multiplicative models (e.g., logistic regression). Because statistical interactions are a result of measure or scale and the model chosen, they should not be confused with biological interactions, which refer to a mechanistic action.

As with exposure and disease, confounders can be misclassified. Nondifferential misclassification of a dichotomous confounder causes a bias in the direction of the confounding variable. The result will be between the unadjusted association and the association that would have been obtained if the confounder had not been misclassified. This is referred to as residual confounding and occurs when the stratum of a confounder is overly broad and/or open-ended (e.g., age ≥65). Confounding can occur within the stratum. Therefore, more narrowly defined strata reduce residual confounding. Differential misclassification of confounders also occur with less predictable results—similar to differential misclassification of exposure and/or disease. The differential misclassification of a confounder may result in adjusted associations that are not even within the range of an unadjusted and correctly adjusted association.

Confounding can be addressed through study design or data analysis, or a combination of both. The former may be accomplished by randomization, restriction, or matching. Randomization can only be performed in experimental studies (e.g., clinical trials). As an example of restriction, if smoking is a confounder for a particular disease (i.e., those who smoke get the disease more frequently than those who do not smoke, but those who do not smoke still get the disease), confounding by smoking can be dealt with by restricting the study subjects (both those exposed to the putative agent and the controls) to just those who never smoked. This design strategy simplifies the analysis and interpretation of the data, but it also restricts how broadly the results can be extrapolated. If only nonsmokers are studied, the results derived from the sample usually only apply to the

larger population of nonsmokers. Comparable information about smokers must come from another study restricted to exposed and unexposed individuals, all of whom smoked. *Matching* of potential confounders (e.g., age, race, gender, smoking) is an intuitively attractive way of addressing confounding that combines elements of both study design and data analysis; however, it is not a panacea [114]. Not only may it be difficult to do properly, but it also places certain constraints on the types of information that can be developed. Also, it may lead to overmatching (i.e., to matching on surrogates of exposure or health outcome) [57]. Matching may be accomplished by individual subjects or by frequency of groups.

Alternatively, if a certain number of subjects is being evaluated and it is known that a proportion of those in the exposed and unexposed groups were smokers, controlling for confounding could be attempted at the data analysis stage of the research, possibly by means of a *stratified data analysis* whereby different strata of smokers are analyzed and the results combined across strata. The other statistical technique would be through the appropriate regression analyses based on study design.

No matter what method is used to prevent or control confounding (e.g., stratification, covariate adjustment, matching), decisions about which specific potential confounders might be important must be made at the stage of protocol development, if for no other reason than to ensure that adequate data are collected. Obviously, it would be impossible to adjust for smoking during the analysis stage of the research if no data concerning cigarette smoking had been collected. Therein lies an excellent reason to draw a causal diagram as part of the protocol development (see section on *Causal Inference*). In experimental studies, the number of variables is purposefully kept to a minimum and ostensibly all of them are under the control of the investigators. Those who conduct observational studies of humans do not have the same advantages. The number of variables is limited only by life itself. Each participant in an epidemiology study has his or her own unique genetic makeup and own unique pattern of extraneous exposures (e.g., diet, medications, personal habits). Although either or both may be only weak confounders for a particular health outcome under investigation, they may be one reason why epidemiology research, especially any single study, has difficulty in reliably identifying putative agents with lesser biological potency, with true relative risks less than 3 or so. This is because even in the absence of selection and misclassification biases, the signal may be swamped by the noise of uncontrolled confounding. In epidemiology, the signal-to-noise ratio is improved via more research, especially more targeted research. As the exposure–disease associations become more focused, the relative risks should increase in size. If they do not, be suspicious of claims of causation. Also, be suspicious of etiologic interpretations based on one study unless supporting evidence is available.

Confounding is not restricted to epidemiology research. It also occurs in toxicology; for example, Hart and associates [68] have explored the impact of feed intake in laboratory animals. They noted that animals fed ad libitum have poorer health and longevity than those whose diet has been restricted. The total caloric load appears to play a role, but trace contaminants may also be important. As reported recently by Paolini and colleagues [101], most standardized diet formulations used by cancer research laboratories worldwide "contain the well-known mutagenic carcinogenic element manganese at the same level and, in some cases, at an even higher level (up to ninefold) compared to that used to study the carcinogenicity of manganese itself." Obviously, the more animals they eat, the higher their caloric load and the higher their dose of this carcinogen; however, the amount ingested could be an unintended consequence of the experiment (e.g., ever larger amounts of the test chemical mixed with the feed may make the feed less and less palatable). For those experiments in which ingestion varied by dose level of the experimental agent, it is quite possible that the results reflect a measure of confounding and perhaps effect modification. Paolini et al. [101] also summarized a number of problems using historical controls; for example, "$B6C3F_1$ mice have a higher natural incidence of tumors than humans, and this incidence has also changed over time, increasing in excess of 50% over a period of just 10 years."

Although it is impossible to consider all possible confounders in any single study, the reader of epidemiology reports should determine whether attempts were made to control for those factors that likely would have had the greatest impact on the results. As with other types of potential bias, a paper can offer a number of clues as to how well this issue was or was not properly managed. If confounding was ignored or obviously inadequately addressed, be skeptical of the information. Look for confirmation in other work that did try to minimize confounding.

Chance

Within the mantra of selection, information bias (misclassification), confounding, chance, and causation, the rubric *chance* covers all things mathematical and statistical and some that are methodological. For example, did the investigators add, subtract, multiply, and divide properly? Were the numbers of subjects consistently the same in the abstract, results, discussion, and tables? With much more complex statistical procedures, it is possible for even the most seasoned epidemiologist to inadvertently ignore a key assumption and thereby produce erroneous results. If numbers are inconsistent within a report, do the authors explain why? And, if they do, does the explanation seem appropriate? If the answer to either question is no, then look for confirmation of the results elsewhere. Or look for a correction published as an errata in a subsequent issue of the journal.

Also, determine how the data were aggregated for analysis. Does it make sense, in particular, biological sense? By way of example, in 2001, investigators affiliated with the Food and Drug Administration published a study entitled *Silicone gel breast implant rupture, extracapsular silicone, and health status in a population of women* [25]. This paper has been represented as demonstrating a causal association between *leaking silicone gel implants* and fibromyalgia (FM) [144]. The original article, its conclusions, and subsequent interpretations have been severely criticized for a variety of reasons [19,45].

For the purposes of this discussion, the point of interest is how the investigators chose to make their key comparison. Through the use of explant surgery, they determined the prevalence of implant status among women with breast implants (no women without implants were included in the research). Three categories of exposure were defined: extracapsular rupture (obvious silicone adjacent to or remote from the outside of the tissue capsule that surrounds every implant), intracapsular rupture (silicone outside the medical device but apparently confined inside the tissue capsule), and intact implants. The authors reported a statistically significant excess (OR, 2.8; 95% CI, 1.2–6.3) of self-reported symptoms consistent with FM, but only when they compared the complaints of women who had extracapsular rupture with those in the aggregate group who either had intracapsular rupture *or* intact implants.

It was pointed out in subsequent letters to the editor that the strategy made no biological sense. As one critic noted, "…if an association exists between implant status and FM, one would hypothesize … the true gradient would be: intact < intracapsular rupture < extracapsular rupture." Reformatting the data presented in the paper so that ORs not reported in the published paper could be calculated, he noted that the OR between FM and ruptured status reached statistical significance largely as a consequence of the way the comparison group was structured [19]:

> The OR between FM and extracapsular rupture (compared to intact devices) is 1.88 and not statistically significant. The OR between FM and intracapsular rupture (compared to intact devices) is 0.50 and also not statistically significant. The OR for any rupture versus intact devices is 0.87 … the largest difference in FM risk is between extracapsular and intracapsular rupture. If a gradient in risk exists, these data seem to suggest a gradient for FM that is: intracapsular rupture < intact < extracapsular rupture … suggesting that intracapsular rupture may protect women against FM!

p Values, Confidence Intervals, and *p*-Value Functions

A major difference between toxicology and epidemiology is in the use of *p* values to describe research findings. Unlike toxicology journals, epidemiology journals often inform its contributors that *p* values are not to be used except in certain circumstances (e.g., tests for trend). Whereas toxicologists will almost always offer measures of variation (e.g., standard deviation) to accompany their point estimates (e.g., arithmetic means), epidemiologists prefer the use of *confidence intervals* for their point estimates (e.g., relative risks, ORs). Why such differences between the two disciplines? A satisfactory explanation requires understanding random error—assuming the biases of systematic error have been controlled—a large assumption indeed.

By definition, a *p* value is the statistic obtained from hypothesis testing. It is the probability, *assuming the null hypothesis is true*, that the obtained study data are as far (or farther) from the null hypothesis than that observed. In experimental studies, the hypotheses that are tested are primarily formulated a priori. The *p* value is conditional on the null hypothesis, which is usually the following: there is no relation between exposure (treatment) and disease. Small *p* values are consistent with the rejection of the null hypothesis. *p* values of 0.05 or 0.01 are often (arbitrarily) used to reject the null hypothesis; however, the null hypothesis could still be true, despite the low *p* value. If the *p* value is above this arbitrary value, the null hypothesis is not rejected; however, the null hypothesis could still be false, despite the higher *p* value. Under either circumstance, the *p* value does not offer an estimate of the random error in the point estimate.

A confidence interval, still set at an arbitrary level usually 95% or 90%, provides precision around a point estimate. A 95% confidence interval of a point estimate (e.g., OR) implies that if the study (data) could be hypothetically obtained many more times, the interval within the confidence limits should include the *true value* 95% of the time. Assumptions include that the only difference in the repeated studies is chance in the collection of data and systematic biases are not present. Although in observational epidemiologic research such assumptions are impossible to fulfill, the confidence interval still provides an estimate of the precision of the data. Wide confidence intervals indicate imprecise point estimates. Narrow confidence intervals indicate the opposite. Neither though prove the validity, or lack thereof, of the data. Some epidemiologists use an informal statistical hypothesis test by stating that if the null hypothesis (e.g., OR = 1.0) is not included in the confidence interval, then the findings are *statistically significant*. However, this is essentially no different than a test of the null hypothesis but at least the width of the confidence interval is still provided to examine precision.

Ideally, a *p*-value function (or confidence interval function) could be offered for important point estimates [104]. Unfortunately, available journal space per article prevents this practice although technical reports could offer it. A *p*-value function plots various *p* values (*y*-axis) for a range of hypotheses with the estimates of effect (*x*-axis) (e.g., relative risk, OR). The shape of the curve results in a visual of the strength of the association and precision. Nested confidence intervals can then be read from the curve; therefore, the reader could better understand a range (indeed an infinite range) of confidence intervals, not just an arbitrary one (e.g., 95%).

As noted previously, a study result may have a wide confidence interval and still be valid. A result from a small study relatively unbiased by selection, misclassification, and confounding

may be more valid than the result from a larger study that has a narrower confidence interval. Although the former may have limited utility in and by itself to support or refute causation as a consequence of its low power, when combined with the results of other studies of comparable quality, it may prove to be very valuable. This is the rationale underlying *meta-analysis.*

Exploratory Data Analysis and Multiple Comparisons

Scientific discovery is not a destination. It is a journey with many side trips along the way. It starts with a hypothesis, a theory whose genesis may be any number of things ranging from the subjective (clinical observations that seem unusual for intuitive reasons) to the super quantitative (statistically significant findings derived during *exploratory data analysis* of a large medical dataset, such as the health claim files of a private insurance company or of Medicare/Medicaid). Before these findings can be accepted as even provisional truth, they have to be confirmed by additional research, preferably well-focused hypothesis-testing research.

In both hypothesis-generating and hypothesis-testing exercises, the same statistical tools and the same levels of statistical significance may be used; yet the findings of the former do not carry the same interpretive weight as those from the latter. That's because the former, in addition to uncontrolled confounding, are subject to a *multiple comparisons bias* [129]. Exploratory statistical analyses of large and diverse datasets that may selectively report only those results that support their own theories are viewed as post hoc reasoning [90,120,129]. Because few comparisons are presented, the reader is given the erroneous impression that only those few were considered and therefore they must have been of some a priori concern. Investigators who are guilty of post hoc reasoning are sometimes derisively called *Texas sharpshooters*. In most target shooting, one shoots at a preexisting bull's eye. The Texas sharpshooter first shoots at the side of the barn (perhaps from very close to the building) and then draws the bull's eye around the holes.

The statistical tests used in health research factor in both a type I and a type II error. A type I is the error of rejecting a null hypothesis, of concluding that a difference exists when, in truth, it does not. By convention, the alpha level (the probability of a type I error) is usually set at 0.05 (which equates to a 95% CI). This means that a certain predictable number of statistically significant findings are incorrect, about 1 in 20. The greater the number of comparisons, the greater is the number of spurious associations that may be found (i.e., the larger is the multiple comparisons bias). Various techniques have been developed to address this bias, the simplest perhaps being the *Bonferroni correction* in which the putative alpha is divided by the total number of comparisons and the *corrected alpha* is used to determine the presence or absence of statistical significance [88]. For example, if the study alpha level was preset at 0.05 and 10 comparisons were made, a Bonferroni-corrected 95% CI would, in essence, be a 99.5% CI. This method has many detractors who argue that much more suitable methods are available to address multiple comparisons especially those incorporating Bayesian statistics where specifying a *prior* is required in the analytical computation of the posterior distribution [63]. Besides the *prior* input parameters, the other input is a function that shows the probability of the observed data for any given set of parameter values (i.e., the likelihood function). In other words, assign a level of probability to the premise of the prior argument. The *posterior* probability will depend on what was used as the *prior* probabilities. A range of disparate *priors* will eventually converge into similar *posterior* probabilities in the face of consistent data. Toxicologists should expect to see more Bayesian analyses presented in the epidemiology literature in the future.

In summary, even the most precise results may be wrong, a consequence of simple mathematical errors, technical bias, or less innocent intent. Although exploratory data analysis is a valuable tool, more is not always better. This maxim applies equally well to epidemiology, toxicology, and clinical medicine (Figure 11.16). To be interpreted properly, the results of tests must be put in the context of the size of the dataset, the number of tests that were performed, the body of knowledge that is already available, and, if possible, the mind-set of the investigators at the inception of the research.

You seem to be in fine health but let's run a few tests.
I'm sure we can find something wrong with you.

FIGURE 11.16 Multiple comparisons bias in clinical medicine.

The latter may be obvious from the introduction of the paper or from the protocol, but sometimes, it can only be surmised.

Meta-Analysis

Meta-analysis refers to the use of statistical tools to combine the results of different studies. Originally, it was confined to randomized controlled clinical trials, to combining results of multiple small studies of the equivalent design (i.e., those with identical dosing regimens and comparable, well-defined outcomes). It is increasingly being used to aggregate the findings of multiple epidemiology studies, even when their results were derived by means of disparate methods (e.g., cohort and case–control studies), the sample sizes varied by orders of magnitude, the categories of exposure differed, and the disease outcomes were similar but not equivalent [14]. Although some decry the use of meta-analysis for this purpose, others view it as an important adjunct to the traditional, more subjective literature review. Essentially, is the primary purpose of meta-analysis to produce a combined estimate of effect that, when done properly, has a narrower confidence interval than each individual study or is the primary purpose of meta-analysis to identify the heterogeneity in study results? The more insightful answer is that understanding the reasons for the heterogeneity of the estimates of effect is indispensable to understanding the literature, regardless of whether a meta-analysis estimate of effect is calculated.

Meta-analysis is not the same as *data pooling*. Whereas meta-analysis depends on the research results as obtained from epidemiology reports, pooling refers to the aggregation of the actual raw data from many different studies and the subsequent analysis of this larger, single dataset. Conceptually, pooling has some advantages over meta-analysis, but in practice, it also has a number of disadvantages, a major one being access to the data. Unlike meta-analysis, where the results have been distributed publicly via the scientific journals, data are not as readily available. In part, this is because of concerns related to protecting the privacy of individual study subjects and the confidentiality of their data [9].

The validity of a meta-analysis is dependent on the validity of the studies included in the exercise. To address this problem, some have suggested that a priori rules must be established with respect to which studies to include or exclude. Unfortunately, these rules may reflect the personal biases of the one doing the meta-analysis. For that reason, a type of sensitivity analysis is arguably a better approach [102]. In this type of analysis, the results of all available studies are first evaluated together and then various combinations are used to better understand how the different methods, number of study subjects, classifications of exposure, or definitions of health outcome may have influenced the calculations. It can even be used to compare and contrast the results of different studies that may have different types of bias and to explore whether potential bias is a likely explanation for why one or just a few of the studies seem to be outliers. If a comprehensive sensitivity analysis is conducted and the results published, readers also have the opportunity to make their own interpretations, something that can be difficult to do with the traditional literature review or even with pooling.

One particular type of bias to which both literature reviews and meta-analyses are particularly susceptible is *publication bias*. Publication bias is a type of selection bias. It refers to the tendency of authors to submit and editors preferentially to accept studies with provocative positive findings [7,42,77]. This has also been referred to as *positive results* bias. Should these results ultimately be shown to not be causal, the findings are considered to be false-positive results. Example 11.B.4 recaps the simmering debate within epidemiology circles regarding false-positive and false-negative results and their role in publication bias. A number of different approaches can be used to assess the possibility of publication bias, but the best way to avoid it is to aggressively search for pertinent research reports, including those in the form of dissertations, abstracts, and publications in obscure journals [107]. The use of funnel plots allows the meta-analyst to visually examine for symmetry of the available studies [126]. In a meta-analysis, the funnel plot is a graph of the estimates of risk for every study with their corresponding precision (standard error). Publication bias may lead to an asymmetrical graph if smaller studies that lack effects are not included in the meta-analysis.

Causation

Understanding disease etiology depends on a complex, iterative course of inquiry called the *scientific method* [131]. To quote Hazen [69], "The scientific method is an elegant process for learning about the natural world, but it is neither intuitive nor obvious." This method can be idealized as a cycle of observation (data collection), synthesis (data analyses), hypothesis (reasoned conjecture based on the interpretations of patterns derived during the data analyses, often as interpreted in the context of other information), and prediction. The prediction then has to be tested (sometimes referred to as the hypothesis-testing stage of scientific inquiry) with a new round of observation and synthesis, providing results that, given they replicate the original findings, reinforce the initial hypothesis. Alternatively, the results might not support the hypothesis, in whole or in part; therefore, the original hypothesis might be dismissed outright or modified and retested by means of a new round of prediction, observation, and synthesis. This goes on until there is some level of consensus that a provisional truth has been identified. As a rule of thumb, the more provocative the association, the more imperative the need to replicate the findings.

It is important to recognize that the prediction must be constructed in a form that is both unambiguous and refutable; therefore, although the hypothesis may be stated as "exposure to agent *X* is associated with an increased risk to disease *Y*," the prediction has to be stated in the null (i.e., exposure to agent *X* will not be followed by an increased risk of disease *Y*) and the null refuted. Refuting the null lends support for the hypothesis, but in science, the default is always the null; consequently, a theory of cause and effect may be disproved by an unfulfilled

prediction, but it can never be completely proved—thus the caveat about *provisional truth*. Alternatively, an anomaly (an exception to a prediction) may lead to new insights; for example, it may suggest a prediction that restricts causal actions of agent *X* to higher levels of exposure.

It is also important to recognize that at the core of any scientific inquiry is the prevailing expectation about the workings of the natural world. For example, the current paradigm regarding dose–response for carcinogens is (1) linear and (2) nonthreshold. This paradigm is now being challenged. For example, Calabrese [26] has shown that for a large number of chemicals, both attributes of the current paradigm are wrong, particularly at lower ranges of exposure. This indicates a necessary reevaluation of the current paradigm in risk assessment and risk management.

Causal Inference

A causal mechanism has many component causes [117]. For example, a concussion incurred by someone hitting his forehead against the car windshield could have the following components: (1) driving too fast, (2) not wearing a seatbelt, (3) talking on the cell phone, (4) distraction by a passenger, (5) oncoming car not dimming their lights, (6) alcohol, (7) turning the radio to a different station, (8) poor brake pads, (9) driver not wearing corrective lenses, and (10) inexperience at driving a motor vehicle. Both genetic and environmental (nongenetic) components may apply. On a population basis, some of these component causes are more strongly associated with the adverse event, whereas some are weaker causes. Some of these components are likely to have interacted such as driving too fast and poor brake pads resulting in a longer distance required to stop. Because of these interactions, the sum of component causes attributable to an effect (disease) is greater than 100%. Bottom line: diseases have multifactorial etiologies. A sufficient cause is defined as those minimum components necessary for the effect (disease) to occur.

The induction period is defined as the period of time beginning at the first component cause and ending when the final component cause acts and the disease (concussion) occurs. Although it appears in the aforementioned example that most component causes occurred simultaneously, the poor eyesight occurred well before this accident and is likely genetic. Thus, induction period is not readily known for many diseases including cancers. The latent period is defined as the time frame from disease occurrence until its detection. In the instance given earlier, the concussion might have occurred immediately but was not detected for a few hours or days. In the case of cancer, component causes result in the initiation and promotion of cancer. The cancer may not be detected, however, until there are marked clinical symptoms (abdominal pain). Or the cancer is detected much earlier via a colonoscopy. Epidemiologists often combine these two time periods in their data analyses (induction–latency period) since they may not be individually well defined.

It is beyond the scope of this brief review to discuss causal inference theory. Scientific philosophies have revolved around inductivism, refutation, consensus, and Bayesianism [117]. As important as understanding causal theory, the toxicologist should familiarize himself with the use of causal diagrams. To draw a causal diagram, it is critically important to distinguish causal relations from associations. Causal diagrams allow nonmathematicians to graph causal relations among the exposure, outcomes, and covariates. Example 11.B.5 shows how researchers determined, through the use of a causal diagram, that an association between concentrations of serum perfluoroalkyls measured during pregnancy and time to pregnancy (TTP) (i.e., subfecundity) was, in fact, the likely result of the interpregnancy interval (which included TTP) affecting the serum perfluoroalkyl concentrations and not vice versa as proposed by the original investigators, otherwise referred to as *reverse causation*.

Establishing causal inference, the weight of evidence inferring that exposure to a substance can cause an effect in humans, is a common goal of epidemiology and toxicology. Adami et al. [1] noted that a principle underlying the philosophy of science is that causality cannot be *proven*; it can only be inferred with different degrees of certainty. Hill introduced a list of nine aspects of associations that should be considered before inferring causation [72]. Often erroneously referred to as the Bradford Hill criteria (guidelines is a more appropriate descriptor as stated by Hill), several of these aspects benefit from evidence that may be collected in toxicological investigations (Table 11.1).

Even when selection, information bias (misclassification), and confounding are minimal, identification of the causes of human disease is not simply an exercise of calculating which exposure–disease associations are statistically significant. Sir Bradford Hill, a British statistician/epidemiologist, presented his guidelines for subjectively considering causation in the mid-1960s [69]. They are still in wide use although with increasing reservations. In interpreting data, he noted that an investigator must deal with two basic problems: *significance* (the statistical reliability of a finding) and *inference* (the interpretations one might make from such a finding). With the former, he cautioned against either over- or underinterpreting the importance of statistical significance—noting that, if absent, *chance is not an unlikely reason* for an apparent difference, for an apparent association, or for an apparent elevated relative risk, but, if present, *chance is still a possible, though unlikely, explanation*. He also advised that conclusions related to a new

TABLE 11.1
Hill Guidelines for Causation

Strength of the association
Consistency
Specificity
Temporal relationship
Biological gradient
Biological plausibility
Coherence of the evidence
Experiment
Reasoning by analogy

finding have to be *more guarded* and stress the *limitations* of the data (size of the sample, potential for bias, etc.). As for inference, he offered nine criteria for differentiating between *causation or merely association* when faced *with a clear and significant association between some form of sickness and some feature of the environment* (Table 11.1).

Despite the widespread use of these guidelines, the nine points have been criticized as misguided and epidemiologists have not agreed on a set of causal criteria [116]. Nor are they likely to do so. For example, scientific refutation of causal hypotheses (inferences) could be considered a more meaningful approach [116]. Nevertheless, these nine points offered by Hill are popular and often cited in review papers. Therefore, a brief explanation is in order despite the sound reservations offered by others. *Strength of the association* refers to the size of the relative risk or OR. *Consistency* refers to the finding of similar relative risks for the same condition and exposure in different epidemiology studies conducted by different investigators on different groups of participants. *Specificity* suggests that elevated risks should be to a single or small number of well-defined health problems. *Temporal relationship* means exposure precedes disease. If the disease occurs before the exposure, it cannot have been caused by the exposure. *Biological gradient* refers to dose–response. *Biological plausibility* depends on the biological knowledge of the day. Some consider this necessary to prove causation; others do not. They are satisfied if a meaningful association is found for a risk factor even if the exact causal agent and the process by which it works are unknown. *Coherence of the evidence* is the amalgamation of what is known concerning the natural history and biology of the disease, the presumptive actions of the etiologic agent, the results of experimental research on animals, and the contributions of other types of information. The evidence can come from within a single study or across studies from many different disciplines. The next attribute was *experiment*, but not necessarily in the context of a laboratory experiment. He also considered the removal of the presumptive etiologic agent a type of experiment. *Reasoning by analogy* refers to the situation where if agent *X* can cause disease *Y*, perhaps a material similar to *X* can cause a disease comparable to *Y*.

From a toxicological perspective, Hill's second viewpoint, consistency of the association, while referring primarily to epidemiological associations, can be strengthened if observations from toxicological studies support the causality assumption. The fourth aspect, temporality, must be achieved and can be strengthened by toxicological investigation, which, by its nature, is prospective and designed to evaluate cause and effect. Biological gradient, or dose–response, is Hill's fifth aspect, and dose–response data from toxicological studies can strengthen the interpretation of exposure–response from epidemiological studies, where quantitation of exposures may be more uncertain. Hill's sixth, seventh, and eighth aspects, respectively referring to biological plausibility, coherence with *generally known facts of the natural history and biology of the disease*, and experimental evidence, all may benefit from the results of toxicological experimentation. With respect to experimental evidence, Hill stated that "Here the strongest support for the causation hypothesis may be revealed." Thus, toxicological evidence can make a strong contribution to the process of inferring causation from associations observed in epidemiological studies.

Inferring causation is an uncertain process, and the degree of uncertainty can be reduced by considering the results of both epidemiological and toxicological investigation. Serving a purpose similar to Bradford Hill's viewpoints, from a toxicological perspective, frameworks have been developed to consider human relevance of toxicological observations, and the REACH guidelines have provided a framework for assessing health hazards and derived no effect levels (DNELs) and derived minimal effect levels (DMELs) based on *animal* and human data. These previous frameworks have been valuable in structuring the manner in which experimental observations are placed in perspective for the risk assessment process. However, a process that consciously aims to integrate the observations from epidemiology and toxicology has been lacking until recently.

Building upon previous work [18,33,80,81,121], Adami et al. [1] have provided a framework for integrating toxicological and epidemiological data in evaluating potential causal inference. Their five-step process, called *Epid-Tox*, includes (1) collection of all data, (2) assessment of data quality, (3) evaluation of the weight of evidence, (4) assignment of a scalable conclusion, and (5) placement on a causal inference grid. This process, leading to the casual inference grid and one of four descriptors for potential causality, is best visualized from their paper, reproduced here as Figure 11.17. The grid itself from Adami et al. [1], reproduced herein as Figure 11.18, includes an *x*-axis on which to place causal inference evidence from epidemiological investigation, from *against* to *for* going from left to right, as well as *y*-axis on which to place biological plausibility based on experimental data, from *low* to *high* going from bottom to top. The grid is divided into four quadrants, with an area allocated to *insufficient information* for placement occurring in the center. The four potential causality conclusions corresponding to the four quadrants are *likely*, *uncertain* (based on strength of the epidemiological association but lack of biological plausibility), *uncertain* (based on plausibility from toxicological studies but lack of evidence in epidemiological investigation), and *unlikely* (both epidemiological and toxicological evidence infer a lack of effect). Adami et al. provide several examples of the application of the Epid-Tox process, and the reader is encouraged to consult these examples.

Any process used to infer causality based on the integration of epidemiological and toxicological data should be considered iterative in time. It is important to remember the observation provided by Hill [72] at the end of his article:

> All scientific work is incomplete - whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have, or to postpone the action that it appears to demand at a given time.

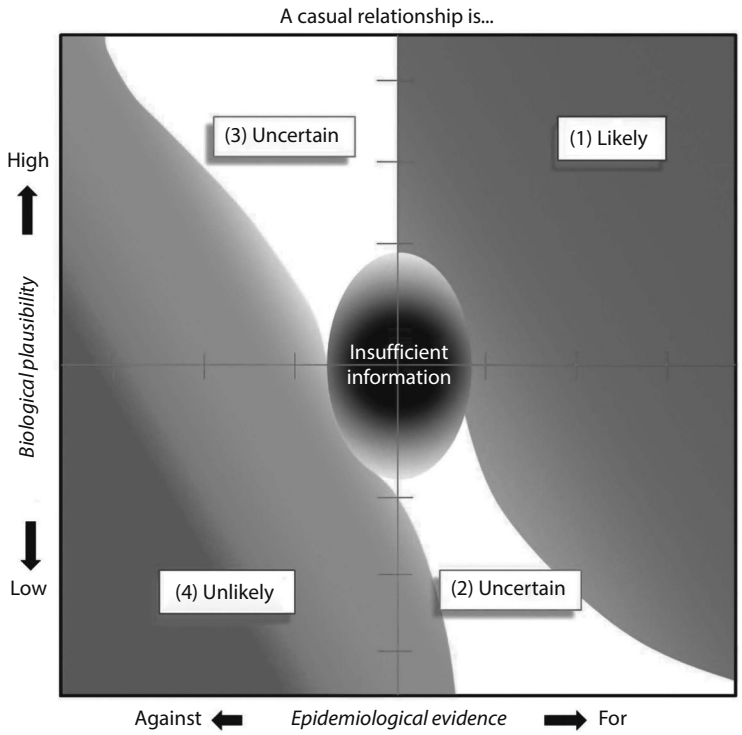


FIGURE 11.17 The causal inference grid: how strong is the evidence for or against a causal relationship in humans? (From Adami, H. et al., *Toxicol. Sci.*, 122, 223, 2011. With permission.)

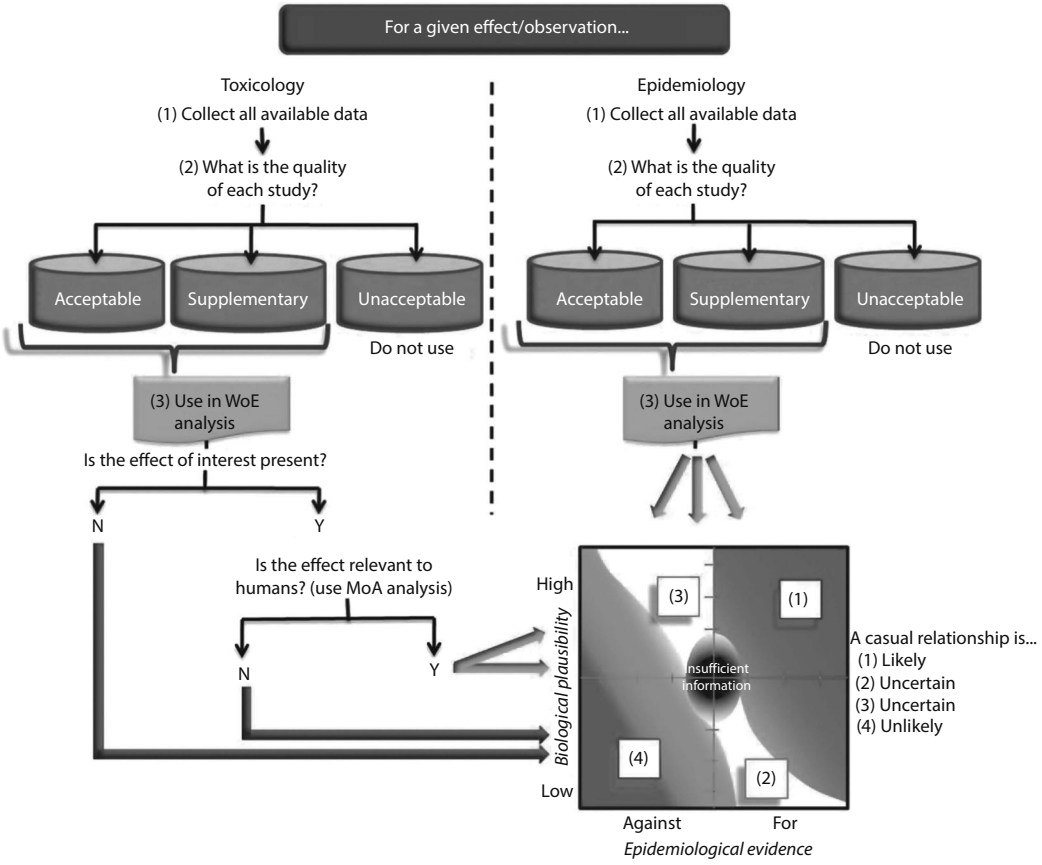


FIGURE 11.18 Schematic representation of the framework for causal inference based upon weight of evidence of animal and epidemiological data. (From Adami, H. et al., *Toxicol. Sci.*, 122, 223, 2011. With permission.)

Legal Causation

At one time, courts tended to disregard epidemiology as simply a statistical exercise that provided information of little probative value; however, within the last 20 years, it has become key to the legal theory of causation as used in the particular type of litigation that deals with tort or product liability [13]. Epidemiology research helps establish not only whether an agent is causally associated with a particular disease but also whether the association supports a finding of *more likely than not*. This equates to an attributable risk percent (AR%) of greater than 50% and, with knowledge of the relative risk (RR), can be calculated with the following formula: AR% = (RR − 1)/RR. For example, a relative risk of 3 would equate to an attributable risk percent of 67%.

As mentioned earlier, the various calculations regarding attributable risk have no meaning until causation for human disease is established—however, that is temporarily accomplished. In theory, therefore, an exposure–disease association has four characteristics that must be demonstrated before a claim of causation logically can be accepted in legal deliberations: (1) The putative agent must be a known cause of the disease, (2) the causal relationship must be more likely than not, (3) the plaintiff must have been exposed to the agent in adequate quantity and for sufficient duration, and (4) the plaintiff must have developed the appropriate disease after the exposure. The first two deal with *general causation*. The last two pertain to *specific causation*. In tort liability cases, the plaintiff has the burden to prove all four, at least in theory. Trials are emotional events and jury deliberations can sometimes be more influenced by the subjective than the objective.

Prior to the 1993 Daubert decision, juries were the triers of fact and judges basically functioned as the umpires of the proceedings [39,53,106]. They made rulings regarding process but few about content. The Daubert case changed that [39]. After a series of appeals that went all the way to the supreme court, judges were given the additional responsibility of serving as *gatekeepers*. Juries retained the role of triers of fact, but judges were charged with determining which body of facts were relevant and reliable versus which were simply junk science—which testimony would assist the jury in their deliberations and "whether the 'probative value' of the testimony substantially outweighed the risks of prejudice, confusion or wasted time" [74]. In practice, this means federal judges now must decide which expert witnesses can and cannot testify and what opinions they will be permitted to convey to the jury. Many state courts are also moving toward a process based on the Daubert principles.

Some courts have done an impressive job in rendering judgments that have included sophisticated legal arguments well infused with scientific principles. Others have accomplished the same result with the help of outside experts employed directly by the court, an option acknowledged in the Daubert decision. Still others at the state level have yet to apply the Daubert principles, in part because some judges feel uncomfortable with their new role and in part because the new rules technically pertain to just the federal judiciary [43].

Lawyers and judges, even at the federal level, are still exploring the limits of the gatekeeper function and how certain statistical and epidemiologic thought might be translated into legal concepts. As an example, statistical significance means that a finding has a lower confidence limit above 1; that is, there is some assurance that the estimate of risk is different than 1. The legal notion of *more likely than not* requires a relative risk above 2, but it is unclear whether the key finding, to be admissible, has to be statistically significantly different than 1 or statistically significantly different than 2. When there is just one or a limited number of epidemiology studies, the latter makes more sense; however, the former is not inconsistent with epidemiology opinion when a large number of reasonably valid studies have similar results.

Clinical Causation

Neither epidemiology causation (what Sir Bradford Hill called *medical causation*) nor legal causation should be confused with *clinical causation*. The primary goal of clinical medicine is diagnosis and treatment. In a sense, the major reason for a diagnosis is to predict which treatment will most successfully reverse, eliminate, or control a patient's troublesome symptoms or signs of pathology. If the diagnosis is correct, the resulting treatment works and the patient is well served. If not, the patient likely gets no better, possibly gets worse, or even may develop additional adverse outcomes as a result of the inappropriate therapy.

Experienced clinicians are adept at the technique of *differential diagnosis*. Through the use of various signs, symptoms, and test results, and factoring in the risks inherent to alternative treatments, they identify the most probable diagnoses, weigh the merits of each, and use the resultant information to help select a treatment that likely will be most successful. If that particular treatment does not work, they move on to the next most likely diagnosis and a different treatment and, if that does not work, to still another, continually balancing benefit and risk.

When clinicians speak of searching for the *cause* of a patient's problems, they usually are referring to identifying the most likely diagnosis, quite possibly one whose underlying mechanisms of action are unknown. Arguably, knowledge regarding the underlying cause of a particular disease is only important in the clinical setting if it materially impacts treatment decisions—for example, if a specific type of bacterial pneumonia is more efficaciously treated by a particular antibiotic—and the underlying causes are not initially discovered by the process of differential diagnosis. Such knowledge is derived from experimental animal research, controlled clinical investigations, and observational epidemiology studies. Contrary to what some clinicians have asserted, differential diagnosis, no matter how sophisticated, does not obviate the need for etiologic research [5,58,62,108]. As the many programs of the National Institutes of Health demonstrate, research regarding cause and effect and that related to diagnosis and treatment are complementary but not equivalent.

Parenthetically, proper diagnoses are made by means of pattern recognition, by what Margolis has called *habits of the mind* [87]. Within the context of clinical causation, this has a number of implications. One, the more extensive a physician's training and experience, the larger the number of mental templates he or she acquires against which he can compare the next patient's combination of signs, symptoms, and test results; thus, even if the underlying etiology for a condition is unknown (i.e., the condition is idiopathic), a physician may develop successful strategies for treating the syndrome. Two, this knowledge, no matter how prodigious, is always finite. Physicians recognize this. They specialize so they might concentrate their energy on developing in-depth knowledge within one sector of medical practice, and even within that specialty they refer patients to their peers, a tacit acknowledgment that another physician may be better suited to diagnose and treat a particular individual. Three, because the number of templates increases as a direct result of experience, the more seasoned the clinician, the greater the clinician's ability to diagnose and, paradoxically, the greater the potential for a multiple comparisons bias. The latter is reflected in case reports.

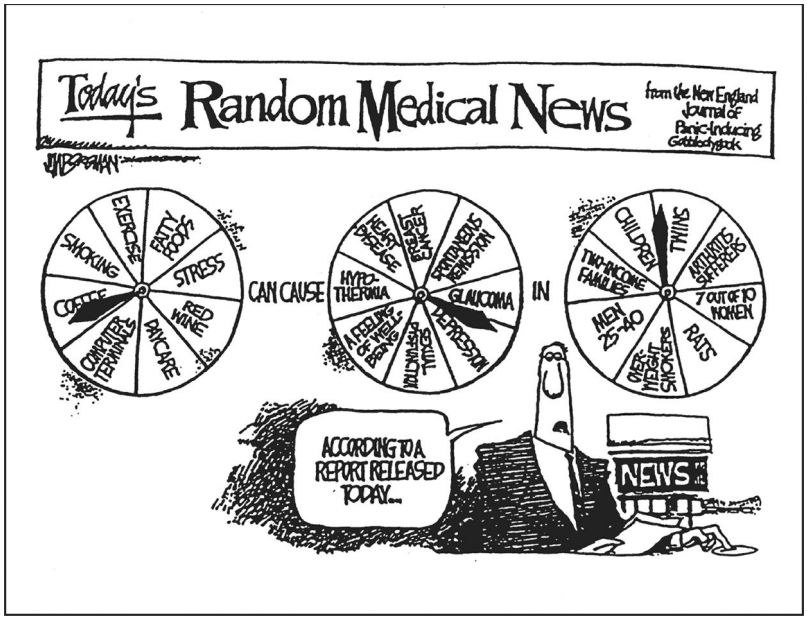
CONCLUSION

As de Grasse Tyson noted in his essay, *Certain Uncertainties*, "The frontier of science is a messy place" [41]. As a consequence, to the uninitiated, science appears to provide contradictory and therefore unreliable findings, regardless of whether the research is experimental, quasi-experimental, or observational, but perhaps more so for the latter (Figure 11.19). Part of the reason for the apparent inconsistencies is related to technical bias (selection, information, and confounding), but part is due to overinterpretation of the findings of any single study, either by the study investigators or by the consumers of research reports.

One of the primary goals of any scientist should be the elimination of bias from his or her research. The first step is to acknowledge that various types of bias exist, the second is to understand how they occur, and the third is to develop methods and procedures to avoid, minimize, or control bias. Over the years, well-trained epidemiologists have found ways to address potential error and improve the validity of their research. The same can be said for toxicologists and clinical investigators. The scientific method has been core to all of these endeavors.

The scientific method is one of the major discoveries in human history [24,133]. It has allowed mankind to gain a more objective view of the universe, to better understand the workings of the atom, and to successfully identify the causes of disease and with that understanding to treat, control, and even eliminate some of the major scourges that once were endemic worldwide. Unfortunately, the scientific method can be laborious, inconvenient, time consuming, and expensive. The temptation to take shortcuts can be great, but history teaches that shortcuts often just lead to further confusion [119]. Identifying the truth can be difficult enough even in the best of circumstances, but it is impossible with biased data, inappropriate methods, or muddled logic. Without a doubt, the scientific method does not lend itself to today's instantaneous news cycle with 30 s sound bites.

One of the responsibilities of the technical journals is to screen research papers and determine which have sufficient rigor in data, methods, and interpretation to warrant publication; however, scientific investigation is a human endeavor and peer review an imperfect process, so biased studies still



By Jim Borgman

FIGURE 11.19 Today's random medical news. (Copyright 1997, The Cincinnati Enquirer, Cincinnati, OH. Reprinted with permission of King Features Syndicate.)

get published, even in the best of journals. And what is the *best* journal? One that has high *impact factor* that is an inherently flawed statistic unto itself [70,71]? For that reason, in epidemiology research, no matter where it is published, the ultimate judgment regarding the value of any single report or group of reports will have to be made by the consumer: the epidemiologist, toxicologist, physician, lawyer, judge, newspaper reporter, or other member of the general public less well versed in the scientific method. This chapter provides a conceptual framework whereby basic understanding of epidemiologic findings might be critically understood by the toxicologist. Within a basic understanding of epidemiology data, measures of association, and methods, this critical assessment is based on the mantra of selection, information, confounding, chance, and causal inference. All are important in determining the validity of epidemiologic studies.

QUESTIONS

11.1 What is the difference between prevalence and incidence?
11.2 Describe two methods used in epidemiology.
11.3 What is literature bias?
11.4 Define sensitivity and specificity.
11.5 What are the three necessary characteristics of a confounder?
11.6 What can be learned from a meta-analysis?
11.7 Are the Hill guidelines for causation universally accepted?

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Gail Olsen in the preparation of this document.

APPENDIX A

STRENGTHENING THE REPORTING OF OBSERVATIONAL STUDIES IN EPIDEMIOLOGY (STROBE) STATEMENT

The Consolidated Standards of Reporting Trials (CONSORT) statement was originally designed to improve the quality of reports of randomized trials [134,136]. Subsequent types of initiatives followed including guidance on how to report data from observational research. To accomplish the latter, an initial workshop was held in 2004 that ultimately produced, 3 years later, the published document *Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement*. Initial financial support for this initiative was obtained from the European Science Foundation.

The STROBE statement should be viewed as a guideline to assist authors when writing reports of analytical observational studies, to support editors and reviewers when reviewing such manuscripts for publication, and to assist the reader when critically reading published papers [136]. The STROBE statement focused on three study designs: cohort, case–control, and cross-sectional. The STROBE statement is a checklist of 22 items that were considered necessary for the reporting of observational studies. The items relate to the article's title (item 1), the introduction (items 2 and 3), methods (items 4–12), results (items 13–17), discussion (items 18–21), and funding (item 22). A total of 18 items are common to all three study designs, while four items (6, 12, 14, and 15) are design-specific items. Accompanying the published STROBE statement checklist is a lengthy explanation and elaboration intended to enhance its understanding.

It is equally important to emphasize what the STROBE statement is not. The STROBE statement is not to be viewed as a prescription for designing or conducting observational research nor mandating requirements for the uniformity of presentations [134]. The STROBE statement should not be interpreted as a means to assess the quality of published observational research.

As with most epidemiological initiatives and studies, the STROBE statement has received its share of criticism. Guidelines for reporting should not evolve into an instrument for judging the quality of observational studies. The STROBE statement does not adequately emphasize the importance of defining study hypotheses, specifying biologic rationale for such hypotheses, testing the hypothesis, and, in particular, requiring careful consideration of biological plausibility [82]. As epidemiology remains a relatively young science whose creativity lies in the development of new methods, the STROBE statement should be time limited with a specific expiration date [118]. Criticism of the STROBE statements also surrounded its affirmative statements on statistical significance testing at the expense of reporting point estimates, confidence intervals, and the need to conduct power analyses on only an a priori basis. And finally, such guidelines should be at the forefront of the design, conduct, and analysis of all studies and not to be considered only at the time of reporting. Poorly conducted studies may be inappropriately triaged to address what should have been done rather than what was done, leading to a less transparent reporting environment, which, of course, is contrary to the founding principle of the STROBE statement [86].

Despite these expressed concerns of some epidemiologists, the STROBE statement can serve as useful guidance to toxicologists to remind them of what data should be reported in epidemiologic studies. The quality of the study, however, remains to be elucidated through the critical evaluation of the study's hypothesis, methods, results, and discussion.

APPENDIX B

EXAMPLE 11.B.1 SELECTION BIAS

The 1995 publication by Robinson and colleagues entitled *Analysis of explanted silicone implants: A report of 300 patients* illustrates a number of potential selection biases [110]. Among the 300 women who Dr. Robinson explanted

TABLE 11.A.1
STROBE Checklist

Item	Recommendation
Title and Abstract	
1. Information	(a) Indicate the study's design with a commonly used term in the title or abstract.
	(b) Provide in the abstract an informative and balanced summary of what was done and what was found.
Introduction	
2. Background	(a) Explain the scientific background and rationale for the investigation being reported.
3. Objectives	(b) State specific objectives, including any prespecified hypotheses.
Methods	
4. Study design	Present key elements of study design early in paper.
5. Setting	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection.
6. Participants	(a) *Cohort study*—Give the eligibility criteria and the sources and methods of selection of participants. Describe methods of follow-up.
	(b) *Case–control study*—Give the eligibility criteria and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls.
	(c) *Cross-sectional study*—Give the eligibility criteria and the sources and methods of selection of participants.
7. Variables	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable.
8. Data sources/ measurement	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group.
9. Bias	Describe any efforts to address potential sources of bias.
10. Study size	Explain how the study size was arrived at.
11. Quantitative variables	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why.
12. Statistical methods	(a) Describe all statistical methods, including those used to control for confounding.
	(b) Describe any methods used to examine subgroups and interactions.
	(c) Explain how missing data were addressed.
	(d) *Cohort study*—If applicable, explain how loss to follow-up was addressed.
	(e) *Case–control study*—If applicable, explain how matching of cases and controls was addressed.
	(f) *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy.
Results	
13. Participants	(a) Report the numbers of individuals at each stage of the study—e.g., number potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analyzed.
	(b) Give reasons for nonparticipation at each stage.
	(c) Consider use of a flow diagram.
14. Descriptive data	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders.
	(b) Indicate the number of participants with missing data for each variable of interest.
	(c) *Cohort study*—Summarize follow-up time (e.g., average and total amount). Report numbers.
15. Outcome data	(a) *Cohort study*—Report numbers of outcome events or summary measures over time.
	(b) *Case–control study*—Report numbers in each exposure category or summary measures of exposure.
	(c) *Cross-sectional study*—Report numbers of outcome events or summary measures.
16. Main results	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (95% confidence intervals). Make clear which confounders were adjusted for and why they were included.
	(b) Report category boundaries when continuous variables were categorized.
	(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period.
17. Other analyses	Report other analyses done—e.g., analyses of subgroups and interactions and sensitivity analyses.
Discussion	
18. Key results	Summarize key results with reference to study objectives.
19. Limitations	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of potential bias.
20. Interpretation	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.
21. Generalizability	Discuss the generalizability (external validity) of the study results.
Other information	
22. Funding	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based.

over the course of 3 years, 214 (71.3%) reportedly had *disruption* (defined as frank rupture of an implant or severe silicone bleed). Interestingly, these authors noted that there was "virtually no difference in the disruption rates between those patients relating symptoms to their implants and those who did not (71.8% vs. 70.9%)," suggesting that health complaints were not a consequence of implant status. Nonetheless, they extrapolated from this sample to predict that most implants will lose their integrity somewhere between 8 and 14 years and recommended that all gel-filled implants be removed, *preferably before 8 years from implantation*.

Robinson et al. based their rates and their interpretations and formulated a policy of explantation on data from a denominator of 300, but that was not the group they actually studied (Figure 11.20). According to the paper, Dr. Robinson saw 495 women who would have been eligible for this investigation, 101 who had been implanted by other surgeons and 394 of his own patients. The 300 were drawn from the 495, but note that, even if he had studied all 495, he still would not have been able to develop rates that were free of potential selection bias. Even with 100% participation of his sample, he would not have been able to develop rates that meaningfully could be extrapolated back to a larger group. That's because the 495 were a convenience sample, an ill-defined and likely highly biased sample of the larger population from whence they came. The larger population included all of Dr. Robinson's implant patients and, by implication, all the breast implant patients of the other 15–20 plastic surgeons who practiced concurrently in the same community. Court records indicate that Dr. Robinson implanted approximately 4000 women and it is quite possible that at least some of the surgeons in his community implanted comparable numbers [109].

So what can we make of the Robinson information? The data collected for this report were prevalence data. Although gathered over a 3-year period, for the individual study subjects they were obtained at a single point in biological time—the time of surgical explantation. Because they had prevalence data, the researchers could not differentiate between events that occurred at the time of surgical implantation, during the period the implant was within the body, or at explantation. Their interpretation, therefore, that implant failure is a function of the aging of the device, presumptively

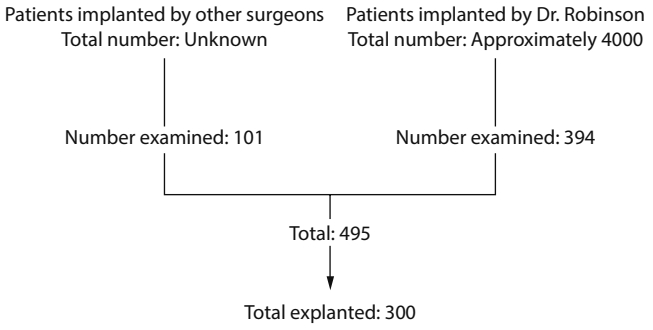


FIGURE 11.20 Convenience sample. (From Robinson, O.G. et al., *Ann. Plast. Surg.*, 34, 1, 1995. With permission.)

related to biological degradation of the silicone elastomer shell, required assumptions (e.g., the incidence time of rupture was just before explantation) that were not adequately addressed in this research.

Based on the work of others, at least some of those assumptions appear to be incorrect. Rapaport et al. [105] found that an appreciable number of implant ruptures occur secondary to micropunctures caused by needles or other medical devices used during the implant procedure. Others have done work that expands on this observation [21]. Brandon and colleagues [22], using lot-matched controls, reported that the material properties of the silicone shell are not affected by implantation for time periods up to 21 years and concluded "that the silicone elastomer undergoes little or no change during implantation." Robinson et al. [110] noted that approximately 25% of the implant ruptures they observed occurred during the explant procedure. At least two other mechanisms contribute to implant ruptures in vivo: closed capsulotomies (manual compression of the breast to rupture the tissue capsule surrounding the medical device) and the so-called fold flaws (disruption of the elastomer by excessive flexing at the site of folds in the shell). Both involve mechanical trauma. Obviously, different approaches might better be used to prevent, control, or otherwise address implant ruptures caused by different mechanisms.

Setting aside the questions of the validity of the data and the causes of implant rupture, if the 300 who were explanted are a representative sample of the ever implanted, then it is quite possible that a high proportion of implanted women have *disrupted* implants. Further, if *disruption* equates to implant rupture, either overt or occult, it suggests that there may be a high rupture rate for these medical devices, at least for those brands and models favored by Dr. Robinson and his colleagues [34]. On the other hand, if the 214 with disrupted implants are the majority of those in the numerator of a true rate, especially if disruption does not equate to rupture, then it is likely that the actual rupture rate is quite low, quite possibly a single-digit phenomenon. Of course, if neither scenario is correct, then the information is invalid and has no utility at all. Furthermore—and in spite of the question about rupture rates—if these authors are correct in their observation that there is a lack of association between implant integrity and health outcome, a conclusion reached independently by others, then is it good public health policy to expose all implanted women to the predictable risks of explant surgery [26,142]? Probably not.

A number of lessons can be learned from this report: One, not understanding the difference between prevalence and incidence data can lead to flawed interpretations [35–37,59–61]. Two, selection bias can occur even when 100% of those selected for the study participate because the selection process itself may be flawed. Three, anytime there is less than 100% participation among those originally selected, even in a descriptive study of just the exposed group, the results are susceptible to an additional selection bias. Particularly troublesome are those situations in which the participation rates differ between the groups (i.e., among

the exposed and the unexposed for a cohort study or the diseased and the healthy in a case–control study) because this suggests that the reasons for participation may not have been equivalent and therefore there may have been a spurious correlation between health outcome and exposure among one group or the other. The consequence of selection bias is an incorrect measure of association, possibly an underestimate of risk but often an overestimate. Complicating the situation still further, the dynamics of selection bias can change over time as a result of a well-publicized environmental controversy, a lawsuit, a provocative news program, or any number of other things; thus, different types of selection bias can wax and wane. Four, biased studies can lead to flawed policies, policies that ironically may put those whom they are designed to protect at greater risk.

EXAMPLE 11.B.2 INFORMATION BIAS

2,4-D is a commonly used herbicide applied in agricultural fields, on golf courses, right-of-ways, and residential lawns for the control of a variety of weeds. It has been extensively researched from a toxicological, epidemiological, exposure assessment, and environmental standpoint. Several scientific reviews have been written over the past 20 years of this literature including a panel hearing report issued by the U.S. Scientific Advisory Board (SAB). After a 17-year deliberative process, 2,4-D was reregistered for use by the U.S. Environmental Protection Agency in 2005 [29,30,47,56,94].

The purpose of this case example is to show data from three NCI case–control studies that suggested the epidemiologic associations reported between 2,4-D and NHL were likely the result of information bias, that is, recall bias by the type of respondent. That is, proxies of cases tended to report more exposure than the direct informants. Proxies were not used with controls. The NCI case–control studies were designed subsequent to Swedish epidemiologic studies that suggested associations between soft-tissue sarcoma, Hodgkin's disease, and NHL with exposure to phenoxyacetic acid herbicides. However, these Swedish studies had their own methodological limitations.

The first NCI case–control study was conducted in Kansas by Hoar et al. [73]. It was a population-based case–control study of male cases of soft-tissue sarcoma, Hodgkin's disease, and NHL. Controls were white men selected from the general population of Kansas. Exposure information, including use of herbicides and insecticides in farming practices, were obtained by a telephone interview. ORs were reported for NHL in relation to *duration, frequency, and latency of 2,4-D use*. Statistically significant trends were reported for all three analyses. In particular, the OR for NHL for frequency of use (days/year) ≥21 days was 7.6 (95% confidence interval 1.8–32.3) compared to having never farmed. Limitations of the study were discussed and the authors suggested the evidence introduced would "likely be similar for cases and controls. This random misclassification of exposure would tend to dilute risk estimates, rather than produce spurious association." However, the questionnaire was not specific for 2,4-D despite the inference otherwise and therefore a correction was issued to clarify this error in the tabular data presented in the original paper. The questionnaire inquired only about herbicide use in general. There appeared to be no difference in ORs based on the vital status of the cases. Information was obtained from next of kin for those cases who were deceased. The authors concluded that since phenoxyacetic acid herbicides were widely used on U.S. farmlands, it meant the carcinogenic effects suggested by this study may have important public health implications.

The second NCI investigation was a population-based case–control study that involved cases of Hodgkin's disease, multiple myeloma, chronic lymphocytic leukemia, and NHL among white men and women in 66 counties of eastern Nebraska [143]. Controls were selected from the same source population that gave rise to the cases. In this study, the questionnaire specifically asked questions about 2,4-D use (not just herbicides in general). Days of application and years of use were inquired. An OR of 3.3 (95% CI 0.5–22.1) was reported for mixing or applying 2,4-D 21 or more days per year. The investigators reported the risk of NHL associated with personal handling of 2,4-D was higher among proxy interviews (1–5 days per year: OR = 2.2; 6–20 days OR = 2.2; 21+ days OR = 2.4) than among self-respondents (1–5 days per year OR = 1.0; 6–20 days OR = 1.6; 21+ days OR = 1.4). Upon finishing their review of other epidemiologic and experimental evidence, the authors concluded, "we believe that the weight of evidence indicates that the user of 2,4-D in an agricultural setting increases the risk of NHL among persons handling the chemical frequently."

The third investigation was the Iowa–Minnesota case–control study [27]. Again, this was a population-based case–control study and was the largest of the three NCI case–control investigations. Questions pertained to *ever handled* various herbicides or insecticides but not about the frequency of handling 2,4-D or any other herbicide (or insecticide). Subsequent to the completion of the study, the investigators reinterviewed a subsample of cases and controls for this information. In a letter to the editor, Cantor et al. reported [28] ORs of 1.3, 1.0, and 0.9 for 1–4, 5–9, and 10+ days per use of 2,4-D (compared to no use) from a resurvey of Iowa farmers from this study. The authors concluded, "The time delay, different method of data collection, and participation of more proxies likely introduced substantial exposure misclassification that is likely to mask exposure response gradients. We therefore consider these findings to be very weak evidence either for or against the possibility of a causal association with any single pesticide exposure." However, the authors only adjusted for type of interview by using its main effect. An interaction term was not included in the model. This was subsequently done by Olsen and Bodner [97] who showed there were differences based on the type of respondent. Among direct informants, the ORs for the three frequency categories of 2,4-D use were 0.5 (95% CI 0.2–1.2), 0.2 (95% CI 0.1–0.8), and 0.7 (95% CI 0.3–1.9), respectively, suggesting recall bias.

For proxy respondents, the ORs were 0.8 (95% CI 0.2–2.7), 1.0 (95% CI 0.3–3.4), and 2.5 (95% CI 0.8–8.0), respectively. Similar differences in magnitudes of association, higher for proxy respondents, were also observed for other herbicides. Olsen and Bodner concluded "if direct informant data are considered more valid than proxy data, which we believe is a reasonable assumption, then the Nebraska study and the Iowa resurvey, using the comparable frequency of use categorizations for 2,4-D among direct informants do not suggest an association between frequency of 2,4-D use and NHL."

Because of the controversy surrounding the use of proxy interviews to obtain information about herbicide usage among cases, Johnson et al. [79] conducted a methodological study of obtaining information about pesticide exposures and the need to assess the reliability and validity of such data. Their study was particularly insightful as it was designed to demonstrate the effect on the OR if there was exposure misclassification. They compared data obtained from direct informants from the original Iowa–Minnesota study to information collected 8–10 years later from proxy respondents of direct informants who died or became incompetent since the initial interview. Their overall findings indicated that both nondifferential and differential misclassification can occur when proxy respondents provide pesticide exposure data and thus misclassification may not always be biased toward the null value. The differential misclassification occurred in approximately 1/3 of the estimates. Pesatori et al. [103] from NCI interpreted these results by saying "on balance, nondifferential misclassification appears to be a greater problem than differential misclassification." Olsen and Bodner, however, disagreed with this conclusion as it quickly dismissed the much greater threat to validity caused by differential misclassification despite it occurring *only* 1/3 of the time in the Johnson et al. methodological study. Olsen and Bodner agreed with the advice offered by Morrison et al. [93] who concluded, after their review of the herbicide and cancer literature, that agricultural pesticide data obtained from case–control studies should be reported stratified by the type of respondent.

The Kansas [73], Nebraska [143], and Iowa-Minnesota [27] NHL case-control studies findings provided some impetus for the NCI's decision to create the Agricultural Health Study (AHS) [135]. The AHS is sponsored by the NCI, U.S. Enviromnetal Proection Agency (EPA), and the National Institute of Environmental Health Sciences (NIEHS). It is a prospective cohort study of pesticide (farming and commercial) applicators in Iowa and North Carolina and began follow-up in 1996 [2]. AHS investigators have yet to publish a peer-reviewed paper on the cohort's experience between NHL and exposure to 2,4-D [3]. However, an abstract presented at the 2013 International Epidemiology Conference in Occupational Health (see Freeman et al. abstract #458) reported no association between NHL and 2,4-D in this prospective study. See Example 11.B.4 for further insights regarding the reporting of false positive, false negative, and publication bias in the epidemiology literature.

EXAMPLE 11.B.3 OCCUPATIONAL CANCER CLUSTER AND EXPOSURE ANALYSIS

More than two decades ago, the U.S. Centers for Disease Control and Prevention published *Guidelines for Investigating Clusters of Health Events* (113). These guidelines primarily focused on *cancer clusters*. A four-stage process was recommended when inquiries of excess cancers were reported to health departments: (1) an initial response to gather information, (2) an assessment of the occurrence of the health event, (3) a feasibility study, and (4) an epidemiologic investigation. Despite numerous efforts to investigate clusters of cancer occurrence in communities, few underlying causes have ever been elucidated. Reasons have included: (1) disease clusters are too small, (2) vague definition of the disease(s), (3) selection of the population studied is flawed by a posteriori reasoning, (4) exposures are poorly defined, (5) difficult data collection due to publicity generated about the cluster, and (6) perhaps the most likely explanation, the fact that identification of cancer clusters is *unexpectedly expected* statistical events. Unlike community clusters of cancer, occupational clusters are more well defined both in terms of location (a specific plant, production process, exposure(s)) and time, although the latter can still be affected from a post priori reasoning. The following case example demonstrates the quality of exposure reconstruction in a complicated environment—that of a research laboratory at a major chemical company. It further shows the difficulty of attributing a specific cause to a well-described excess of cancer (brain) in this research laboratory workplace—despite the rigorous effort put forth with historic exposure reconstruction. A brief review of this exposure reconstruction is illuminating unto itself.

Near the turn of this century several young chemists were diagnosed with brain cancer at the Rohm and Haas Company's research and developmental facility located in Spring House, Pennsylvania. An epidemiologic investigation ensued, which determined between 1963 and 2007 among 5284 workers that there were 14 males who died from brain cancer compared to 5.6 expected based on Pennsylvania mortality rates (SMR 2.39, 95% CI 1.30–4.01) [4]. There were 0 female deaths from brain cancer compared to 1.1 expected. Excluding the brain cancer, this population demonstrated the healthy worker effect. Altogether there were 486 observed deaths versus 952.9 expected (SMR 0.51, 95% CI 0.47–0.56) with statistically significant deficits for all cancer, cardiovascular disease, and diabetes. The histologic types determined for the brain cancers were four astrocytomas, five glioblastomas, one glioma not otherwise specified, one oligodendroglioma, two primary brain cancers histology not provided, and one case with no primary confirmed.

A nested case–control study was conducted with five controls selected for every case based on incidence density sampling. Controls were selected from risk sets of cohort members who were eligible for follow-up, alive at the time of death of the index case, and born within a 5-year birth interval.

Prior to the conduct of the epidemiologic component of the study, a tiered exposure assessment process was developed. Acknowledging the potential for *tens of thousands* of chemical exposures that could have occurred in this workplace, Chen et al. designed a strategy of reconstructing cumulative historical exposures for multiple chemicals and chemical groups [32]. Chemicals of interest were selected on the basis of the plausibility of penetrating the blood–brain barrier and the uniqueness of the chemical's biological activity. Briefly, the strategy was as follows. Employment records were obtained that identified job title, department, and dates of employment that were then used to classify workers into similar exposed groups (SEGs). To create SEGs, Chen et al. [32] categorized jobs to 10 functions: three primary functions involved either synthesis of new chemicals, formulating new chemicals, or handling chemicals and custom tailoring them for customers (tech services). Seven additional functions included administrative, analytical services, applications, synthesis and/or applications, synthesis, and/or formulation, maintenance, and toxicology services. Next, the numerous chemicals used in the research laboratory were narrowed by four decision criteria: (1) physical and chemical properties that focused on chemicals likely to cross the blood–brain barrier, (2) chemicals cited in the literature with central nervous system effects, (3) chemicals that had not been extensively studied in other settings, and (4) advice from technical staff and stakeholders. Using these criteria, five chemical groups were identified: acrylates, bis-chloromethyl ether (BCME), chloromethyl methyl ether (CMME), isothiazolones, and nitrosamines. Common solvents known to affect the CNS were excluded since their use in other industry sectors was more prevalent. Sources of exposure for these five major exposure groups included exposure monitoring data obtained by industrial hygienists, general ventilation data, and procurement data by department.

A Bayesian framework for quantitative exposure reconstruction was used for acrylates, BCME, and CMME. Historical monitoring, exposure modeling, and professional judgment data provided an industrial panel the information to reconstruct the likelihood exposures for acrylates, BCME, and CMME for each SEG over the time periods of interest. Where monitoring data were available, reconstructed exposures for a SEG were based on the terms of the probabilities of the arithmetic mean exposure lying within each of four categories of exposure relative to reference concentrations. For instances when monitoring data were unavailable and exposure modeling was performed, a simple general ventilation model was used. When information was unavailable, professional judgments by current and former industrial hygienists were used that provided a probability analysis of the category of exposures where the arithmetic mean of the SEG most likely belonged and the degree of confidence surrounding this decision. Relative exposures for each SEG were developed by considering information from an employee panel that considered six general determinants of exposure: general ventilation status, hood ventilation status, use of gloves, use of safety glasses, use of personal protective equipment (e.g., respirators, protective equipment), and general health, safety, and cleanliness. Only hood ventilation status and use of gloves had significant changes over time. In addition, relative exposures for all SEGs were estimated by using formaldehyde as an inhalation exposure surrogate. Formaldehyde was chosen due to its ability to cross the blood–brain barrier, CNS toxicity, extensive use at the research facilities, and large amount of monitoring data available. Information obtained allowed for the construct of exposure modifiers for the five chemical exposures. The final job exposure matrix was created by combining the SEGs with the corresponding quantitative or semiquantitative exposure estimates for the five chemical groups. For each employee, the person's cumulative exposure is weighted by the employment history and duration at the research facility with the exposure for each time period of the SEG.

Despite incorporation of this rigorous reconstruction of this exposure matrix, analyses from the nested case–control study were unable to explain the brain cancer cases [4]. Few brain cancer cases were associated with any specific SEG.

This example demonstrates that under the best of circumstances for reconstructing exposure histories for the purpose of disease cluster analysis, it is a difficult undertaking that may still yield minimum etiologic insights. Because of this inability to often provide answers, the source population for any cancer cluster should be provided a reasonable understanding of the likelihood of identifying etiological factors [11].

EXAMPLE 11.B.4 FALSE POSITIVES, FALSE NEGATIVES, AND PUBLICATION BIAS

In 1995, Taubes [130] wrote a highly controversial (within epidemiology) news article published in science that questioned whether epidemiology was facing its limits of being able to identify true positive findings at low effects. The article featured quotes from several prominent epidemiologists and journal editors. One was from Sander Greenland (UCLA) who said, "Remember, there is nothing sinful about going out and getting evidence, like asking people how much do you drink and checking breast cancer records. There's nothing sinful about seeing if that evidence correlates. There's nothing sinful about checking for confounding variables. The sin comes in believing a causal hypothesis is true because your study came up with a positive result, or believing the opposite because your study was negative."

The article by Taubes unleashed currents of support and criticism, internal and external to the discipline of epidemiology. In particular, this quote by Greenland, which was the last statement made in the Taubes article, highlighted the issue of false-positive and false-negative findings. During the past 15 years, this controversy has been a focus of reflection in the epidemiology literature. Some of this discussion is highlighted in the succeeding text.

In 2005, Ioannidis [78] provided a quantitative analysis why he thought most published research findings were

false positives. In other words, once a research result has been established as statistically significant, the poststudy probability that it is true is the positive predictive value (PPV) where PPV = $(1 - \beta)R/(R - \beta R + \alpha)$ where R = the ratio of the number of *true relationships* to *no relationships* among those tested in the field, α = type I error, and β = type II error. Because most investigators rely on $\alpha = 0.05$, this equation results in the inference that a research finding is more likely true than false if $(1 - \beta)R > 0.05$. This relationship, of course, holds assuming no study bias. In the increasing presence of bias (μ), however, Ioannidis shows that PPV = $([1 - \beta]R + \mu\beta R)/(R + \alpha - \beta R + \mu - \mu\alpha + \mu\beta R)$ such that PPV decreases with increasing μ unless $1 - C \leq 0.05$. Thus, with increasing bias, the chances that a research finding is a true positive diminish considerably. Ioannidis offered six corollaries about the probability that a research finding is true:

Corollary 1. The smaller the studies conducted in a scientific field, the less likely the research findings are to be true. Explanation: Small sample size means smaller power. PPV for a true research finding decreases as power decreases toward $1 - \beta = 0.05$.

Corollary 2. The smaller the effect sizes in a scientific field, the less likely the research findings are to be true. Explanation: Modern epidemiology is increasingly obliged to target smaller effect sizes. Consequently, the percentage of true research results is expected to decrease.

Corollary 3. The greater the number and the lesser the selection of tested relationships in a scientific field, the less likely the research findings are to be true. Explanation: Because PPV depends on R (prestudy odds), then research findings are more likely true in confirmatory designs than in hypothesis-generating analyses.

Corollary 4. The greater the flexibility in designs, definitions, outcomes, and analytical modes in a scientific field, the less likely the research findings are to be true. Explanation: Flexibility increases the potential for transforming what would be negative results into positive results as a consequence of increasing bias.

Corollary 5. The greater the financial and other interests and prejudices in a scientific field, the less likely the research findings are to be true. Explanation: Financial and nonfinancial conflicts of interest may lead to distorted reported results and interpretations. Epidemiologists may be prejudiced purely because of their belief in a scientific theory or commitment to their own findings.

Corollary 6. The hotter a scientific field (with more scientific teams involved), the less likely the research findings are to be true. Explanation: Extreme opposite findings are often reported early in any research area. The PPV of isolated findings decreases when many investigator teams are involved. "Positive" results are prioritized as most important to publish. "Negative" results become attractive for dissemination after a positive association has been report. This rapid change from 'positive' to 'negative' research findings has been termed the *Proteus phenomenon*.

In 2009, Boffetta et al. [17] issued a plea for *epistemological modesty* when it came to the false-positive reporting of results in cancer epidemiology studies. They cited two specific examples, one each from environmental and occupational epidemiology, and performed a cumulative meta-analysis of the initial study and subsequent published studies. The two initial papers were on 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) and breast cancer and acrylonitrile and lung cancer. In the DDE and breast cancer cumulative meta-analysis, the initial study by Wolf et al. [141] published in 1993 reported a relative risk of 3.7 (95% CI 1.0–13.5) for the highest to lowest 20% of the DDE distribution in serum. By 7 years later, with the addition of eight more research studies, the cumulative meta-analysis relative risk approximated 1.0. The cumulative meta-analysis for acrylonitrile and lung cancer began with the initial study published in 1978 that showed a relative risk of 4.0 (95% CI 1.7–7.9). Twenty-one years later with the addition of nine more published studies, the final pooled meta-analysis relative risk was 1.1 (95% CI 0.9–1.4). Boffetta concluded there are multiple reasons for positive results, particularly initial results, to be false. These included absence of a specific a priori hypothesis, small magnitude of associations, absence of dose effects, systematic biases including selection, information and confounding biases, and failure to consider multiple comparisons. They also discuss preferential publication bias of studies with positive findings—especially with initially new associations. They criticized the tendency by authors to hype new findings citing a few examples. They stressed the need for journals to require authors to discuss the limitations, as well as results, in the summary and abstract of published papers. They proposed that a *note box* of the study limitations become standard practice.

Blair et al. [15] provided their own commentary on the *rhetoric* of false positives published in a different journal. They were highly critical of the position taken by Boffetta et al. They argued that Boffetta et al. only provided a few examples of false positives in the literature, and thus if they (false positives) were "such a widespread and serious problem in epidemiologic research, one would expect that it would be relatively easy to provide many more examples." Blair et al. didn't acknowledge the potential limitations of word length in journal articles as a likely reason why Boffetta et al. limited their paper to a few examples. Blair et al. argued that multiple comparisons are more than just the number of comparisons but on how hypotheses and priori probability of associations are developed and that the lack of subsequent support after initial leads demonstrates how the early phase of this process may be *set aside after further investigation*. Blair et al. felt a rigid a priori listing of hypotheses would be particularly restrictive

and counter the use of long-term prospective studies to evaluate risk factors or outcomes that were not initially specified in the original protocols.

Blair et al. argued that biases from selection and information are more likely to result in false-negative than false-positive associations and thus tend to diminish the association due to nondifferential misclassification. Exposure categorizations that are binary would only result in nondifferential misclassification and diminish the relative risk. When more than two levels of exposure are evaluated, nondifferential misclassification could result in an increase in relative risks for exposure categories other than the highest. Blair et al. suggested that although the potential of uncontrolled confoundings exist, among occupation and lung cancer studies, smoking has been found to be a relatively infrequent confounder despite the well-known association between smoking and lung cancer. Blair et al. agreed that the media may *hype* study findings but criticized Boffetta et al. as they did not provide indication of when scientists' interpretations of their results are *hyped* or simply legitimate disagreements. They felt Boffetta et al. provided only anecdotal evidence of *hyping* and countered it by stating that the cited examples by Boffetta et al. did not provide *obvious evidence of hype*. Using the example regarding DDT and breast cancer [141], Blair et al. quoted an accompanying editorial on the DDT breast cancer paper by Hunter and Kelsey [76]. Hunter and Kelsey wrote, "These data [141] although limited, do suggest the plausibility of an association between organochlorines and increased risk of breast cancer. However, at this stage these mechanisms are incompletely understood, and they will require considerable additional refinement before becoming truly compelling." Blair et al. surmised that this quote and editorial placed the Wolff et al. results in the appropriate context. Blair et al., however, failed to mention the title of the accompanying editorial by Hunter and Kelsey [76] of the Wolff et al. [141] paper. The editorial was entitled, "Pesticide residues and breast cancer: The harvest of a silent spring?" Is this title hyping the findings by Wolf et al. [141]? A counterpoint offered by Blair et al. of the Boffetta paper was their suggestion that Boffetta and colleagues failed to offer no more than few examples of publication bias. Although Blair et al. suggested publication bias may exist, its frequency was unknown. Blair et al. wrote, "We would note that today researchers are highly motivated to publish even negative results because of the expense of conducting studies and the intense public debate that surrounds health issues." This opinion, however, does not necessarily support the fact the NCI has offered only one abstract through 2013 (International Epidemiology in Occupational Health Conference 2013 meeting; see Freeman et al. abstract #458), that has provided results of the Agricultural Health Study (AHS) pertaining to NHL and 2,4-D use. The NCI's initial NHL case-control studies related to 2,4-D [72, 143, 27] were published a quarter of century earlier (see Example 11.B.2 regarding Information Bias). The AHS was a prospective study of a large cohort of pesticide applicators (farmers and commercial applicators) in Iowa and North Carolina. Results? Overall, 78% of the 52,324 applicators provided information on 2,4-D use. There was no association with NHL and 2,4-D use overall (p-trend = 0.84) or any sub-type of NHL with intensity-weighted lifetime days suggesting the earlier case-controls findings were likely false positive results. The lesson for the toxicologist is that consensus based on epidemiologic research is likely to take many years, if not decades.

Another example of potential publication bias was criticism [16,140] leveled at Danish researchers [122] who chose not to report 15 years of government-funded sperm count data that they had collected that appeared not to support their original hypotheses of declining sperm counts? Interestingly in their defense, Skaekaeaek et al. [15] wrote the following, "In 2008, we tried to publish an article containing a figure similar to the one Bonde et al. [16] have now included in their commentary, showing stable sperm counts in Denmark, contrasting with a downward trend in sperm counts in Finland. However, our paper was rejected by 2 leading journals in the field. One referee said frankly: "Most results reported in this new article are not very original and are confirming previous data." As part of the Danish material had been published before, we cut the Danish data out of the manuscript and the Finnish data were subsequently published separately. But now Bonde et al. and Wilcox are attacking our scientific integrity by raising suspicions that we have disingenuously published the decreasing Finnish trend, and have withheld the data showing the unchanged Danish sperm count." Have they? Is this, or is this not, publication bias? If so, by whom? By the investigators for not continuing to try to publish their (nonpositive) Danish results and only publish the positive Finnish findings? By the journal's reviewer by judging the need to publish a paper by the originality of the data? By the editor by not supporting the investigators decision to publish the data despite the reviewer's comment? By all parties?

In conclusion, hyped or not, false-positive and false-negative associations, along with publication bias, are important points of contention within the epidemiology community. The authors do not anticipate a consensus anytime soon, if ever.

EXAMPLE 11.B.5 APPLICATION OF A CAUSAL DIAGRAM

Fei et al. [50] reported maternal levels of perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA), measured at 4–14 weeks of pregnancy, were associated with subfecundity in an analysis of 1240 women from the Danish National Birth Cohort (DNBC). The DNBC was an open cohort selected between 1996 and 2002 and was established by recruitment through general practitioners inviting their pregnant patients to participate in the study. Approximately 50% of general practitioners participated and 60% of invitees participated. Two blood samples were provided during pregnancy with the first taken at weeks 4–14 of pregnancy. Any woman who identified her pregnancy as planned or partly planned was asked how long did she try to get pregnant before becoming pregnant.

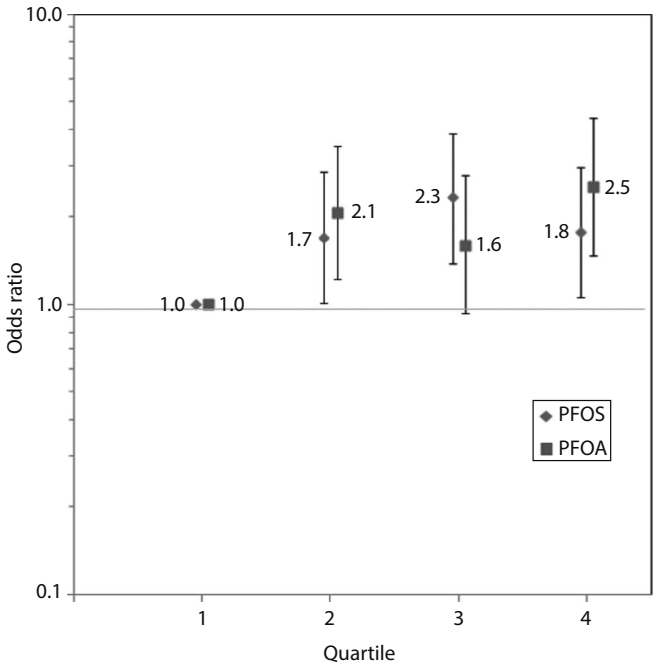


FIGURE 11.21 OR for subfecundity (TTP > 12 months) by PFOA or PFOS quartile. (From Fei, C. et al., *Human Reprod*., 24, 1200, 2009.)

Infertility was defined as having a TTP of >12 months or infertility to establish the current pregnancy. Plasma PFOA and PFOS were measured using high performance liquid chromatography mass spectrometry methods. Potential confounders included maternal age at delivery, parity, prepregnancy body mass index, maternal socio-occupational status, paternal education, paternal age, and alcohol consumption before pregnancy. Provided in Figure 11.21 are ORs for four quartiles of PFOS and PFOA concentrations for TTP (quartile 1 = reference). Trends were significant for PFOS ($p = 0.025$) and PFOA ($p = 0.006$). Fei et al. concluded that general population plasma levels of PFOS and PFOA may increase TTP and could explain some of the fertility differences observed among different populations in developed countries. This study received considerable news media attention at the time of its publication.

As part of their review of the epidemiology literature related to perfluoroalkyls, Olsen et al. [99] questioned the interpretation of the data analyses put forth by Fei et al. Using a causal diagram, Olsen et al. suggested there was no causal association between perfluoroalkyls (PFOA and PFOS) and TTP but rather a *backward* association generally referred to as reverse causation. As shown in Figure 11.22, indicated by the directional lines (follow the arrows), parity is both an outcome of fecundity and a cause of perfluoroalkyl concentrations. This induces a cyclic chain that violates the conditions of causal inference because under the reasonable assumption that perfluoroalkyl levels will be lower after a pregnancy (some placental transfer), a longer interval between births would result in more time for a woman to reaccumulate PFOA and PFOS that would replace the loss incurred from the birth. Assuming two women who begin with comparable perfluoroalkyl concentrations and equal parity, they will have different perfluoroalkyl concentrations at their next birth based on the interpregnancy interval of time. Women with longer time to pregnancies would likely have longer interpregnancy intervals and so may have higher PFOA and PFOS levels prior to the next pregnancy. This would result in longer TTP associated with higher perfluoroalkyl levels but the direction of the causality is backward. The longer time between births (including the TTP) results in the higher PFOA and PFOS concentrations. Unfortunately, Fei et al. did not stratify their analyses by parity to determine whether such an association exists.

Whitworth et al. [139] tested the hypothesis offered by Olsen et al. by analyzing TTP in parous and nulliparous

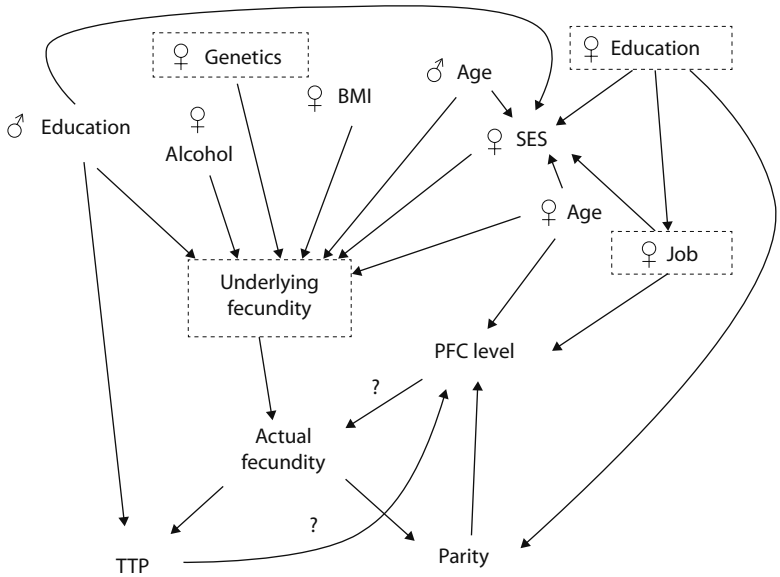


FIGURE 11.22 Causal diagram of maternal perfluorochemical concentrations and TTP. (From Olsen, G.W. et al., *Reprod. Toxicol.*, 27, 212, 2009.)

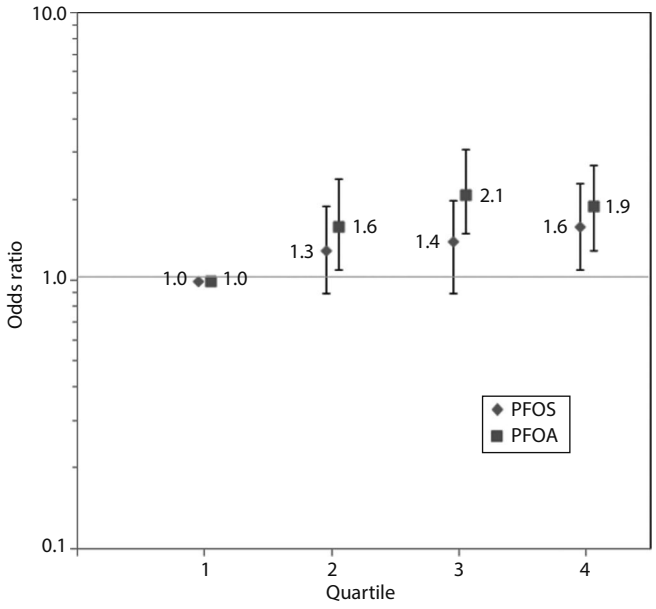


FIGURE 11.23 OR for subfecundity (TTP > 12 months) by PFOA or PFOS quartile. (From Whitworth, K.W. et al., *Epidemiology*, 23, 257, 2012.)

women. If the causal model was likely, then the association between longer TTP and perfluoroalkyl levels would be seen among parous but not nulliparous women since the latter did not have an interpregnancy interval of time. An association observed among nulliparous women would support a subfecundity inference. Whitworth et al. conducted a case–control analysis of 910 women enrolled in the Norwegian Mother and Child Cohort Study. A total of 416 women were cases who self-reported a TTP >12 months. Blood collection for the cohort occurred at approximately 17 weeks gestation. Plasma PFOA and PFOS concentrations were measured by HPLC-MS for cases and controls. Not stratifying for parity resulted in analyses (Figure 11.23) similar to those reported by Fei et al. (Figure 11.21) with perfluoroalkyl levels associated with subfecundity. Stratifying for parity, however, resulted in this association being observed only among the parous women (Figure 11.24), indicating their increased perfluoroalkyl levels may be due to a long interpregnancy interval rather than the cause of long TTP. The results among nulliparous women did not support an association with subfecundity with either PFOA or PFOS.

Upon publication of the Whitworth et al. findings, Fei et al. [51] acknowledged that perfluoroalkyl concentrations can be potentially influenced by the interpregnancy interval for parous women through accumulation of PFOA and PFOS. Adjusting for parity in their original model [50] did not adequately control for it. Upon stratifying their analyses for parity as done by Whitworth et al. [139], Fei et al. [51] reported among nulliparous women a diminished TTP association with PFOA, but not PFOS (Figure 11.25). Fei et al. concluded that there was *limited* evidence for reverse causation based on their reanalysis and welcomed additional studies in populations with elevated and variable levels of PFOA and PFOS exposure.

In summary, the cited example illustrates how the construction of a causal diagram (referred to as directed acyclic graphs) can be very insightful for the epidemiologist, toxicologist, and clinician to more fully understand the role of confounding and bias as they relate to the calculation of an estimate of effect for an exposure on an outcome of interest.

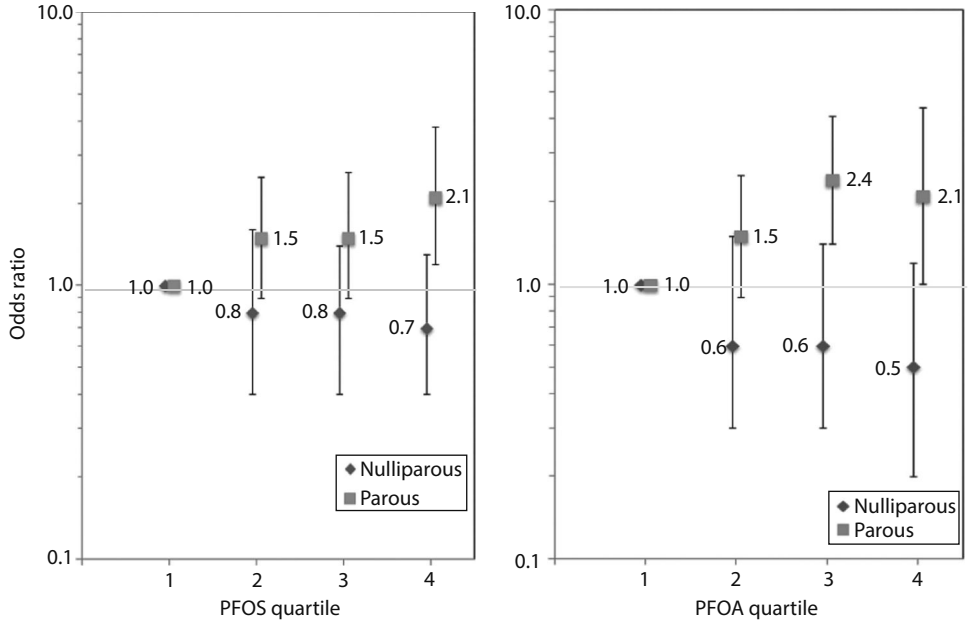


FIGURE 11.24 OR subfecundity (TTP > 12 months) stratified by parity by PFOA or PFOS quartile (95% CI). (From Whitworth, K.W. et al., *Epidemiology*, 23, 257, 2012.)

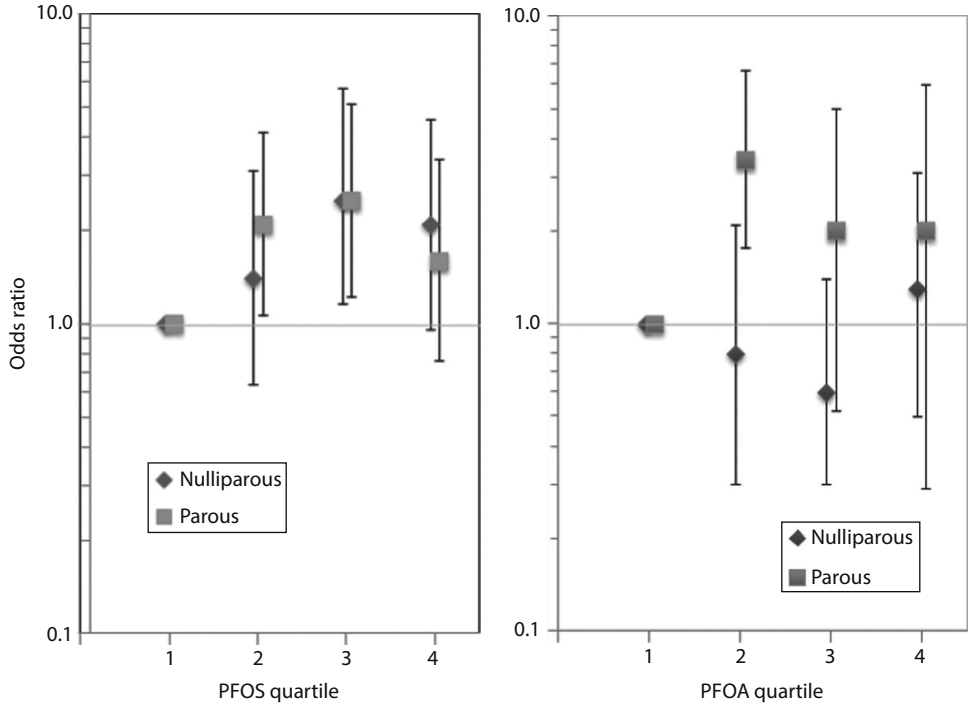


FIGURE 11.25 OR for subfecundity (TTP > 12 months) stratified by parity by PFOA or PFOS quartile (95% CI). (From Fei, C. et al., *Epidemiology*, 23, 264, 2012.)

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12 Principles of Pathology for Toxicology Studies

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CONTENTS

INTRODUCTION

Anatomical pathology findings often define the critical outcomes of the hazard identification process, including primary target organ effects, no-observed-effect levels (NOELs), adversity of exposure, and interspecies relevance of exposure. As such, the anatomical pathology evaluation is essential to the identification and characterization of target organ toxicity.

Pathology is defined as "the medical science, and specialty practice, concerned with all aspects of disease, but with special reference to the essential nature, causes, and development of abnormal conditions, as well as the structural and functional changes that result from the disease processes" [1]. This definition encompasses the traditional role of the pathologist in identifying morphological changes in tissues at the gross and microscopic levels. It also describes the more comprehensive and often more complex aspects of pathology. These include identifying and characterizing the nature (e.g., inflammatory, degenerative, or disturbances of growth) of abnormal findings, as well as their cause and development (pathogenesis).

Toxicologic pathology is the science that integrates the disciplines of pathology and toxicology and is concerned with the effects of potentially noxious substances [2,3]. The Society of Toxicologic Pathology (STP) defines a toxicologic pathologist as follows: "Any person who is a toxicologic pathologist by virtue of training, experience, and/or scientific contributions to the field, and is actively involved in safety assessment, teaching, or research in toxicologic pathology or the administration of these activities" [4]. The role of toxicologic pathologists is to identify pathological changes, to determine the etiology and significance of those changes, and to clearly and accurately report their conclusions to other scientists. This role requires not only an understanding of normal and abnormal tissue morphology but also a sound understanding of general (*whole animal*) physiology, clinical medicine, and the cellular and molecular processes underlying normal and disease states. This understanding necessarily contains a comparative component, as the pathology evaluation usually encompasses multiple laboratory animal species and is typically used as one component of human risk or safety assessment. Accurate interpretation of the significance of the multiple components of a toxicological study demands that the pathologist draw from a broad spectrum of disciplines in the biological and medical sciences.

The gold standard of the pathology evaluation in toxicity studies has been the examination of paraffin-embedded, hematoxylin- and eosin-stained tissue sections. These standardized methods are time tested, and accurate diagnosis and reporting of findings derived from such routinely prepared specimens will continue to play a critical role in the hazard identification process. In addition, the pathology assessment must integrate other relevant data, such as in-life study parameters (e.g., clinical signs, body weight changes), clinical pathology findings, and metabolism and pharmacokinetic data. Added to these more traditional parameters are a wide array of new technologies that are continually arising from advances in computer technology and molecular biology. Examples include the various *-omics* (genomics, proteinomics, metabolomics), genetically modified animal models, molecular assays, and special microscopy (e.g., digital, laser capture, confocal). Many of these technologies are, or will become, critical tools in drug discovery and development, as well as in the hazard identification and risk assessment of xenobiotics; however, accurate morphological diagnoses using standard pathology methods will likely remain important guideposts directing the practical application of these newer technologies.

This chapter provides (1) an overview of standard pathology procedures commonly used in toxicity studies; (2) a discussion of important considerations in the evaluation, interpretation, and reporting of pathology findings; and (3) a discussion of quality assurance practices in pathology, including pathology peer review and pathology working groups (PWGs). Comprehensive discussions of general pathology and organ-specific toxicologic pathology are beyond the scope of this chapter and are provided in a number of standard texts devoted to these subjects [5–9].

PATHOLOGY PROCEDURES

The primary goals of the pathology examination are to identify and collect all gross lesions, to collect all tissues listed in the study protocol, to trim and process all required tissues for microscopic evaluation, and to diagnose and report all lesions accurately. All of these procedures must be done in a consistent manner and in accordance with standardized procedures.

Necropsy

Necropsy refers to the examination of a body after death [10]. Although the necropsy is often one of the shortest phases in a toxicology study, it is one of the most critical. Procedures typically performed during the necropsy for toxicology bioassays include the gross examination, determination of organ weights, and collection of tissues for microscopic examination. Other procedures that commonly occur at necropsy include terminal blood and urine collection, preparation of bone marrow smears, and collection of samples for biochemical or molecular biology procedures.

The necropsy represents the beginning of data generation in the postmortem phase of the study and is the link between in-life findings and histopathological findings [11]. Necropsy observations often provide the first evidence of target organ effects, including carcinogenicity and cause of death. In addition, intercurrent disease or procedural factors that could complicate study interpretation may also be discovered at necropsy. Improper or incomplete necropsy examination can negatively impact an entire study, and because most aspects of the necropsy are not reproducible events within a study, errors occurring during this phase of the study generally cannot be corrected retrospectively. It is therefore essential that the necropsy be performed by highly trained technicians operating under the supervision of a qualified pathologist [12]. Also, for careful planning and conduct of the necropsy,

the necropsy team must have the following documents available for review prior to and during the necropsy [11,12]:

- *Study protocol*, including amendments
- Standard operating procedures (SOPs)
- Clinical records

The study protocol sets forth the study objectives and study-specific procedures and takes precedence over other documents. SOPs provide study personnel with information on the conduct of specific procedures described in the study protocol that may be performed during the necropsy. The clinical records for each animal must be available to the necropsy technician to ensure that any unusual findings observed in life are identified and collected for microscopic evaluation. In addition, every animal presented for necropsy should have an individual necropsy record for recording body and organ weights, gross findings, and tissues collected. Euthanasia date, time, and method and signature lines for all personnel involved in the necropsy of the animal should also be included [13].

The procedures documented in SOPs are followed universally within an institution for every toxicology study in order to standardize necropsy technique. These SOPs are required by regulatory agencies and contribute to an efficient and comprehensive necropsy [3]. Technicians should be knowledgeable about the procedures documented in the SOPs prior to beginning the necropsy and should be able to consult the SOPs during the necropsy if questions arise. Inconsistencies in the performance of procedures, including those for euthanasia; tissue dissection, retrieval, and weighing; use of descriptive terminology for gross findings; and tissue fixation at necropsy can introduce variables that may significantly complicate subsequent evaluation of organ weight data and may produce tissue artifacts that confound the subsequent histopathological evaluation. Sources of artifact are many, but some common ones include freezing rather than refrigerating animals that die on study, autolysis resulting from prolonged intervals between death and tissue fixation, and inadequate tissue fixation, such as occurs with immersion fixation of tissues that are too large or from the use of improper or compromised fixative.

Euthanasia Procedures

Methods of euthanasia should adhere to the recommendations of the American Veterinary Medical Association *Guidelines on Euthanasia* [14] and the *Guide for Care and Use of Laboratory Animals* [15]. The euthanasia procedure should seek to minimize pain and distress, should be easy to perform consistently, and should minimize tissue artifacts. The selection of specific agents and methods for euthanasia will depend on the species involved and the objectives of the study. Some common methods of euthanasia are given in Table 12.1. Generally, inhalant or non-inhalant chemical agents are preferable to physical methods such as decapitation [15]. Although the selection of specific agents and methods may be species and protocol dependent, all methods of euthanasia should be reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

TABLE 12.1
Methods of Euthanasia

Asphyxiation
Carbon dioxide
Anesthesia
Isoflurane
Sodium pentobarbital
Methoxyflurane
Halothane
Cervical dislocation
Decapitation (guillotine)

A number of pathology parameters may be influenced by the method of euthanasia and the choice of euthanasia agent. For example, barbiturate euthanasia agents are known to cause pooling of blood in the spleen, resulting in gross enlargement (splenomegaly) and congestion of the spleen in dogs. If the spleen were anticipated to be a primary or secondary target of a test compound, the use of an alternative method of euthanasia should be considered, or at least the known effects of the barbiturate on the spleen would have to be considered in interpreting the gross and organ weight data. The choice of euthanasia procedure may also produce specific histopathological changes that should not be confused with treatment-related changes. For example, carbon dioxide asphyxiation may produce focal, acute alveolar hemorrhage in the lung (Figure 12.1).

To limit postmortem autolysis and the potential introduction of confounding artifacts, the interval between death and necropsy should be minimized. Prolonged intervals between euthanasia and necropsy may produce significant alterations in organ weights and histology. For example, increased liver weights (both absolute and relative to body weight) and microscopic vacuolation of the liver may occur with a

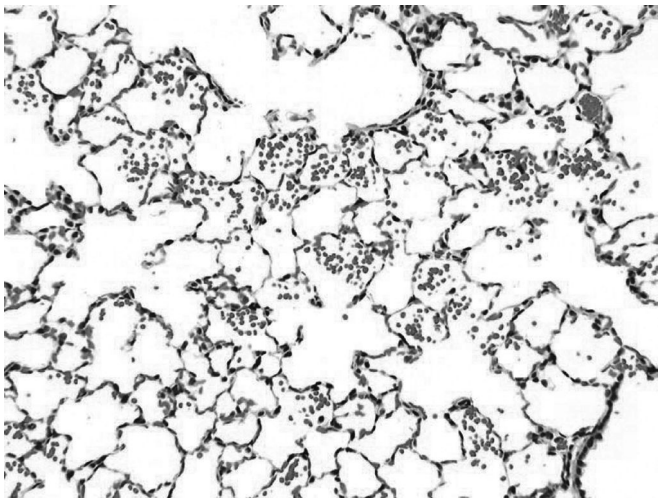


FIGURE 12.1 Focal acute alveolar hemorrhage in the lung of a rat euthanized by CO_2 anesthesia and exsanguination.

delay of only 25 min between euthanasia and necropsy [16]. Dissection should begin no longer than 5 min after euthanasia, and dissection time should not exceed 20 min.

Dissection and Gross Examination

Necropsy personnel performing the gross evaluation should be trained in the anatomy of the test species and in postmortem dissection procedures. Guidelines for the necropsy of laboratory animals have been published [17,18] and should be consulted if needed. During the postmortem examination, the prosector must handle unfixed tissues carefully. Excessive tissue manipulation, including excessive digital pressure or crushing or puncturing tissues with dissection instruments, may create artifacts that could complicate the microscopic examination. Normal saline should be used when rinsing tissue or to keep tissue moist; hypotonic tap water may produce tissue artifacts [12]. To avoid critical and irreversible errors in organ retrieval, the protocol should be strictly followed and consulted as necessary with regard to the tissues and organs that are to be collected. Table 12.2 provides a list of tissues recommended by the STP for subchronic and chronic toxicity studies [19]. This list is a minimum core list for all types of repeat-dose studies. Additional tissue may be added based on exposure route, species or strain of test animal, or known targets. To ensure tissue accountability, the individual necropsy record should include a list of all protocol (and nonprotocol) tissues to be collected and each tissue should be checked-off as it is placed in fixative.

The detection and an accurate description of gross lesions are essential aspects of the necropsy examination, as the gross examination guides subsequent tissue trimming and histopathology. Gross identification and retrieval of lesions is particularly important for nonprotocol tissue, which would otherwise not be examined microscopically. To ensure consistency between prosectors, both within and across studies, gross observations should be identified according to standard descriptive terms. Abnormal gross findings should generally be described using some or all of the following criteria: location, number, size, color, consistency, distribution, and any special features that characterize the lesion. A list of selected terms that can be used in the gross description is given in Table 12.3. The gross descriptions of tissue changes should be concise and descriptive (rather than diagnostic), with special attention given to consistency throughout the necropsy. An example of a gross description is given in Figure 12.2.

TABLE 12.2
STP-Recommended Core List of Tissues to Be Examined Histopathologically in Repeat-Dose Toxicity Studies (for All Species where Applicable)

Adrenal gland	Peripheral nerve
Aorta	Pituitary
Bone with bone marrow[a]	Prostate
Brain	Salivary gland
Cecum	Seminal vesicle
Colon	Skeletal muscle
Duodenum	Skin
Epididymis	Spinal cord
Esophagus	Spleen
Eye	Stomach
Gallbladder	Testis
Harderian gland	Thymus
Heart	Thyroid gland
Ileum	Trachea
Jejunum	Urinary bladder
Kidney	Uterus
Liver	Vagina
Lung	Gross lesions
Lymph node(s)	Tissue masses
Mammary gland[b]	Tissues relevant to route of exposure[c]
Ovary	Tissues unique to the species or strain
Pancreas	Known target tissues
Parathyroid gland	

Source: Adapted from Bregman, C.L. et al., *Toxicol. Pathol.*, 31, 252, 2003. With permission.

[a] For nonrodents, either rib or sternum; for rodents, femur including articular cartilage.

[b] Females only.

[c] Such as nose and larynx for inhalation studies.

Organ Weight Determinations

As with other end points collected during the necropsy, organ weights cannot be reproduced at a later date, so consistency and accuracy are critical to generating meaningful organ weight data [20]. Organ weight changes can be sensitive indicators of target organ toxicity, and significant changes in organ weights may occur in the absence of changes in other pathology parameters [21]. For example, increased liver weight associated with hepatic cytochrome P450 induction is a common finding in toxicology studies, and liver weight increases of up to 20% relative to controls may occur without microscopic evidence of hepatocellular hypertrophy or changes in serum chemistries [22]. Similarly, modest dose-related changes in kidney weight commonly occur in toxicology studies without histopathological evidence of cellular alteration and may indicate test substance effect [23]. However, while changes in organ weight parameters may be useful indicators of test substance-related effects, a change in organ weight as the sole indicator of target organ toxicity is uncommon, and organ weight changes must be interpreted in an integrated fashion with other study parameters including gross pathology, clinical pathology, and histopathology. Detectable organ weight changes occurring absent correlative changes in these other parameters may not necessarily be indicative of treatment-related or adverse findings and should be interpreted with caution [20].

Given the importance organ weight data may play in the overall interpretation of pathology findings, standardized methods for organ collection and weighing should be in place to ensure consistency and avoid artifactual weight changes. The animal necropsy order should be randomized or appropriately rotated to prevent bias, as organ weights may change

TABLE 12.3
Gross Lesion Description

General Location	Number	Consistency	Special Characteristics
Cutaneous	Single	Brittle	Area
Subcutaneous	Two	Caseous	Adhesion
Peritoneal	Four	Fibrinous	Circumscribed
Abdominal	Greater than *x*	Firm	Depressed
Thoracic	Multiple	Friable	Distended
Cranial	Size	Fluctuant	Flat
Sacral	Small	Gelatinous	Irregular
Lumbar	Enlarged	Granular	Layered
Cervical	Increased in size	Greasy	Linear
Axillary	Decreased in size	Gritty	Lobulated
Inguinal	Exact measurement	Hard	Macule
		Mucoid	Mass
Specific Location	**Color**	Oily	Nodule
Ventral	Black	Rough	Oval
Dorsal	Blue	Rubbery	Papillary
Lateral	Brown	Scaly	Papule
Medial	Clear	Soft	Pedunculated
Distal	Cloudy	Thin	Perforated
Proximal	Dark	Viscous	Pitted
Deep	Green	Watery	Plaque
Hilus	Gray		Polypoid
Wall	Mottled	**Distribution**	Prominent
Lumen	Opaque	Focal	Umbilicated
Mucosa	Pale	Multifocal	Raised
Superficial	Pink	Diffuse	Round
Serosa	Purple	Patchy	Spherical
Cortex	Red	Bilateral	
Medulla	Tan	Symmetrical	
Parenchyma	Transparent	Confluent	
Peripheral	Translucent	Unilateral	
Margin/Edge	White	All lobes	
Anterior	Yellow	Random	
Posterior			
Right			
Left			
Cranial			
Caudal			

throughout the day, particularly in fasted rodents. Similarly, prosector assignments should be rotated to ensure that one prosector does not disproportionately necropsy animals from one dose group, as differences in organ removal and trimming techniques might impact results [20]. More consistent results for organ weight determinations can be attained if the animal is bled out prior to weighing organs [12]. At weighing, care should be taken to remove extraneous tissue and blood clots and to prevent tissue dehydration. For some small tissues, such as rodent thyroid glands or pituitary glands, weighing tissue after fixation may help minimize artifacts associated with the handling of fresh tissue [3,20]. Interpretation of organ weight findings requires evaluation of

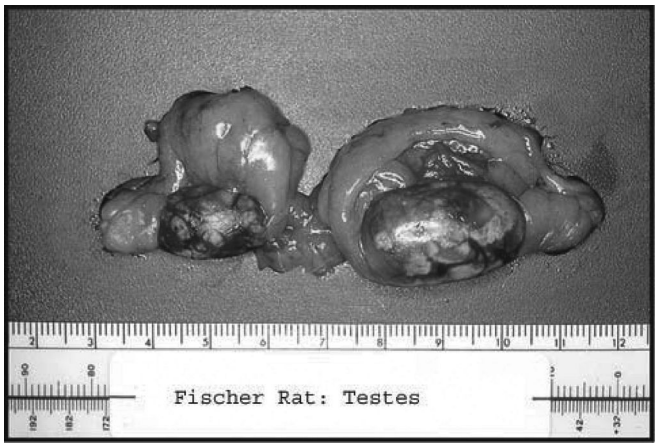


FIGURE 12.2 Gross description: testes. Masses, bilateral, multiple, firm, tan, 0.2–0.5 cm in diameter.

individual animal values, as well as group means for absolute organ weights and organ-to-body and/or organ-to-brain weight ratios. To minimize weighing errors during necropsy, a range of expected normal weights for each organ (matched to the species, strain, sex, and age of the test animal) should be available. Any weights that are outside this range should be verified before recording.

Terminal body weights collected at necropsy should be used to calculate organ-to-body ratios to control for potential variations that may be induced by stressful handling, diurnal fluctuations, or fasting. Organ-to-brain weight ratios may also be useful in some circumstances and can be calculated routinely or can be requested on a case-by-case basis. As noted below (see Primary versus Secondary Effects), understanding the effect of body weight changes on the organ weight for a given organ is important in the overall assessment of organ weight changes, as changes in body weight may lead to increases, decreases, or no change in organ-to-body weight ratios, depending on the organ affected.

The STP has provided recommendations for organs to weigh in repeated-dose toxicity studies of 1-week to 1-year duration (Table 12.4). In addition to those organs listed in Table 12.4, additional organs may be weighed on a case-by-case basis. These organs include uterus, ovary, lung, lymph nodes, gastrointestinal tract, pancreas (nonrodent species), seminal vesicles, salivary glands, and thymus (rodent species). Weighing lymph nodes is not recommended due to marked intra- and interanimal variability and because these tissues may be difficult to isolate from adjacent fat [20]. Organ weight determinations are not recommended for carcinogenicity studies, including those using alternative mouse models, as aging changes and intercurrent disease contribute to greater interanimal variability that can confound interpretation of organ weight changes. Similarly, due to confounding factors such as changes in nutritional status, lack of exsanguination, and absence of matched control, organs from animals that die or are euthanized prior to the scheduled termination should generally not be weighed. Organ weights are also considered to be of limited value in single-dose (acute) studies or in dose escalation studies [20].

TABLE 12.4
Recommended Organs for Weighing[a] in Multidose Toxicology Studies with Study Durations of 7 Days to 1 Year

Organ	Species	Comment
Adrenal glands	Rat, mouse, nonrodent	
Brain	Rat, mouse, nonrodent	
Epididymides		• Weighed in nonrodents and mice on a case-by-case basis. • Weight more valuable when assessed in mature animals.
Heart	Rat, mouse, nonrodent	
Kidneys	Rat, mouse, nonrodent	
Liver	Rat, mouse, nonrodent	
Lungs		
Pituitary gland	Rat, nonrodent	• Optional in mice, as the collection/weighing process may produce artifacts that can complicate microscopic assessment. • In rodents, fixation prior to weighing may provide more accurate weight measurements and improve morphology.
Prostate gland	Rat	• Weighed in nonrodents and mice on a case-by-case basis. • Weight more valuable when assessed in mature animals.
Spleen	Rat, mouse	• Weighed in nonrodents on a case-by-case basis. • Weight may be influenced by method of euthanasia and consistency of exsanguination.
Testes	Rat, mouse, nonrodent	• Weight more valuable when assessed in mature animals.
Thymus	Rat, mouse	• Weighed in nonrodents on a case-by-case basis.
Thyroid/parathyroid glands	Rat, nonrodent	• Optional in mice, as the collection/weighing process may produce artifacts that can complicate microscopic assessment. • In rodents, fixation prior to weighing may provide accurate weight measurements and improve morphology.

[a] Paired organs should be weighed together.

Tissue Fixation

A wide array of fixatives and fixation procedures is available and no universal fixative exists because no one fixative is perfect for all applications. The specific fixation procedure should be determined by the study objectives and study protocol. However, the most common fixation method is immersion fixation in neutral-buffered formalin. Prior to immersion fixation, tissues should be trimmed to approximately 0.5 cm thickness. They are then placed in formalin for at least 24–48 h at a 10:1 volume ratio of fixative to tissue; however, ratios as low as 3:1 are adequate if tissues are properly prepared for fixation. Formalin provides relatively rapid fixation, is easy to use, and is inexpensive, but formalin is potentially toxic and thus requires proper ventilation and disposal. Also, tissue artifacts, such as retinal detachment, may occur secondary to tissue shrinkage. Nevertheless, for most routine studies and for most tissues, 10% neutral-buffered formalin is typically the fixative of choice. Other fixatives and fixation methods may be used for specific tissues or procedures. A common example is the use of modified Davidson's fixative for fixation of the eye and testes [24]. Glutaraldehyde-based fixatives are often employed for ultrastructural studies. Inflation is the preferred method for fixation of the lung and may also be used for hollow organs, including the urinary bladder, stomach, and intestines [12], and involves gentle instillation of fixative into the organ prior to immersion into fixative. Perfusion may be used in special target organ toxicity studies. For example, whole body intravascular perfusion with Karnovsky fixative is commonly employed in neurotoxicity studies [25].

Histology

All changes to tissue that occur after fixation are, in effect, artifact. The goal in processing slides for microscopic (or ultrastructural) examination is to control the artifactual change so it is consistent across organs and across animals [13]. As noted for the necropsy, clearly written SOPs for histology procedures and strict adherence to these SOPs are essential for consistency in histological slide preparation. It is also important that each batch of tissues processed includes tissues from animals in all study groups to avoid apparent compound effects that are actually the result of variation in processing, embedding, or staining. Artifacts may be introduced at any stage in the preparation of tissue for microscopic evaluation including tissue dissection, weighing, and fixation; tissue processing for paraffin embedding; and microtomy, mounting of tissue sections onto glass slides, slide staining, and coverslipping [26,27].

Tissue Trimming

The first step in processing tissues for microscopic evaluation is tissue trimming. Trimming should be performed by

trained technicians with knowledge of gross anatomy and medical terminology and an understanding of the meaning of gross observations made during necropsy. The technician should have the study protocol, as well as the gross findings, available prior to trimming. Tissue trimming is yet another procedure where inconsistencies may result in biased sampling and poor comparability across different groups within a study or across many different studies. Thus, for each organ, SOPs should be available that outline standard trimming procedures to be followed, including the plane of trim (e.g., transverse or longitudinal), and, for larger organs such as the liver or lung, the number of sections and the specific areas to be trimmed. Guides for tissue trimming in rodents have been published in an attempt to standardize tissue trimming across laboratories [28–30]. Tissues should be trimmed to a maximum thickness of 0.3 cm for processing. Smaller tissues can be embedded intact. When trimming masses, adjacent normal tissue should be included where possible, and multiple sections should be taken from masses that are large or variable in appearance.

Tissue Processing, Embedding, and Staining

Following fixation and trimming, the tissue is most commonly processed and embedded in paraffin for microscopic evaluation. The steps involved in tissue processing and paraffin embedding are given in Figure 12.3. Although paraffin embedding is suitable for most forms of light microscopy, embedding media composed of acrylic or epoxy resins are commonly used for high-resolution light microscopy and electron microscopy. Further information on these advanced techniques can be found in other textbooks [31].

Paraffin-embedded tissue blocks are routinely cut with a microtome to produce sections with a thickness of about 4–6 μm (microns). Paraffin tissue sections are most commonly stained with the hematoxylin and eosin stain; however, other histochemical and immunohistochemical stains may also be used to identify specific properties of cells, intracellular structures, or microorganisms [31,32]. Examples of some histochemical special stains used to identify specific structures or materials are given in Table 12.5.

HISTOPATHOLOGY EXAMINATION

Overview

Histopathology is the study of morphological changes in tissues at the light microscopic level [33]. Histopathological findings frequently form the basis of the no-observed-adverse-effect level (NOAEL) in toxicology studies and are a critical part of hazard identification and risk assessment of pharmaceuticals, chemicals, biologics, and medical devices. The STP has published best practices guideline for toxicologic histopathology [33], which is aimed at identifying and defining the fundamental elements of the histopathological examination and appropriate techniques to minimize observer bias. Some of the recommendations given in this guideline are summarized in Table 12.6, and selected topics are discussed in more detail as follows.

Qualifications and Responsibilities of the Pathologist

The histopathological evaluation is the responsibility of the pathologist who must clearly communicate the results of that evaluation not only to other pathologists but also to

Dehydration

- Necessary because paraffin is not miscible with water
- Graded alcohols most commonly used

↓

Clearing

- Clearing reagents must be miscible with both the dehydrant (alcohol) and paraffin
- Commonly used reagents: xylene, toluene, D-limonene
- As the alcohol is removed, the tissue clears
- Must be carefully regulated to avoid excess hardening of the tissue

↓

Impregnation

- Complete removal of clearing reagents by substitution with paraffin
- Temperature of paraffin baths is critical; temperatures >5°C above the melting point of 56°C–58°C will cause excessive hardening of the tissue
- Vacuum removes air, gases, and remaining clearing reagent and draws paraffin into all areas of the tissue

↓

Embedding

- Orientation of tissue in melted paraffin
- Provides a firm medium for tissue sectioning
- Orientation and location of tissue in paraffin block must be consistent across animals

FIGURE 12.3 Procedures for tissue processing and embedding in paraffin.

TABLE 12.5
Selected Histochemical Stains Used in the Histopathological Evaluation

Stain	Uses
Hematoxylin and eosin (HE)	Most commonly used stain for routine histopathology
	Stains nucleus basophilic (blue) and cytoplasm eosinophilic (pink to red)
Periodic acid–Schiff (PAS)	Stains PAS positive structures (magenta); examples include
	Glycogen and mucin
	Basement membranes
	Microorganisms, including some fungi and protozoa
Masson's trichrome	Stains collagen (blue) and muscle (red)
Oil red O	Stains lipids (red); requires fresh smears or cryostat sections
Perl's iron stain	Stains iron (hemosiderin) (blue)
Luxol fast blue	Stains myelinated nerve fibers (blue)
Von Kossa's	Stains calcium (deep purple)
Toluidine blue	Stains mast cell granules (violet)

toxicologists and other scientists. Unlike the approach used in classic diagnostic pathology, which is characterized by lengthy detailed descriptions of each morphological abnormality, toxicologic pathologists must record their findings in a manner that allows for meaningful tabular summaries of the data. A comparison of the diagnostic and descriptive approaches used in classic and toxicologic pathology is given in Figure 12.4. The pathologist should then use the pathology narrative to place the tabulated microscopic findings in proper perspective relative to treatment-related effects and their biological significance; thus, histopathology is interpretive as well as descriptive [33,34].

A uniform accreditation standard for toxicologic pathologists does not exist, and the basic requirements to qualify as a toxicologic pathologist vary by country; however, a general consensus is that practicing toxicologic pathology requires formal training in a biomedical field and postgraduate training in toxicologic pathology. A review of the regional standards for the training and accreditation of toxicologic pathologists has been conducted by the International Federation of Societies of Toxicologic Pathologists [4].

One of the most challenging aspects of the histopathological evaluation in toxicologic pathology is achieving consistency among pathologists both within and across studies, as well as over time. Bucci [3] noted that, other than inaccuracy, inconsistency was the most undesirable characteristic of toxicology data. Histopathological diagnoses typically include some degree of subjectivity; therefore, although the diagnostic terms used by different pathologists for the same lesion should be comparable, they are not expected to always be identical [33,35]. Nevertheless, even though no two pathologists can be expected to produce identical findings (diagnoses, lesion grades) across all tissues examined in a study, qualified pathologists should be able to identify the same treatment-related lesions and the NOAEL for those lesions.

TABLE 12.6
Some Fundamental Elements of the Histopathological Evaluation

Information that should be available to the pathologist prior to the microscopic evaluation

The nature of the test substance and the class of compounds to which it belongs
Results of previous studies with the test compound in the same or different species, including target organ effects
All details of the experimental design (study protocol)
In-life data, including clinical signs, body weight, and nutritional data
Clinical pathology data (hematology, clinical chemistries, urinalysis), as well as hormone and enzyme induction data
Gross findings and organ weight data for individual animals

The process of histopathological evaluation

Assess specimen quality (reflects adequacy of tissue collection, fixation, trimming, processing, and staining)
Ensure that appropriate sections of tissues or organs are present on the slide and request recuts if necessary
Use concise, standardized diagnostic nomenclature and diagnostic criteria for tabular summaries
Use detailed free text as needed to better define complex lesions
Evaluate tissues either animal by animal (allows a more comprehensive assessment of animal's health) or organ by organ (provides a more focused examination of changes and greater consistency in severity grading)
Use a severity grading system that is definable, reproducible, and meaningful
In carcinogenicity studies,
- Distinguish hyperplasia, dysplasia, and neoplasia
- Classify neoplasms as benign or malignant and as primary or metastatic
- Provide evaluation of cause of death

Procedures that may be used to enhance accuracy and consistency of the histopathology evaluation

Informal reevaluation of specific changes in specific tissues; may be conducted using masking techniques
Peer review by a second pathologist of defined subsets of animals and tissues, as well as study conclusions
Review by a PWG consisting of experts for the target tissues of interest

Source: Adapted from Crissman, J.W. et al., *Toxicol. Pathol.*, 32, 126, 2004.

Procedures for the Histopathological Examination

In most circumstances, best results are achieved when all tissues from a study are evaluated by one pathologist [33]; however, in some circumstances, such as large studies or critical studies with short time lines, it may be necessary for two or more pathologists to read different subsets of tissues. Most commonly this involves different pathologists evaluating tissue from males and females. Peer review by a single pathologist is important in achieving consistency in studies where more than one pathologist has evaluated a study. The order of slide review among study groups may vary based

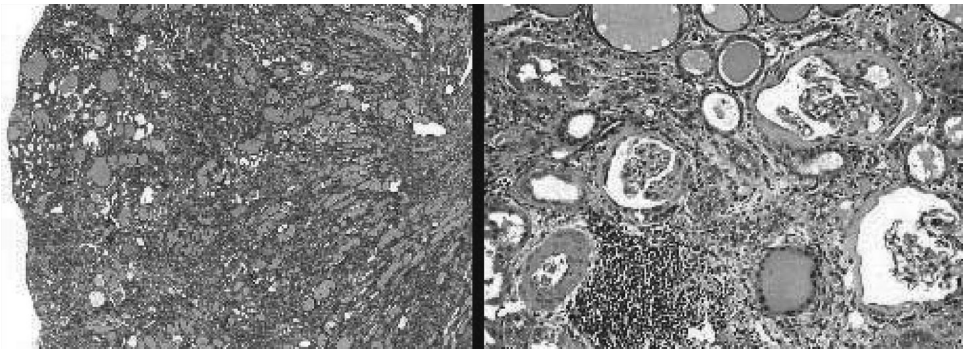


FIGURE 12.4 Morphological diagnoses in classic and toxicologic pathology. Morphological diagnoses based on classic descriptive pathology: Kidney, nephropathy, progressive, chronic, characterized by basement membrane thickening, tubular degeneration and regeneration, tubular dilatation, proteinaceous casts, mixed interstitial inflammatory infiltrate, interstitial fibrosis, mesangial proliferation, dilatation of Bowman's space, proliferation of parietal cells, glomerular adhesions, and sclerotic glomeruli. Morphological diagnosis for tabular summaries: Nephropathy, chronic, progressive, severe.

on study type and personal preference of the pathologist. For some studies, especially those with small group sizes, all controls may be evaluated first to establish the spectrum of *normal*. This is followed by the evaluation of high- and intermediate-dose groups as required by the protocol. Alternatively, the histopathological evaluation may proceed through alternating subsets of animals from each group.

Coded (Blinded, Masked) Histopathology Evaluations

The practice of conducting the initial histopathological evaluation with the pathologist having no knowledge of treatment status of individual animals is a controversial and much debated issue [36,37]. The controversy exists primarily between pathologists and nonpathologists, as most practicing toxicologic pathologists do not endorse coded evaluations for the *initial* slide review [36,38]. The position of the STP on coded evaluations is as follows [39]:

> The Society of Toxicologic Pathologists unequivocally supports open or non-blinded microscopic evaluation of tissues from experimental animals obtained from toxicology studies. The Society supports the long-standing diagnostic pathology practice that a pathologist, when making a microscopic evaluation of tissues, must have access to all available information about the animals from which the tissues were derived. Over the years, this method has been proven as an efficient and effective way of generating accurate data in a setting where time, costs, and productivity must all be considered.

Similarly, the American College of Veterinary Pathologists (ACVP) stated the following position [40]:

> In the opinion of the ACVP, such procedure [coded initial evaluation] is not appropriate for the routine evaluation of slides from toxicology studies.

The foremost objection to a coded slide evaluation concerns the initial histopathological examination of slides in a study. Coded examination will result in a loss of knowledge of the range of normal that exists in known controls. The pathologist uses the concurrent controls to establish a baseline for what is expected in a particular study for a given species, strain, sex, and age of animal. Without this baseline, subtle differences between treated and control groups may be difficult to detect. Another objection to coded examination is that knowledge of the treatment groups during the initial slide review allows the pathologist to assess the spectrum of related morphological changes and determine the most appropriate diagnostic terminology, including combining related diagnoses when indicated, to more accurately describe an observed treatment-related effect. The use of multiple diagnoses for a single disease process may obscure treatment-related effects by introducing unnecessary and confusing detail to the tabular summaries. In addition to these considerations, the additional procedures required to code and decode data and the additional effort necessary for the pathologist to record essentially all observations (including those well within *normal*) would increase study costs and timing; thus, the disadvantages of coded evaluations for the initial slide evaluation are both scientific and economic [38,40].

Although coded evaluations are not recommended for the initial slide review, a coded reexamination of selected target organs is commonly undertaken by toxicologic pathologists to confirm subtle changes or to clarify slight treatment-related effects on the incidence or severity of common background lesions. Thus, the histopathological evaluation has been described as a two-stage process, the components of which are the *identification stage* and the *confirmation stage*. In the identification stage, potential treatment-related findings are initially discovered, and in the confirmation stage, treatment-related associations are validated. The identification stage, as discussed earlier, is traditionally performed as an uncoded examination to minimize the chance that subtle, treatment-related findings will be missed. The confirmation stage is conducted in a coded or masked manner and is used as an informal *check* by the pathologist to guard against the possibility of reporting false-positive or

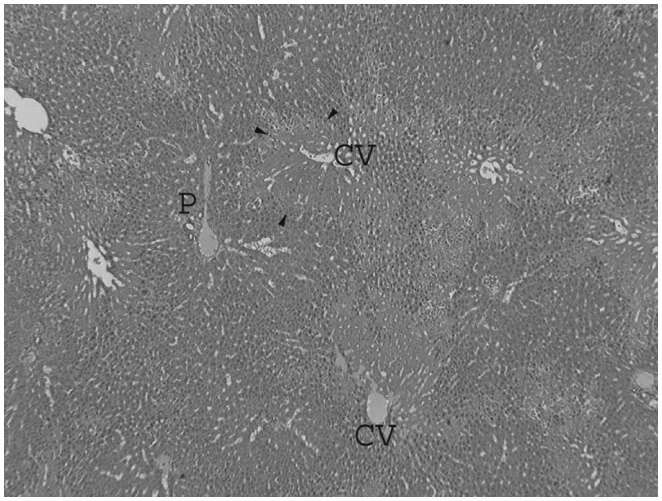
false-negative results. The confirmation stage may not be required in all cases, such as when there are no findings that are potentially treatment-related (negative studies), or for studies in which the evidence of treatment-relatedness is obvious. However, confirmation steps are necessary when, for example, causality for a finding is uncertain, when criteria used for severity grading are especially nuanced, or when determining a NOEL at the lower, less conclusive end of the dose–response for the histopathological finding [41].

Diagnostic Nomenclature

The histopathological diagnosis is the primary means of communicating the results of the microscopic evaluation. The goal of the microscopic examination is to identify test article effects through a comparison of treated animals with control animals. It is important that microscopic observations be recorded in a consistent, objective manner that readily allows tabulation and comparison of group effects. Different grading scales, whether a change should be graded or only noted as present, and the use of modifiers and thresholds all provide opportunities for variation between pathologists. Therefore, standardized terminology should be chosen that clearly communicates the important aspects of tissue changes. Internationally recognized standards for diagnostic nomenclature have been developed with the aim of harmonizing nomenclature and diagnostic criteria in toxicologic pathology [42]. These include th*e Standardized System of Nomenclature and Diagnostic Criteria (SSNDC): Guides for Toxicologic Pathology* [43] and, more recently, the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) [44]. A number of toxicologic pathology reference texts also provide guidance on diagnoses and diagnostic criteria [7–9,45].

The construction of a morphological diagnosis is typically hierarchical and includes the topography or site (organ or tissue), the major pathological process, and qualifiers. Qualifiers may specify subsites within the organ, distribution, duration, character, and severity [12,35,46]. An example is given in Figure 12.5. Qualifiers are used as needed to make distinctions that are toxicologically relevant. Except for the severity grade, different terminology for topography, process, or qualifiers defines a separate and distinct diagnosis; thus, the hierarchical approach is highly flexible and allows for an almost unlimited number of diagnoses. Overuse of this flexibility, however, by selecting different combinations of diagnostic terms to describe similar lesions can potentially obscure a treatment-related effect or create the appearances of an effect where none is present. In Figure 12.5, for example, some possible diagnoses might include the following:

- Liver. Necrosis, acute, centrilobular, moderate
- Liver. Necrosis, moderate (no duration or distribution qualifier)
- Liver. Necrosis, acute, coagulative, centrilobular, moderate (character qualifier added)



Organ/Tissue	Primary Pathological Process	Qualifiers		
		Duration	Distribution	Severity
Liver	Necrosis	Acute	Centrilobular	Moderate

FIGURE 12.5 Liver from a Sprague–Dawley rat. Acute coagulative necrosis of hepatocytes (arrowheads) is centered about central veins (CV); hepatocytes in portal areas (P) are unaffected.

Although describing the same primary process, each of these diagnoses as constructed would be summarized and tabulated separately when, instead, a single diagnosis would be more appropriate; however, liver necrosis with a distinctly different distribution may be indicative of a different pathogenesis for the necrosis and thus should be diagnosed separately from the centrilobular lesion presented in Figure 12.5. As an example, subcapsular, rather than centrilobular, necrosis of the liver has been reported to occur following drug-induced liver microsomal enzyme induction with associated hepatomegaly and compression of hepatocytes subjacent to the liver capsule [22]. It is the responsibility of the pathologist to appropriately group lesions of similar morphology, location, and pathogenesis under a single diagnosis that best allows for the detection of treatment-related changes.

Neoplasia has been defined as an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of normal tissue and persists in the same excessive manner after cessation of the stimulus that evoked the change [47]. For neoplastic lesions, the diagnosis should indicate whether the neoplasm is benign or malignant and whether it is primary to the tissue being examined or is metastatic. Tumor multiplicity and their bilateral or unilateral presence in paired organs may also be noted. Lesions occurring secondary to the neoplasm, such as inflammation and necrosis, are generally not recorded, as these secondary changes seldom provide useful information.

Although there are exceptions, most neoplasms are classified as to their predicted biological behavior and their cell

of origin. To designate biological behavior, the suffix *-oma* typically indicates that the lesion is benign, while *carcinoma* and *sarcoma* indicate malignant neoplasms of epithelial or mesenchymal origin, respectively. For histiogenesis, prefixes such as *adeno-* (glandular tissue) and *fibro-* (fibrous or connective tissue) are used to designate the cell or tissue of origin. Thus, mammary gland adenocarcinoma refers to a malignant neoplasm of glandular tissue, specifically of the mammary gland. Selected examples of taxonomy of neoplasms are given in Table 12.7 [48].

TABLE 12.7
Selected Taxonomy of Neoplasia

Tissue	Benign Neoplasia	Malignant Neoplasia
Epithelium		
Squamous	Squamous cell papilloma	Squamous cell carcinoma
Transitional	Transitional cell papilloma	Transitional cell carcinoma
Glandular	Adenoma	Adenocarcinoma
Liver cell	Hepatocellular adenoma	Hepatocellular carcinoma
Connective tissue		
Fibrous	Fibroma	Fibrosarcoma
Embryonic fibrous	Myxoma	Myxosarcoma
Cartilage	Chondroma	Chondrosarcoma
Bone	Osteoma	Osteosarcoma
Fat	Lipoma	Liposarcoma
Muscle		
Smooth muscle	Leiomyoma	Leiomyosarcoma
Skeletal muscle	Rhabdomyoma	Rhabdomyosarcoma
Endothelium		
Lymph vessels	Lymphangioma	Lymphangiosarcoma
Blood vessels	Hemangioma	Hemangiosarcoma
Hemolymphatic		
Lymphoid cells	Not recognized	Lymphoma (malignant)
Hematopoietic cells	Not recognized	Leukemia (granulocytic, erythroid)
Neural tissue		
Schwann cells	Schwannoma, benign	Schwannoma, malignant
Oligodendrocytes	Not recognized[a]	Oligodendroglioma, malignant (low or high grade)
Astrocytes	Not recognized[a]	Astrocytoma, malignant (low or high grade)
Embryonic cells	Not recognized	Neuroblastoma

Source: Modified from Haschek, W.M. and Rousseaux, C.G., Chemical carcinogenesis, in *Fundamentals of Toxicologic Pathology*, Haschek, W.M. and Rousseaux, C.G., eds., Elsevier, Amsterdam, the Netherlands, chapter 2, Section II, 2010.

[a] Well-differentiated lesions may be designated *malignant, low grade* rather than benign.

The classification of proliferative lesions as hyperplasia, benign neoplasia, or malignant neoplasia is based on *predictive* biological behavior. This prediction is based on historical correlation of certain microscopic features of neoplasms to clinical behavior. For example, neoplasms are diagnosed as malignant if there is evidence of invasion or metastasis or if histological features of the neoplasm have been shown historically to correlate with invasion or metastasis. It is important to remember, however, that these are operational terms based on evaluation at one point in time, and the future progression of the lesion cannot be definitively predicted unless metastasis has already occurred. Furthermore, the morphological distinction between hyperplasia and neoplasia or benign and malignant neoplasia is not always clear; thus, although the designations hyperplasia, benign neoplasia, or malignant neoplasia have practical utility, they do represent simplifications of a complex process [47].

In toxicologic pathology, diagnostic criteria for proliferative lesions that are relatively objective and reasonably predictive of biological behavior are ideal. At some points along the morphological continuum for some proliferative lesions, however, clear features that differentiate, for example, hyperplasia from adenoma are difficult to identify. In these cases, the size of the lesion may be the central (albeit not the only) criterion used to differentiate hyperplasia from adenoma. Although size should not be the only diagnostic criterion, the use of size as a central feature for some lesions adds consistency to the application of diagnostic terms and thus facilitates comparisons across studies and with historical incidence data. It is important to recognize that in these cases, size may not be a reliable predictor of biological behavior. For example, for proliferative lesions of pancreatic acinar cells, the 2D size of the lesion has been proposed as an important criterion, with lesions less than 5 mm in diameter considered hyperplasia and those greater than 5 mm considered benign neoplasms [44]. The use of size as one of the main criteria in this instance facilitates consistency in diagnosis across studies and laboratories, and some relationship between size and biological behavior for proliferative pancreatic acinar cell lesions has been suggested [49]. However, the differential diagnosis between hyperplastic acinar cell lesions approaching 5 mm in diameter and adenomas slightly greater than 5 mm in diameter is somewhat arbitrary and not necessarily reflective of meaningful differences between the respective lesions.

Severity Grading of Lesions

Severity grading is the semiquantitative application of a defined severity score to specific lesions [35,50]. Although a wide array of morphometric methods is available to quantitate changes in tissue, semiquantitative rating systems, if adequately defined and consistently applied, are fully capable of detecting treatment-related changes. Comparisons of results from quantitative and semiquantitative methods often show no relevant difference between the two methods in identifying a treatment-related effect or determining the NOEL for

that effect; thus, the routine histopathological evaluation is typically conducted using semiquantitative methods [12,50].

Severity grading is used as a diagnosis qualifier, primarily for nonneoplastic lesions, and is especially useful in identifying treatment-related effects that are not clearly incidence based; for example, a treatment-related effect may manifest only as increased severity of a common spontaneous lesion, such as chronic nephropathy. Severity grading is subjective, and systems for grading may vary among pathologists; therefore, reproducibility of results from severity grading requires that the grading scheme be clearly defined. Clear definitions not only provide the reviewer with an image of the spectrum of changes observed for a particular lesion but also aid in the peer review process, which should include an evaluation of the consistency of grading within the study by the peer review pathologist. As long as severity grades are consistently applied across a study, a one-point difference of opinion regarding severity is acceptable, given the semiquantitative nature of severity scoring [42]. For some lesions where severity grading adds no useful information, pathologists may not assign a severity grade. In such cases, changes are typically recorded as present (P), rather than having a severity grade assigned. Examples include neoplasms, cyst(s), autolysis, and congenital anomalies.

There are no standardized guidelines for lesion grading; however, grading schemes most commonly use four or five severity grades, designated by descriptive terms or numerical grade, to denote the extent of tissue involvement and/or the degree of tissue damage. Some commonly used grading schemes are given in Table 12.8.

TABLE 12.8
Some Commonly Used Severity Grading Schemes

Grading Scheme I

0 = Not present
1 = Minimal (<1%)
2 = Slight (1%–25%)
3 = Moderate (26%–50%)
4 = Moderately severe/high (51%–75%)
5 = Severe/high (76%–100%)

Grading Scheme II	**A**	**B**
Grade 1 = Minimal	(<10%)	(0%–25%)
Grade 2 = Mild	(10%–39%)	(26%–50%)
Grade 3 = Moderate	(40%–79%)	(51%–75%)
Grade 4 = Marked	(80%–100%)	(76%–100%)

Grading Scheme III

Grade 1 = Minimal
Grade 2 = Slight (same as mild)
Grade 3 = Moderate
Grade 4 = Marked (same as severe)
Grade 5 = Massive (same as very severe)

Source: Shackelford, C. et al., *Toxicol. Pathol.*, 30, 93, 2002. With permission.

Diagnostic Thresholds

In addition to recording treatment-related alterations and notable spontaneous lesions, the pathologist must determine whether or not to document minor variations in normal tissue morphology. Thresholding refers to the practice of determining which variations in normal morphology will be recorded and which variations are below a threshold and will not be recorded. These variations may result from minor age-related changes or they may represent normal anatomic variability within a population of animals. Some degree of thresholding of these common subtle variations in tissue morphology is needed to provide a meaningful compilation of microscopic pathology data. However, care must be taken in determining thresholds for common spontaneous changes, as the potential for a treatment-related effect on the incidence or severity of those changes may be more difficult to determine if they are not recorded. Nonetheless, setting an appropriate threshold can aid in streamlining the number of diagnoses produced in a study, so that treatment-related changes are clear [42].

Diagnostic Drift

Diagnostic drift refers to gradual changes in nomenclature or application of severity grading scales that may occur in a single study group or across several groups in a single study or when several studies are compared. The use of multiple terms or many different qualifiers to diagnose different morphological changes that are essentially the same is one source of diagnostic drift. Terminology and severity grading may also change over the course of evaluating the study as the pathologist becomes better aware of the full spectrum of treatment-related effects. Diagnostic drift cannot be appreciated by observing a single event but rather requires numerous data points separated by time. It is more commonly a problem in large studies containing large numbers of animals and tissues that must be evaluated over a relatively long period of time. Diagnostic drift is a source of variation that, if severe enough, may falsely create or mask treatment-related changes or may complicate determination of the NOEL. A slide evaluation method used to minimize diagnostic drift is to evaluate replicates of animals across all groups—for example, five controls, five high-dose, five low-dose, five mid-dose, and so on. If diagnostic drift is clearly identified in a study, the tissue affected should be reevaluated. In these circumstances, a coded evaluation of the specific tissue and lesion in question may be beneficial [12,33,35].

EVALUATION OF PATHOLOGY DATA

The interpretation of pathology findings requires a comprehensive assessment of gross, organ weight, and histopathological data, as well as in-life and clinical pathology findings. As noted previously, careful attention to the quality and consistency of the processes used to generate pathology data will minimize confounding factors, such as tissue sampling bias or tissue artifacts, that may mimic histopathological lesions.

The pathology findings must, in turn, be considered in the context of other study factors such as study design (dose, duration, route of exposure, postexposure recovery), test animal (species, strain, age, sex, mode of death), and animal husbandry (group vs. individual housing, ad libitum vs. diet optimization, caging, bedding) [51].

As with other data generated in toxicology studies, the evaluation of pathology data is primarily concerned with identifying changes that are due to treatment and determining if those changes are adverse. Although the focus of routine bioassays typically involves comparison of end points across distinct treatment groups, the evaluation of factors such as statistically significant differences between group means, or percent change of a treated group mean from control, should not occur at the expense of a careful examination of individual animal data. This is especially important in shorter-term studies, which typically have group sizes of 10 or fewer animals, and for end points whose measurements are inherently imprecise. Outlined in the following are some of the issues that frequently must be addressed in the evaluation of pathology findings in routine toxicology studies.

Assessing Cause–Effect and Adversity of Pathology Findings

A number of factors should be considered when assessing whether differences between treated and control groups are due to chance or represent an effect of the test article and if treatment-related effects are adverse. Lewis et al. [52] have proposed a list of factors that can be considered for such determinations. These are summarized in Table 12.9. However, none of the factors listed in Table 12.9 should be considered in isolation. Rather, in assessing cause–effect or adversity, these general factors should be considered in combination with specific information for a given study, such as study design and known effects of the test article from previous studies (weight-of-evidence approach). For example, as for other end points in a study, dose–response is an important factor in determining if differences observed in pathology end points between controls and treated groups are likely to represent true effects or are due to chance. An otherwise expected dose–response may not occur in some circumstances even when the response observed is treatment related. For example, test article effects at lower doses may be obscured or absent at higher doses due to overt toxicity, including lethality. Or, dose-dependent mechanisms of action, wherein different (even opposite) mechanisms of action may exist for a compound depending on dose, may also alter an expected dose–response [52]. And while consistency between sexes of potential test substance-related effects may be supportive of a causal association, gender differences in the pharmacokinetic or pharmacodynamic response to a test substance may result in gender-specific target organ effects or severity of effects.

Outliers are extreme deviations in an individual finding from the group norm, as well as from historical values [52]. In the context of assessing test article-related versus chance effects, the term *outlier* also assumes the deviation from norm is not due to the test article. Outliers that are determined to be due to technical errors or that occur secondary to disease states unrelated to test article administration do not reflect a group effect of the test article and should be removed from the analysis. Statistical outliers, however, may represent low-incidence occurrences of compound-related effects; therefore, as previously noted, a weight-of-evidence approach must be taken when determining whether to include or exclude putative outliers.

Although concurrent study controls are the first and best reference for comparison to treated groups, historical data can be a valuable tool in assessing causality (or adversity) of apparent treatment-related effects in a study. A robust historical control dataset for a given parameter may provide better insight into the true incidence and variability of a lesion within an untreated population. As noted by Lewis et al. [52], however, the use of historical data "should not be seen as a convenient device for discounting unwanted or difficult findings." The fact that an altered value for a given parameter falls within historical values would not, in isolation, indicate a chance finding but instead may be more indicative of nonadversity of the effect. For assessing causality, historical data may be helpful in identifying aberrant values within concurrent controls or in assessing potential compound-related

TABLE 12.9
Discriminating Factors for Assessing Cause–Effect Relationships and Adversity of Pathology Findings

Discriminating factors for assessing cause–effect relationship
There is no obvious dose–response.
The group change is due to an outlier in one or more animals.
The measurement of the end point is inherently imprecise.
The change is within normal biological variation (historical control or reference values).
There is a lack of biological plausibility (e.g., the difference is inconsistent with class effects, mode of action, or what is known or expected of the test material).
Discriminating factors for assessing adversity
The effect causes no alteration in the general function of the test organism or of the organs/tissues affected.
The effect is adaptive.
The effect is transient (i.e., resolves in the course of treatment vs. reversibility, which refers to resolution with cessation of treatment).
The severity of the effect is limited (below threshold of concern).
The effect is isolated and independent. Changes in other parameters usually associated with the effect of concern are not observed.
The effect is not a precursor (i.e., not part of a continuum of changes known to progress with time to an established adverse effect).
The effect is secondary to other adverse effects.
The effect is a consequence of the experimental model (e.g., stress associated with restraint or reactions to physical properties of the test substance, such as taste or odor).

Source: Adapted from Lewis, R.W. et al., *Toxicol. Pathol.*, 30, 66, 2002.

effects whose incidence within the control population is very low or very high and variable [52]. The use of historical control data (HCD) in carcinogenicity studies is discussed in more detail below (see Use of Historical Control Data in Carcinogenicity Studies).

Several definitions of adversity have been proposed [52–54]. Based on a review of the literature addressing the question of adverse versus adaptive responses, the following definitions for an adverse effect and for an adaptive response were proposed [55]:

Adverse Effect: A change in morphology, physiology, growth, development, reproduction, or life span of a cell or organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences.

Adaptive Response: In the context of toxicology, the process whereby a cell or organism responds to a xenobiotic so that the cell or organism will survive in the new environment that contains the xenobiotic without impairment of function.

Evaluating the adversity of an effect that has been determined to be due to exposure to a test article is complicated by a number of factors. First, the terminology associated with adverse effects is varied, with words and terms such as *adverse*, *toxicologically significant* (or *relevant*), and *biologically significant* (or *relevant*) used interchangeably by some or having distinct meanings by others. In addition, most definitions of adversity are not accompanied by criteria for determining adversity. Finally, determining the adversity of effects usually requires case-by-case expert judgment that often precludes rote approaches, but some of the factors given in Table 12.9 may be considered in a weight-of-evidence approach to determining adversity. The NOAEL for a study must be determined within the context of the particular study, and not be based on knowledge of possible compound effects or results of future studies.

Primary versus Secondary Effects

Some changes in organs or tissues that are observed following exposure to a test compound may be the result of primary target effects on some other organ or tissue or due to general systemic toxicity. These secondary effects are generally not considered adverse (Table 12.9); however, in some cases, such as massive iron accumulation in the spleen secondary to hemolysis, the secondary response may produce adverse effects in the affected organ.

As noted previously, changes in some organ weight parameters occurring in association with a decrease in body weight are commonly encountered as secondary effects in toxicology studies. Failure to consider the relationship between an organ weight and the associated decrease in body weight may lead to misinterpretation of organ weight findings. A common example is failure to consider the effects of body weight decrements on the organ weight/body weight ratio. The absolute organ weight of some organs, such as brain and testes, is relatively unaffected by modest decrements in body weight. In these cases, because the numerator (organ weight) is constant but the denominator (body weight) is decreased, the organ weight relative to body weight for these organs is increased in association with treatment-related decreases in body weight. Absent other evidence of primary effects, this increase in organ weight relative to body weight should not be interpreted as a primary pathological change in the affected organ. In contrast, many organs, most notably the liver, decrease in weight with decreased body weight, so the ratio of liver weight to body weight may remain normal relative to controls under conditions of decreased body weight. An increase in the liver weight relative to body weight, even in association with decrements in body weight, may indicate a primary weight increase in the liver [56,57].

Bailey et al. [21] investigated the effects of body weight changes on organ weights and ratios of organ weight to body or brain weight. Organ weight relative to body weight was the most appropriate parameter to evaluate organ weight effects in liver and thyroid gland, and the organ/brain weight ratio was most appropriate for adrenal gland and ovary. For other organs, alternative methods, such as analysis of covariance, were recommended. Whereas these results focused on the rat, the body and organ weight correlations observed were considered to be generally applicable to other species [21].

Many histopathological changes may also occur secondary to severe systemic toxicity, and these may complicate the interpretation of compound-related effects; for example, atrophy and weight decrements in lymphoid organs, especially the thymus, may occur in response to general stress, which may be produced at high doses in routine bioassays. Differentiating primary immunomodulating effects from a high-dose generalized response to stress may be problematic; however, careful examination of the dose–response for lymphoid organ changes may help in the evaluation. Immunosuppressive drugs often produce dose-related effects on lymphoid organs at doses not associated with other significant effects. In contrast, lymphoid changes occurring secondary to stress are expected to be limited to high doses and to occur in association with other signs of toxicity such as weight loss or general clinical suppression [58].

Another common secondary microscopic observation is atrophy of female reproductive organs due to nonspecific general toxicity associated with stress or reduced feed intake, which in turn result in reduced gonadotropin secretion [59]. This may make it difficult to distinguish some primary effects in female reproductive organs from nonspecific secondary effects at doses of a compound that produce severe stress or body weight effects. Nevertheless, making such distinction between primary and secondary effects on reproductive organs can have important implications for reproductive hazard classification. As discussed previously, the significance of

organ weight or histopathological changes must be considered in the context of other study findings, as well as any other information known about the test compound or its class.

Effects Associated with Extreme Doses or Severe Cytotoxicity

Some histopathological changes, including neoplasia, may occur only at high doses of a test compound that overwhelm normal physiological defense mechanisms or that produce severe cytotoxicity with resultant regenerative hyperplasia. Although these effects certainly represent adverse findings to the test species, their relevance to the hazard identification process is questionable, and it is important for the pathologist to fully characterize and contrast these findings with those observed at lower, more relevant doses.

One example of histopathological changes due to excessive doses is the constellation of lung lesions that have been reported to occur in rats following chronic exposure to very high concentrations of particulates that are poorly soluble and of low inherent toxicity (*lung overload*) [60,61]. Microscopically, lung changes are characterized by marked accumulation of particle-laden macrophages in alveolar spaces, interstitial inflammation, hyperplasia of type II pneumocytes, and bronchiolarization and squamous metaplasia of alveolar ducts. With increased duration of exposure, squamous metaplasia and the formation of large cystic, keratinizing squamous lesions or squamous neoplasms may occur in the lung (Figure 12.6). The pathogenesis of these lesions is thought to involve alteration in macrophage clearance and persistent inflammation due to the large surface dose of dust in the lungs. At lower dust concentrations that are not associated with marked inflammatory and adaptive responses, proliferative squamous lesions are not observed. This finding underscores the role of high dust concentrations or surface dose and the associated inflammatory and proliferative changes in the pathogenesis of the lesions.

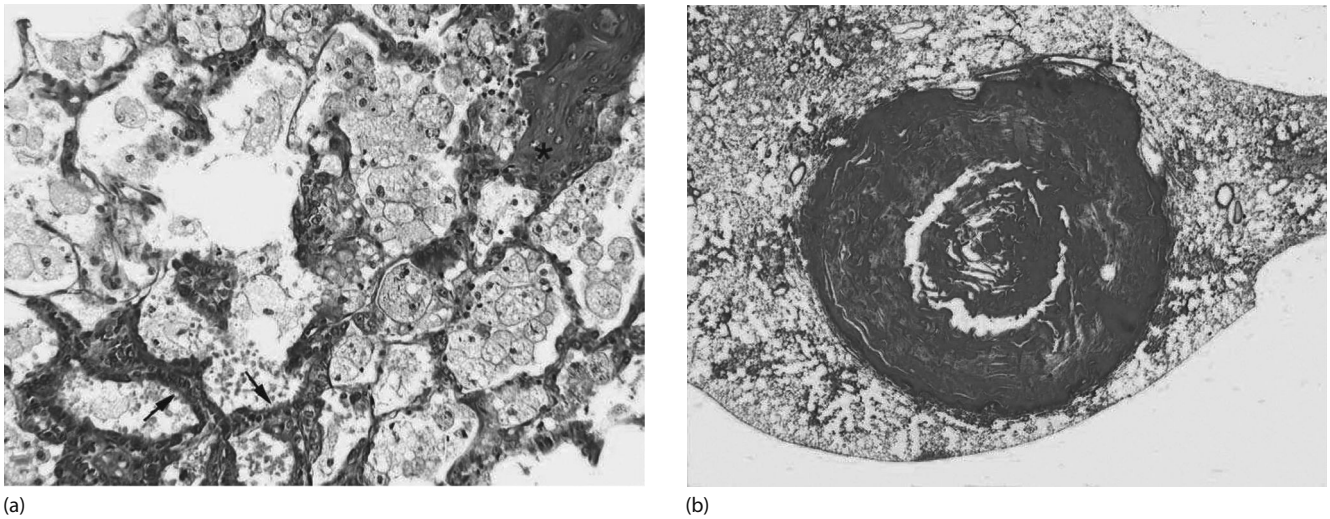
It is important to determine if proliferative lesions such as hyperplasia or neoplasia are due to a direct effect of the compound or are a response to a primary degenerative or necrotic event leading to regenerative hyperplasia. If hyperplasia can be clearly associated with tissue toxicity, then exposures that do not produce the primary toxic event are unlikely to produce cancer in the affected tissue [62].

Selected Data Evaluation Considerations in Rodent Carcinogenicity Studies

Some additional issues that are most commonly encountered in carcinogenicity studies are discussed in Chapters 24 and 25 and in several reviews on the subject. These include general texts and reviews on design and interpretation of long-term studies [63,64] and application of statistics [65–67]. An overview of other selected issues that may be encountered when assessing the result of rodent carcinogenicity studies are discussed in the following.

Combining Neoplasms in Rodent Carcinogenicity Studies

Many of the more common neoplastic processes observed in rodent carcinogenicity studies likely occur through progression from hyperplasia through benign lesions to malignant lesions. In evaluating potential treatment-induced proliferative responses occurring along a morphological spectrum, it may be appropriate in some cases to combine the incidence of neoplasms in the same organ or tissue or in different



(a) (b)

FIGURE 12.6 Lung from a Sprague–Dawley rat exposed by inhalation to high concentrations of a dust. (a) Lesions are characterized by marked accumulation of dust-laden macrophages in alveolar ducts and alveoli, with interstitial inflammation, hyperplasia of type II pneumocytes, and bronchiolarization (arrows) and focal squamous metaplasia (*) of alveolar ducts. (b) With chronic exposure, proliferative keratinizing squamous lesions, including proliferative keratin cysts, may develop.

organs or tissues in which the morphology of the tumors is comparable [68,69]. Brix et al. [69] proposed the following criteria for combining neoplasms:

1. Substantial evidence exists for progression of benign to malignant neoplasms of the same histomorphogenic type. Progression is considered more important if demonstrated within the study in question than if comparisons must be made with past experience (although this knowledge is valuable).
2. The occurrence of hyperplasia may be used as supporting evidence alone, but more so when the criteria for differentiating hyperplasia from benign neoplasia are not clear (i.e., borderline lesions) or when they are arbitrary and do not reflect the biological potential of a given lesion.
3. Most neoplasms of the same histomorphogenic type are combined even if they occur in different anatomic sites.
4. Neoplasms of different morphological classification may be combined when their histomorphogenesis is comparable.

Guidelines for combining neoplasms based on a specific organ or tissue are given in Table 12.10. In addition, neoplasms of the same histomorphogenic type from different but related anatomic sites are generally combined for evaluation, although there are exceptions. For example, neoplasms of larynx, trachea, and major bronchi may be combined while those in the peripheral lung (bronchiolar–alveolar neoplasms) would be analyzed separately. Similarly, neoplasms of the nasal mucosa would not normally be combined with those found in other regions of the respiratory tract. In the alimentary tract, squamous cell neoplasms of the tongue, esophagus, and forestomach (nonglandular) are often combined for evaluation, as are epithelial neoplasms from various regions of the small or large intestine. Morphologically similar neoplasms occurring in both the small and large intestines may also be combined to evaluate the intestinal tract as a whole. However, neoplasms of the glandular stomach are usually evaluated independently. Smooth muscle neoplasms are generally combined for all sites of the body except the gastrointestinal and reproductive tracts, where they are evaluated independently. Systemic neoplasms that have a common histogenesis but may arise in various tissues may also be combined for evaluation of carcinogenicity. Examples of systemic neoplasms include all blood cell neoplasms, including histiocytic sarcoma, as well as neoplasms in which the cell type is present in many different organs, such as hemangiosarcomas and malignant mesotheliomas [69].

Other combinations of neoplasms for comparison between groups may be considered on a case-by-case basis. However, one comparison that is not appropriate is the total number of tumor-bearing animals across groups—without regard to specific morphological tumor types or whether the tumor is benign or malignant. Since most groups of rats and mice (control as well as treated) in 2-year studies will have a very high incidence of neoplasms, the usefulness of such a nondiscriminate grouping would not be additive to the evaluation of carcinogenicity.

Assessment of Hyperplastic Lesions in Rodent Carcinogenicity Studies

Evaluation of a potential neoplastic response in a rodent carcinogenicity study should also include an assessment of hyperplastic changes as they relate to neoplasia since, as noted previously, exposure to chemicals may result in a spectrum of proliferative changes ranging from hyperplasia to neoplasia. Evaluation of the relationship between hyperplastic and neoplastic changes must be done with careful consideration of the multiple factors that impact such a correlation. Some questions to consider when assessing the relevance of hyperplastic lesions to a coexisting neoplastic process are given in Table 12.11. The foremost consideration is that hyperplasia must be viewed within the context of the specific study (and compound) under consideration. Many factors that contribute to an accurate weight-of-evidence assessment of the relevance of hyperplasia to a coexisting neoplastic process are often study-specific. For example, it is important to determine if hyperplastic lesions are a direct effect of compound action or occur secondary to a primary degenerative or necrotic change, with associated reparative (regenerative) hyperplasia. Focal hyperplasia that appears morphologically similar to neoplasia in a given tissue, but occurs without evidence of concurrent tissue injury, may be indicative of a potential direct treatment-related neoplastic response. In contrast, hyperplasia and cancer in a tissue with compound-induced inflammation or degeneration/regeneration suggest that the proliferative lesions are occurring secondary to chronic tissue injury. Even when evidence suggests that hyperplastic lesions are a primary effect of the test article, there are different implications depending on the type of change (e.g., diffuse or focal, with or without cellular atypia or dysplasia), the biochemical mechanism(s) underlying cellular proliferation, the nature of any associated concurrent lesions, the tissue(s) involved, and the type of neoplastic findings [62].

Use of Historical Control Data in Carcinogenicity Studies

HCD for proliferative lesions are useful in the interpretation of long-term rodent carcinogenicity bioassays, especially in the interpretation of rare tumors, tumors that occur spontaneously in high incidences or in highly variable incidences, and in studies where only a marginal increase in tumor incidence in a treated group relative to concurrent controls occurs. HCD are also useful in the interpretation of unexpected increases or decreases in tumor incidences in study control animals or when there is a need for quality control of intercurrent factors that may have compromised the survival of the control or treated animals. The major prerequisites to compare HCD with a specific study under evaluation are the validity and consistency of the respective databases to be compared [70–72].

TABLE 12.10
Guidelines for Combining Neoplasms in the Rat and the Mouse

Organ/Tissue	Combine	Do Not Combine
Adrenal gland	Cortical adenomas and cortical carcinomas	Cortical neoplasms and subscapular neoplasms (mouse)
	Pheochromocytomas and malignant pheochromocytomas	Cortical neoplasms and medullary neoplasms
		Subscapular neoplasms and medullary neoplasms (mouse)
Cardiovascular system	Hemangiomas and hemangiosarcomas	
Intestines, small and large	Adenomatous polyps, adenomas, and adenocarcinomas	Benign mesenchymal neoplasms—various types
	Benign and malignant mesenchymal neoplasms of the same type (if a continuum is observed in a given study)	Malignant mesenchymal neoplasms—various types
Hematopoietic system—mouse	Malignant lymphomas—all types	Malignant lymphomas—all types—and histiocytic sarcomas
	Malignant lymphomas—all types—and lymphocytic leukemia	Leukemias—all types
Hematopoietic system—rat	Large granular lymphoma (mononuclear cell leukemia) and leukemia—other types	Malignant lymphomas—all types—and histiocytic sarcomas
	Malignant lymphomas—all types	
	Large granular lymphoma (mononuclear cell leukemia) and malignant lymphomas—all types	
Kidneys	Tubular cell adenomas and tubular cell carcinomas	Tubular cell neoplasms and transitional cell neoplasms
	Transitional cell adenomas and transitional cell carcinomas	Mesenchymal neoplasms and epithelial neoplasms
Liver	Hepatocellular adenomas, hepatocellular carcinomas, and hepatoblastomas	Bile duct neoplasms and hepatocellular neoplasms
		Hepatocellular neoplasms and vascular endothelial neoplasms
Lung	Bronchioalveolar adenomas and bronchioalveolar carcinomas	Squamous cell neoplasms and bronchioalveolar neoplasms
Mammary gland	Carcinomas—various types	Adenomas and fibroadenomas (but an adenoma or carcinoma arising from a fibroadenoma should be combined with other mammary gland adenomas and carcinomas)
	Adenoma, adenocarcinoma, and carcinoma (if a continuum is observed in a given study)	
	Mammary gland fibroadenomas and fibromas/fibrosarcomas of the subcutis (when the fibroma/fibrosarcoma arises in the mammary gland region)	
Nervous system	Gliomas—various types	Gliomas and medulloblastomas
		Granular cell neoplasms and gliomas
		Nerve cell neoplasms and gliomas
		Meningiomas (all types) and other central nervous system neoplasms
Nose	Squamous cell papillomas and squamous cell carcinomas (if a continuum is observed in a given study)	Squamous cell neoplasms and glandular cell neoplasms
		Esthesioneural epithelial neoplasms and other neoplasms
Oral cavity (and esophagus)	Squamous cell papillomas and squamous cell carcinomas	
Ovary	Germ cell neoplasms—all types	Germ cell neoplasms and stromal neoplasms
	Stromal neoplasms—all types	
Pituitary gland	Adenomas and carcinomas	
Pancreas	Islet cell adenomas and carcinomas	
	Acinar cell adenomas and acinar cell carcinomas	
Prostate	Adenomas and carcinomas	
Skeletal system	Bone neoplasms and cartilage neoplasms (if a continuum is observed in a given study)	
Skin/subcutis	Basal cell neoplasms—all types	Benign mesenchymal neoplasms—various types
	Squamous cell papillomas and squamous cell carcinomas	Malignant mesenchymal neoplasms—various types
	Squamous cell neoplasms and keratoacanthomas	
	Squamous cell neoplasms and adnexal neoplasms (if a continuum is observed in a given study)	
	Benign and malignant mesenchymal neoplasms of the same type (if a continuum is observed in a given study)	

(*continued*)

TABLE 12.10 (continued)
Guidelines for Combining Neoplasms in the Rat and the Mouse

Organ/Tissue	Combine	Do Not Combine
Stomach	Glandular stomach adenomas and adenocarcinomas	
	Forestomach squamous cell papillomas and squamous cell carcinomas	
Testis	Germ cell neoplasms—all types	
	Stromal neoplasms—all types	
Thyroid gland	Follicular cell adenomas and follicular cell carcinomas	Follicular cell neoplasms and C-cell neoplasms
	C-cell adenomas and C-cell carcinomas	
Urinary bladder	Transitional cell papillomas and transitional cell carcinomas	
Uterus (and cervix)	Glandular adenomas and glandular carcinomas	Stromal neoplasms and glandular neoplasms
	Stromal polyps and stromal sarcomas	
Zymbal's gland (and clitoral and preputial glands)	Adenomas—various types	
	Carcinomas—various types	
	Adenomas and carcinomas	

Source: Modified from Brix, A.E. et al., Combining neoplasms for evaluation of rodent carcinogenesis studies, in *Cancer Risk Assessment*, Stedeford, T. and Hsu, C.H., eds., John Wiley & Sons, Inc., Hoboken, NJ, chapter 28, 2010.

TABLE 12.11
Questions to Consider When Assessing the Relevance of Hyperplasia to a Coexisting Neoplastic Process

- Is there a common cell of origin for hyperplastic and neoplastic processes?
- Is there a morphological continuum between hyperplasia and neoplasia within the study?
- Are the hyperplastic and neoplastic lesions histologically similar?
- What is the incidence and severity of spontaneous chronic diseases that may influence development of hyperplastic and neoplastic lesions?
- What are the incidences of hyperplasia and neoplasia?
- Is there other evidence for treatment-related toxicity?

HCD may be limited by variability and drift over time in animal- and study-related factors. These include such factors as animal genetics, the experimental environment, and the macroscopic and microscopic pathological interpretations. Progress has been made in recent years by various groups to reduce some of the sources of variability through international efforts to improve harmonization and standardization of terminology, trimming procedures, and study designs. Guidance has been provided from a variety of regulatory agencies on the use of HCD in carcinogenicity studies [70]. Best practices recommendations of the STP for use of HCD of proliferative rodent lesions are given in Table 12.12.

HCD is just one of many considerations in a weight-of-evidence approach to assessing the potential carcinogenic effect of a compound. Other data to consider include the incidences of other lesions of similar cell lineage, body weight effects, survival, time of tumor onset, concordance of the tumor response across both sexes or different species, and the presence of a positive dose-related response [72].

Assessing Cause of Death

Determining the cause of death in individual animals may be an important consideration in some toxicity studies. The pathologist is responsible for determining the cause of death or morbidity in animals that die prior to the scheduled necropsy and should attempt to identify a cause of death whenever possible. The pathologist should also determine if overall mortality and differences in mortality among groups are the results of the test compound. A review of the assigned causes of death should be included as part of the pathology peer review [73].

Determining the cause of death requires a thorough knowledge of the systemic pathology of the test species and professional judgment. The pathologist should have all study data for the individual animal available, including clinical observations and clinical pathology, as well as pathology data, to make this judgment [73,74]. The cause of death for each animal should be based on the primary disease process judged to have led to morbidity or mortality. The World Health Organization defines cause of death as "the disease or injury which initiated the train of events leading directly to death, or the circumstances of accident or violence which produced fatal injury [75]." If there are several potential causes of death, the one judged to have most likely led to mortality should be chosen. Not all animals have a clear cause of death; if the cause of death cannot be determined from the information available, it should be recorded as undetermined [73].

REPORTING PATHOLOGY FINDINGS

The pathologist's findings and conclusions must be accurately and completely reflected in the study report. The pathology findings must also be integrated with other study data to provide a comprehensive discussion of study findings including

TABLE 12.12
Recommendation for Use of Historical Control Data for Proliferative Rodent Lesions

- The concurrent control group is the most relevant point of comparison for determining treatment-related effects in a study.
- HCD may be useful in the interpretation of the following:
  - Rare tumors
  - Marginally greater incidences (and/or severity) of proliferative changes in treated groups compared to controls
  - Unexpected increases or decreases of tumor incidences in study control animals
  - Disparate findings in dual concurrent control groups
  - Trends in tumor biology and behavior that may evolve over time in rodent models
- HCD should be considered as one of many sources of information that add to the weight-of-evidence approach when assessing the potential carcinogenic effect of a compound.
- HCD from the laboratory that conducted the study under review will likely be more comparable than that compiled from several laboratories.
- Study design parameters should be considered when selecting the appropriate studies for the HCD. These include laboratory, species/strain, route of administration, vehicle, feed, feeding practices, study duration, and housing.
- HCD is more valuable if pathology practices, including necropsy and trimming procedures and application of diagnostic criteria, are standardized.
- HCD from peer-reviewed studies are generally more reliable than those not peer reviewed.
- Published HCD may provide guidance in evaluating data but should be reviewed carefully, as there may be difficulties in assessing the quality of published data.
- HCD may be presented as a range of incidences or percentages and as a mean and standard deviation for a given proliferative lesion. Reporting of incidences per study allows assessment of potential influences of outlier populations.
- The collection of HCD within a limited time span of 2–7 years has been proposed in many guidance documents, but wider intervals may be appropriate if tumor types are stable over a longer period.

Source: Keenan, C. et al., *Toxicol. Pathol*., 37, 679, 2009.

TABLE 12.13
Best Practices for Pathology Report Generation

Study Protocol and Accountability

- The study pathologist(s) should contribute to generation of the study protocol.
- The study pathologist should have access to the study protocol and all protocol amendments.
- Individual accountability and responsibility of all study personnel including the study pathologist(s) should be clearly defined in the study protocol and/or final report.
- The study pathologist(s) should have primary responsibility for interpretation of assigned pathology data, including necropsy findings, microscopic findings, and ultrastructural findings (and usually clinical pathology and organ weight findings).

Data Accessibility and Data Review

- The study pathologist should have access to all study data including
  - The intended pharmacologic target and mechanism of action
  - In-life study data
  - Clinical pathology data
  - Organ weight data
  - Necropsy findings
  - Toxicokinetic information data from previous studies with the same test article
- The study pathologist should review all pathology individual and summary data tables.

Study Report and Pathology Report

- Either an integrated study report containing the pathology narrative and tables or a separate pathology report can provide appropriate accountability and integration of all study data.
- The study pathologist should assist the study director in writing and/or reviewing the final study report, including the summary, discussion, and conclusion.
- The study pathologist must sign a separate pathology report or an integrated study report.

relationship of any toxicity findings to exposure to the test substance and the significance of those findings (e.g., adverse or adaptive).

Toxicology laboratories generally issue either an integrated final study report or a separate pathology report that is appended to the final study report. Regardless of the reporting format, the study pathologist and other contributing scientists should provide an integrated assessment of significant study findings including clinical findings, clinical pathology information, organ weight data, anatomic pathology findings, and pharmacokinetic data. The pathology report should include the necessary individual animal data tables, the summary data tables, and the pathology narrative containing integration of pathology findings with all other study data. The pathology report should also clearly note accountability for the primary evaluation and interpretation of pathology data. It is also important that the main study report preserve the integrity of the interpretation of pathology findings [76]. Some recommended best practices for pathology report generation are given in Table 12.13 [76].

QUALITY ASSURANCE AND THE PATHOLOGY PEER REVIEW

Because pathology data often provide important information used in making regulatory decisions on the health hazard and risk to humans of many drugs, industrial chemicals, and pesticides, clearly defined procedures must be in place to ensure the accuracy of pathology data, including the generation, interpretation, and reporting of the data. These quality review procedures usually include a quantitative pathology data review (data audit or pathology materials review) and a pathology peer review. In addition, review of pathology findings for some studies may include a PWG review [12,77,78]. These aspects of the pathology quality assurance are discussed in more detail as follows.

Pathology Data Review

The purpose of the pathology data review is to ensure the quality of the materials and procedures used to generate histopathological data, as well as other pathology data. The data review is most commonly conducted by a quality assurance unit. The pathology materials reviewed include individual animal necropsy records, histology laboratory worksheets, fixed tissues, blocks, and microscopic slides. A complete (100%) inventory of slides, blocks, and bags of remaining wet tissue should be conducted to ensure proper identification and labeling, and all slides and blocks should be matched. A random subset of slides should be examined macroscopically and microscopically to evaluate slide quality, including cover slipping, tissue placement, staining, and presence or absence of artifacts. A random subset of residual wet tissues should also be examined to verify the animal identification number. In addition, residual wet tissue may also be examined by a pathologist to ensure that no additional gross lesions are present that were either not identified at necropsy or not trimmed. Items that may be discovered in the data review include untrimmed lesions, incorrectly identified animals, and slides that do not correspond to their respective blocks [12,77].

Pathology Peer Review

Pathology peer review is a procedure whereby a second pathologist reviews a subset of tissues and other data from the initial pathology evaluation. The primary purpose of the peer review is to verify the accuracy of toxicologically significant microscopic findings; however, it is not intended to corroborate every detail of every microscopic finding in a study [79]. A pathology peer review serves to ensure the integrity of the pathology evaluation, encourages consistency in the application of diagnostic criteria and terminology, and provides a method of continuing education for participants [77,80]. Documented histopathology peer reviews also increase the confidence of regulatory agencies in the pathology portion of the report [81]. Pathology peer review is recommended when important risk assessment or business decisions may be based on pathology interpretations in toxicity studies, including GLP toxicity and carcinogenicity studies. Pathology peer review may also be valuable for non-GLP studies that inform compound development decisions or dose selection for future studies [82].

The histopathology peer review may be informal (undocumented consultations) or formal, and the formal peer review may be prospective or retrospective. The prospective peer review is conducted prior to finalization of the study and is conducted by an informed reviewer, its procedures are included in the protocol, and the results of the peer review are documented in the final report. A retrospective peer review, such as that conducted by a PWG, generally occurs after the data are finalized. The results of a retrospective peer review should be documented in a separate report [77].

A formal peer review is conducted by an independent pathologist whose objectives are to ensure that diagnoses are accurate, that lesions are diagnosed consistently across animals and groups, and that generally accepted diagnostic criteria and nomenclature are followed [77]. Approaches may vary between study types and study objectives as to the sampling size to be evaluated by the reviewing pathologist, but the review typically includes subsets from high-dose and control groups of both sexes, as well as all neoplasms and all target organs. Recommendations for peer review tissue examination protocols are given in Tables 12.14 and 12.15 for toxicity and carcinogenicity studies, respectively [82]. The reviewing pathologist should confirm all treatment-related findings, including NOAELs. At the end of the review, the final diagnosis should represent the consensus of the study pathologist and the reviewing pathologist. The formal peer review should be fully documented to include the tissues examined, the diagnosis of both the study and peer review pathologists, and the actions taken to resolve any differences [77,79]. Worksheets containing the detailed findings of the primary and review

TABLE 12.14
Recommendations for Pathology Peer Review in Toxicity Studies

Rodent Studies

- Protocol tissue
  - *High-dose group*: Examine all protocol organs in at least 30% of high-dose animals of each sex in the treatment phase.
  - *Controls*: Examination of all protocol organs from a subset of control animals may be performed at the discretion of the peer review pathologist.
- Target organs
  - *Controls*: Examine target organs in all animals (in the affected sex) in the control group.
  - *NOAEL groups*: Examine target organs in all animals at the NOAEL (i.e., in the highest-dose group lacking the finding).
  - *Affected groups*: Examine target organs in a sufficient number of animals (50% or more) in affected groups to characterize the finding.
  - *Recovery groups*: Examine all target organs in all control recovery animals and all target organs in all treated recovery animals in the dose groups and sexes in which the finding was observed at the end of the treatment period.

Nonrodent Studies

- Protocol tissue
  - *High-dose group*: Examine all protocol organs in at least 50% (minimum of two) of high-dose animals of each sex in the treatment phase.
  - *Controls*: Examination of all protocol organs from a subset of control animals may be performed at the discretion of the peer review pathologist.
- Target organs
  - *Controls, NOAEL group, and affected group*: Examine target organs in all animals in the affected sex.
  - *Recovery groups*: Examine all target organs in all control recovery animals and in all treated recovery animals in the dose groups and sexes in which the finding was observed at the end of the treatment period.

TABLE 12.15
Recommendations for Pathology Peer Review in Carcinogenicity Studies

- Protocol tissue
  - *High-dose group*:
    - Two-year studies: Examine all protocol organs in at least 10% of high-dose animals of each sex.
    - Six-month alternative carcinogenicity studies: Examine all organs from five high-dose animals per sex.
  - *Controls*: Examination of all protocol organs from a subset of control animals may be performed at the discretion of the peer review pathologist.
- Neoplasms: Examine all neoplasms in all animals
- Target organs
  - *Neoplastic target organs*: Examine all neoplastic target organs (or suspected target organs) from all animals in all groups. Each sex should be considered separately.
  - *Nonneoplastic target organs*: Examine target organs with nonneoplastic findings from
    - All control animals
    - All animals of the highest-dose group lacking the finding
    - Sufficient animals (at least 30%) in affected groups and sexes to verify the finding

pathologist need not be retained, as these are the equivalent of *pathology work files* and are not raw data [79].

Pathology Working Group

A PWG is a panel of expert pathologists assembled to review a specific question concerning study results. A PWG review is typically conducted for finalized studies and may be convened to review certain pivotal or controversial studies that raise regulatory concern or studies that have critical effects for which diagnostic criteria and terminology have changed since the original review; also, they allow comparison of the results of multiple studies that may have been conducted and evaluated by different laboratories or pathologists. The PWG may also be used to address differences between a study and reviewing pathologist that could not be resolved during the peer review process. Sources of disagreement may include unfamiliarity with a lesion, use of different criteria for tumor classification, use of different thresholds for diagnosis of lesions (especially nonneoplastic aging lesions), use of different terminology for the same lesion, diagnostic drift, and varying pathology reporting system data input and reporting requirements. The technical aspects of the PWG have been reviewed by Mann [77] and are summarized in Table 12.16. The U.S. Environmental Protection Agency has also outlined the procedures and documentation necessary for the results of a PWG to be considered in place of the original reading (Table 12.17) [83].

TABLE 12.16
Pathology Working Group: Technical Approach

Composition

Three to five experienced toxicologic pathologists
A chairperson, usually nonvoting,
- Organizes and presents material so as to resolve issues in an unbiased and scientifically sound manner
- Anticipates and seeks resolution of potential problems with the data that might affect interpretation of the study

The study pathologist and reviewing pathologist may attend as panel members

Procedures

The PWG slide review is coded; members have no knowledge of treatment group or previous diagnosis.
PWG reviews representative slides of the target tissue containing the potential treatment-related changes in question.
PWG reviews all slides for which different diagnoses were recorded between the peer review and study pathologists.
PWG provides a consensus diagnosis for each slide examined:
Consensus diagnosis is based on majority vote.
Discussion and reexamination may be required.
The final consensus diagnosis is recorded by the chairperson.
No changes are made to the consensus diagnosis once the slides are uncoded.
After examination and uncoding of slides, the PWG members may be asked to utilize their expertise to discuss the biological significance of their findings.

PWG Report

The report is assembled by the PWG chairperson.
The PWG narrative summary includes the following:
- Incidence tables
- Comments on the diagnostic terminology used
- Morphological descriptions of the lesions examined
- An evaluation of the study pathologist's report
- Comments on the quality of the histological preparations and tissue availability
- The opinions of the PWG

Source: Adapted from Mann, P.C., *Toxicol. Pathol.*, 24, 650, 1996.

Histopathology Data Collection and Audit Trail

The definition of raw data varies in different regulatory jurisdictions. Raw data have been defined in the U.S. Code of Federal Regulations (21 CFR Part 58; Good Laboratory Practices) as any record (laboratory worksheet, memoranda, or note) that results from an original observation or activity in a nonclinical laboratory study that is necessary for the reconstruction and evaluation of the report of that study. Histopathology observations are based on examination of histopathology slides that are durable and can be reevaluated during the conduct of the study and into the future. Also, in developing the final interpretation and diagnoses, the pathologist must consider a number of dynamic factors during the course of the histopathological evaluation such as histopathology observations in other tissues and in other animals, as well as other types of data from the study. Therefore, the initial histopathology observations, based on evaluation of the tissue(s) present on single glass histopathology slides,

TABLE 12.17
Pathology Working Group Procedures Required by the U.S. Environmental Protection Agency

For any target tissue reevaluated, all slides containing that tissue in all dose groups, as well as the controls, must be reread by the peer review pathologist. This is to include the following:

- Slides previously classified by the study pathologist as within normal limits.
- Slides having tumors, hyperplasia, hypertrophy, foci of cellular alteration, or other nonneoplastic lesions.
- The pathology reports from both the study and peer review pathologists and the original slides are to be submitted to a PWG.
- The PWG will review, as a minimum, all slides about which there were significantly differing diagnoses between the study and peer review pathologists.

A detailed pathology report should be provided that presents the following:

- The PWG findings, including the original diagnosis and the new diagnosis for each slide read.
- A comment column to note any discrepancies, missing slides, etc.

Source: Adapted from USEPA, Requests for reconsiderations of carcinogenicity peer review decisions based on changes in pathology diagnoses, PR Notice 94-5, U.S. Environmental Protection Agency, Washington, DC, 1994.

represent *working interim notes*, and these interim notes are not necessary for reconstruction and evaluation of the report and thus do not represent raw data [84]. Consistent with these considerations, the preamble to the GLP Regulations (http://www.ovpr.uga.edu/qau/resources/glps/fda/preamble) states the following:

> Although the notes taken by a pathologist during histopathological examination of slides are indeed the result of original observations, these notes are not necessary for the reconstruction and evaluation of the final report. The final report is evaluated by an analysis of the pathology syndrome as described in the pathologist's report, which is required under Section 58.185(a)(12). Further, because Section 58.190(a) requires histopathological blocks, tissues, and slides to be retained as specimens, the final report can be reconstructed by verification of the pathology findings by, e.g., a second pathologist or by a team of pathologists.

Based on these considerations, the preamble further notes that

> The pathologists interim notes, therefore, which are subject to frequent changes as the pathologist refines the diagnosis, are not raw data because they do not contribute to study reconstruction. Accordingly, only the signed and dated final report of the pathologist comprises raw data respecting the histopathological evaluation of tissue specimens.

GLP-compliant electronic histopathology data collection computer systems have security measures incorporated into them to protect the integrity of the data throughout the entire data collection and reporting processes. When the histopathology database is locked/completed, an audit trail is activated to track any subsequent changes made. Since the initial histopathology observations/interim notes are not raw data, are not required for study reconstruction and evaluation of the final report, and are not required to be retained, no audit trail is required to track changes to these interim histopathology notes prior to locking the database. Also, for a pathology peer review conducted before a study is completed, the audit trail for the microscopic pathology data should not be initiated until after any changes to the original microscopic observations and interpretations resulting from pathology peer review have been made [76]. Once the histopathology database is locked, a full audit trail entry (who, when, what, and why) is required for each change to the database [84].

TOXICOLOGIC PATHOLOGY IN THE *OMICS* ERA

Advances in genome sequencing and the development of platforms to assess gene and protein expression have made *omics* technologies increasingly important tools in toxicological research [85]. Important areas of impact for toxicogenomics include understanding biological mechanisms, identification of biomarker candidates, and identifying species differences in toxicological responses [86]. Effective application of technologies such as toxicogenomics requires a broad range of skill sets, including molecular biology, biochemistry, pathology, toxicology, bioinformatics, statistics, and mathematics. As such, the interpretation of changes, for example, at the level of the transcriptome should always be undertaken in light of other available study information, such as hematology and clinical chemistry data, histopathology findings, and organ weight changes [87]. In addition, much of the current understanding of adverse effects is linked to apical phenotypic effects such as histological changes. Therefore, phenotypic anchoring of changes in gene or protein expression, or other in vitro end points, is important in determining, for example, whether such changes represent adverse or adaptive effects [55].

As would be expected, many of the factors that may contribute to variability in traditional pathology end points may also be sources of variability in toxicogenomics studies. Thus, gender, fasting, strain, and organ section—important consideration when assessing histopathological changes—are among the most reproducibly prominent biological factors associated with gene expression variance among control animals [88]. As such, established principles of pathology are important considerations in the design and interpretation of toxicogenomic studies. For example, tissues and organs are not uniform in their morphology or function, so gene expression patterns are expected to be different when sampling whole kidney versus renal cortex or renal medulla following exposure to a renal toxin. Therefore, consideration should be given as to what tissues and what portions of tissues need to be collected for gene analysis [85].

The characterization of the morphological changes in a tissue can play an important role in the interpretation of gene expression data. For example, the liver changes following

exposure to a hepatotoxicant may include not only a spectrum of normal, degenerating, and dead cells but also inflammatory cell infiltrates, all of which will be reflected in the RNA from exposed animals. Furthermore, the pattern of changes in gene expression can be expected to vary as the lesions progress from the acute to more chronic phases. And since gene expression in an organ such as the liver may actually reflect primary changes in other targets, such as the red blood cell or intestines, a complete necropsy with histopathologic evaluation of key organ systems can provide an important morphological anchor and corroboration of specific gene expression changes. Other tools of traditional toxicologic pathology, such as electron microscopy and immunohistochemistry, can also provide more detailed information on cellular changes and may provide additional perspective on changes in gene expression. As a discipline based on pattern recognition and requiring familiarity with the dynamics of disease processes and interactions between organs, tissues, and cell populations, toxicologic pathology will be an important contributor to the field of toxicogenomics, from experimental design to data interpretation [89].

QUESTIONS

12.1 What documents should be available to the necropsy team prior to and during the necropsy, and what information is provided in each of these documents?

12.2 What are the disadvantages of coded or blinded histopathological evaluations, and what are some circumstances where a blinded evaluation would be indicated?

12.3 What is diagnostic drift, what are some factors that may cause diagnostic drift, and how can it be minimized?

12.4 What are some discriminating factors to consider in a weight-of-evidence approach to determine if effects are treatment related and if they are adverse?

12.5 What are some scenarios under which HCD might be useful in a carcinogenicity study? Are there any other situations in which HCD can be used and what are they?

12.6 What are the primary purpose and key elements of the pathology peer review?

12.7 Explain phenotypic anchoring and why it is necessary.

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13 The Information Infrastructure of Toxicology

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CONTENTS

SCOPE OF TOXICOLOGY

The U.S. Society of Toxicology's (SOT) *Definition of Toxicology Task Force* developed the following consensus definition of toxicology in 2005:

> Toxicology is the study of the adverse effects of chemical, physical or biological agents on living organisms and the ecosystem, including the prevention and amelioration of such adverse effects.

This definition was further elaborated by a consensus discussion:

> Toxicity is the adverse end product of a series of events that is initiated by exposure to chemical, physical or biological agents. Toxicity can manifest itself in a wide array of forms, from mild biochemical malfunctions to serious organ damage and death. These events, any of which may be reversible or irreversible, include absorption, transport, metabolism to more or less toxic metabolites, excretion, interaction with cellular macromolecules and other modes of toxic action. Toxicology integrates the study of all of these events, at all levels of biological organization, from molecules to complex ecosystems. The broad scope of toxicology, from the study of fundamental mechanisms to the measurement of exposure, including toxicity testing and risk analysis, requires an extensively interdisciplinary approach. This approach utilizes the principles and methods of other disciplines, including molecular biology, chemistry (analytical, organic, inorganic and biochemistry), physiology, medicine (veterinary and human), computer science and informatics.

This definition reflects the broad multidisciplinary nature of toxicology. Toxicology encompasses research studies on animals, humans, and the environment. Furthermore, it consists of an important medical component via the practice of clinical toxicology. Translational science that seeks for ways to translate bench science to bedside clinical practice is helping to bridge the gap in toxicology between research and patient care.

One can consider toxicology from a variety of perspectives. What are the substances involved?—Chemicals (both singly and in mixtures and formulations; as nanoparticles and otherwise; in food, drugs, industrial applications, products, etc.), physical (such as noise and radiation), and biological (such as animal venoms and plant toxins) or consider effects of various target sites—liver, kidney, skin, nervous system, etc. In addition to the agent, one needs to consider the subject and particular susceptibilities due to gender, age, genetics, state of health, etc. Both acute and chronic exposures or effects need to be taken into account. Occupational toxicology puts the science in a new setting and poses unique problems. Regulatory and safety evaluation and the legal framework present toxicology with a host of additional challenges.

While animal experimentation continues to be widespread, it is expensive, and labor and time intensive, and alternative testing approaches are increasingly being employed. Understanding how to map the molecular pathways of toxicity in cells will play a major role in enhancing our understanding of what makes a substance toxic. Furthermore, toxicology has embraced the *omics* revolution. Bioinformatics and computational toxicology are offering new ways to evaluate and assess toxicity.

SCOPE OF INFORMATION RESOURCES AND THE ROLE OF THE WEB

The use of existing, and generation of new, information has always been an essential part of science. Information science and its progenitor, library science, have traditionally been concerned with the collection, organization, classification, archiving, and dissemination of information. As more and more computer processing power and technological innovations have become available, the lines between the sciences, including toxicology, and information science have intersected. The term *informatics* has been increasingly used to represent an academic discipline utilizing a variety of techniques to process, manage, and retrieve data and other forms of information.

Toxicology data and information can be distributed via an array of resources and media, including paper (yes, still), electronic journals and books, databases and portals, websites, professional societies, trade associations, and government and nongovernmental organizations (NGOs). Data are generated from laboratory animal or in vitro studies (e.g., cell culture), or via in silico methods (i.e., using computer programs to estimate the toxicity of chemicals), with increasing reliance upon high-throughput screening and other alternatives to animal testing. In addition, ethically conducted human studies can provide toxicology and safety information.

Finding the best ways to keep current with the vast amount of literature and other information associated with the many aspects of toxicology is both a challenge and an opportunity. Significant advances have been made in recent years in ease of access to toxicological information, with 24 h–a-day access readily available. The vast array of websites, in addition to Internet mailing lists and social media, provides numerous opportunities for training and information sharing.

Information in toxicology is widespread and often diffusely scattered across scientific disciplines despite continuing efforts to integrate, coordinate, and consolidate. Moreover, the information is presented in many forms, including raw data and study reports, texts and monographs, statutes and regulations, journal articles, and more, which presents a significant challenge for those attempting to find and retrieve information relevant to their interests. In the predigital era, information was either oral or written and on a physical support such as paper, clay tablets, parchment, or vellum. Oral information referred largely to the collegial network—colleagues communicating with each other in face-to-face discussions or via telephone calls as a means of diffusing knowledge. Written information took on a discrete number of forms—textbooks and other monographs, technical journals, newsletters, dissertations, abstracting and indexing services, etc. Over time, the task of storing, maintaining, and retrieving this ever-expanding knowledge base became more complicated and labor intensive. Fortunately, with the advent of computers and, particularly, computer databases, the nature of information began to change, and revolutionized the way we store, retrieve, and share toxicological data. Now, with the establishment and unprecedented expansion of the web, information and its infrastructure have taken on a level of complexity hardly dreamt of even by those already conversant with an earlier era of database searching.

Over the years, toxicology has moved from a descriptive science to one that is largely data driven. Beginning in the late twentieth century, quantitative structure–activity relationship (QSAR), high throughput screening, in silico methodologies, computational approaches for predictive toxicology, the -omics revolution, and systems biology have all been responsible for a groundswell of new data. All these, combined with more recently introduced mobile technologies and electronic social networks, plus standard journal articles, books, Internet databases, and collegial networks, now constitute the broader informatics framework. These loosely connected informational tools, known in the aggregate as toxicoinformatics, have become essential to the practice of toxicology. Toxicologists would do well to embrace its capabilities in order to make the best use of existing toxicological information and to plan for as yet undefined, but unstoppable, future developments.

Today, the lines demarcating one kind of information from another are increasingly blurring, as more and more packaged and synthesized information, not to mention raw data, and the invariable unsubstantiated musings of would-be experts are democratically finding their way onto the web. This presents a challenge to those seeking scientifically

credible toxicology and health information, inasmuch as there is no governing body to ensure that information on the web is accurate or true. Therefore, it is becoming ever more important for information seekers to apply critical and skeptical thinking when viewing web-based toxicology and health information. Guidelines for evaluating the reliability of health (and toxicology) information on the web can be found at the following National Library of Medicine (NLM) website: http://www.nlm.nih.gov/medlineplus/evaluatinghealthinformation.html.

Although bibliographic information and summaries of standard technical documentation in books and journals already have a robust history of online access, the goal of *full*-text digital availability is only now being realized. Most scientific journals now offer direct access to full-text articles online for their subscribers. Elsevier's Science Direct (http://www.sciencedirect.com) is one such collection of full-text literature. MEDLINE®, Thomson Reuters' *Web of Knowledge*, and CAS's *SciFinder* and *SciFinder Scholar* also offer full-text access to subscribers for articles retrieved in bibliographic searches.

Online libraries are in our future, and authors seem to be increasingly accepting of the concept of online publication.[10] Project Gutenberg (http://www.gutenberg.org/), begun in 1971, offers over 100,000 free electronic books on the Internet, directly or through their partners and affiliates. It consists largely of older literary works in the public domain in the United States and elsewhere, but a scientific counterpart is inevitable. The issue of who will ultimately pay for this, and how, remains to be resolved and will be explored a bit more at the end of this chapter. More recently, in 2004, the company that created the web search engine Google™ announced that it is working with libraries at the University of Michigan, Harvard University, Stanford University, Oxford University, and the New York Public Library to digitize books in their collections and make them accessible online. The list of libraries has since expanded. This massive scanning project has brought millions of volumes of printed books into the Google Print database (http://print.google.com/) for around-the-clock and around-the-world access. Both free (public domain) and pay-for-access books, including toxicology texts, are available. Interestingly, the European Union (EU) is moving forward with a similar complementary effort to put European literature online (http://www.europeana.eu/portal/).

Virtually every organization of relevance to toxicology, as reflected here, has a web presence. Activities that would once have required reams of paper to describe and would have resulted in a distribution nightmare are usually consolidated on websites where the user comes to the information instead of vice versa. Consider government agencies such as the U.S. Environmental Protection Agency (EPA) and the U.S. Food and Drug Administration (FDA), whose extensive websites are dense with information. Other notable agencies in this regard include the National Institute for Occupational Safety and Health (NIOSH), the National Institutes of Health (NIH), and the U.S. Centers for Disease Control (CDC). The challenge here, if anything, is how to manage and efficiently navigate (and then digest) the large amount of information and data. Utilization of new *push technologies*, as well as mobile devices and applications, along with social media, all of which will be discussed later, offers alternative approaches to delivering information.

The formerly strict lines between multimedia and computerized representation have long been erased. Digital audio or video transmissions via the Internet (i.e., streaming media) are commonplace, and quality continues to improve as bandwidth increases. Computer technologies used for entertainment hold great potential for educational applications. Indeed, many websites provide audio and video access to live meetings, and instructional videos are increasingly incorporating tools such as Flash™ and ActionScript™ to visually enhance the learning process.

Monographs

This section provides a selective list of books in six areas dealing with toxicology, as well as an *other* category. The six main areas considered are general toxicology, clinical toxicology, analytical toxicology, industrial/occupational toxicology, environmental toxicology, and risk assessment. The *other* category is comprised of books on a variety of topics, such as risk communication, forensic toxicology, and more. The following list offers mostly recent books published since 2000 but also includes older books that are considered classics in the field.

General Toxicology

Ballantyne, B., Marrs, T., and Syversen, T. (2009): *General and Applied Toxicology*, 3rd edn., Macmillan, London, U.K.

Brunton, L., Chabner, B., and Knollman, B. (2010): *Goodman & Gilman's the Pharmacological Basis of Therapeutics*, 12th edn., McGraw-Hill, New York.

Derelanko, M. J. and Hollinger, M. A. (2002): *Handbook of Toxicology*, 2nd edn., CRC Press, Boca Raton, FL.

Duffus, J. H. and Worth, H. G. (eds.) (2006): *Fundamental Toxicology*, Royal Society of Chemistry, Cambridge, U.K.

Gilbert, S. G. (2004): *A Small Dose of Toxicology: The Health Effects of Common Chemicals*, CRC Press, Boca Raton, FL.

Hayes, A. W. (2008): *Principles and Methods of Toxicology*, 5th edn., Taylor & Francis Group, Boca Raton, FL.

Hodgson, E. and Smart, R. C. (eds.) (2008): *Molecular and Biochemical Toxicology*, 4th edn., Wiley, Hoboken, NJ.

Illing, P. (2001): *Toxicity and Risk*, CRC Press, Boca Raton, FL.

Kent, C. (1998): *Basics of Toxicology*, Wiley, New York.

Klaassen, C. D. (2007): *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 7th edn., McGraw-Hill, New York (8th edn. in process).

Klaassen, C. D. (2010): *Casarett & Doull's Essentials of Toxicology*, 2nd edn., McGraw-Hill, New York.

Krishnan, K. and Andersen, M. (2010): *Quantitative Modeling in Toxicology*, John Wiley & Sons.

Lewis, R. A. (1998): *Lewis' Dictionary of Toxicology*, CRC Press, Boca Raton, FL.

Lu, F. C. and Kacew, S. (2009): *Lu's Basic Toxicology: Fundamentals, Target Organs and Risk Assessment*, 5th edn., Informa Healthcare USA, Inc., New York.

McQueen, C. A. (ed.) (2010): *Comprehensive Toxicology*, 2nd edn., (14 vols.). Elsevier, Amsterdam, the Netherlands.
Rose, V. E. and Cohrssen, B. (2010): *Patty's Industrial Hygiene and Toxicology*, 6th edn., Wiley, New York.
Saura, C., Casciano, D., Ballantyne, B., Marrs, T., and Syversen, T. (eds.) (2011): *General, Applied and Systems Toxicology*, Wiley, Chichester, U.K.
Timbrell, J. A. (2001): *Introduction to Toxicology*, 3rd edn., CRC Press, Boca Raton, FL.
Vettorazzi, G. and Anadon, A. (2001): *The ITIC International Dictionary of Toxicology*, ITIC Press, San Sebastián, Spain.
Wexler, P. (2005): *Encyclopedia of Toxicology*, 2nd edn., Elsevier, Oxford, U.K. (3rd edn. in preparation, scheduled for 2013 publication).
Wexler, P., Hakkinen, P. J., Mohapatra, A., and Gilbert, S. G. (2009): *Information Resources in Toxicology*, 4th edn., Academic Press, San Diego, CA.
Williams, P. L. and Burson, J. L. (2000): *Principles of Toxicology: Environmental and Industrial Applications*, 2nd edn., Wiley, New York.
Woolley, A. (2003): *A Guide to Practical Toxicology: Evaluation, Prediction, and Risk*, Taylor & Francis Group, London, U.K.

Clinical Toxicology

Barceloux, D. G. (2008): *Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Plants, and Venomous Animals*, 1st edn., John Wiley & Sons, Hoboken, NJ.
Barile, F. A. (2010): *Clinical Toxicology: Principles and Mechanisms*, Informa Healthcare USA, Inc., New York.
Brunton, L. L., Chabner, B., and Knollman, B. (2011): *Goodman & Gilman's the Pharmacological Basis of Therapeutics*, 12th edn., McGraw-Hill, New York.
Dart, R. C. (2003): *Medical Toxicology*, 3rd edn., Lippincott Williams & Wilkins, Philadelphia, PA.
Delaney, K. A., Ling, L. J., Erickson, T., and Ford, M. D. (2000): *Clinical Toxicology*, W.B. Saunders, Philadelphia, PA.
Dreisbach, R. H. and True, B. (2002): *Handbook of Poisoning: Prevention, Diagnosis, and Treatment*, 13th edn., Parthenon Publishing Group, Boca Raton, FL.
Goldfrank, L., Flomenbaum, N., Lewin, N., Howland, M. A., Hoffman, R., and Nelson, L. (2011): *Goldfrank's Toxicologic Emergencies*, 9th edn., McGraw-Hill Medical, New York.
Gupta, S. K., Kaleekal, T., and Peshin, S. S. (2003): *Emergency Toxicology: Management of Common Poisons*, Narosa Publishing House, New Delhi, India.
Luch, A. (2009): *Molecular, Clinical and Environmental Toxicology*, Vol. 2: *Clinical Toxicology*, Birkhäuser, Basel, Switzerland.
Murray, L. (2011): *Toxicology Handbook*, 2nd edn., Churchill Livingstone, Sydney, New South Wales, Australia.
Rossoff, I. S. (2001): *Encyclopedia of Clinical Toxicology: A Comprehensive Guide and Reference*, Parthenon, New York.
Shannon, M. W., Borron, S. W., and Burns, M. (2007): *Clinical Management of Poisoning and Drug Overdose*, 4th edn., W.B. Saunders, Philadelphia, PA.
Sullivan, J. B. and Krieger, G. R. (2001): *Clinical Environmental Health and Toxic Exposures*, 2nd edn., Lippincott Williams & Wilkins, Philadelphia, PA.

Analytical Toxicology

Baselt, R. C. (2011): *Disposition of Toxic Drugs and Chemicals in Man*, 9th edn., Biomedical Publications, Foster City, CA.
Dasgupta, A. (2008): *Handbook of Drug Monitoring Methods: Therapeutics and Drugs of Abuse*, Humana Press, Totowa, NJ.
Flanagan, R. J., Taylor, A. A., Watson, I. D., and Whelpton, R. (2007): *Fundamentals of Analytical Toxicology*, 1st edn., John Wiley & Sons, Hoboken, NJ.
Külpmann, W. R. (2009): *Clinical Toxicological Analysis: Procedures, Results, Interpretation*, Wiley-VCH, Weinheim, Germany.
Moffat, A. C., Osselton, M. D., Widdop, B., and Watts, J. (2011): Clarke's analysis of drugs and poisons. In: *Pharmaceuticals, Body Fluids and Postmortem Material*, 4th edn., Pharmaceutical Press, London, U.K.; Chicago, IL.
Smith, F. P. (2005): *Handbook of Forensic Drug Analysis*, 1st edn., Elsevier Academic Press, Amsterdam, the Netherlands; Boston, MA.
Xu, Q. A. and Madden, T. L. (2011): *Analytical Methods for Therapeutic Drug Monitoring and Toxicology*, 1st edn., Wiley, Hoboken, NJ.

Environmental Toxicology

Baird, C. (2008): *Environmental Chemistry*, 4th edn., W.H. Freeman, New York.
Crosby, D. G. (1998): *Environmental Toxicology and Chemistry*, Oxford University Press, Oxford, U.K.
Hoffman, D. J., Rattner, B. A., Burton, G. A., and Cairns, J. (2002): *Handbook of Ecotoxicology*, 2nd edn., CRC Press, Boca Raton, FL.
Landis, W. G., Sofield, R. M., and Yu, M. (2010): *Introduction to Environmental Toxicology: Molecular Substructure to Ecological Landscapes*, 4th edn., CRC Press, Boca Raton, FL.
Manahan, S. E. (2009): *Environmental Chemistry*, 9th edn., CRC Press, Boca Raton, FL.
Moriarty, F. (1999): *Ecotoxicology: The Study of Pollutants in Ecosystems*, 3rd edn., Academic Press, London, U.K.
Newman, M. C. (2009): *Fundamentals of Ecotoxicology*, 3rd edn., CRC Press, Boca Raton, FL.
Rand, G. M. and Petrocelli, S. R. (1995): *Fundamentals of Aquatic Toxicology*, 2nd edn., Taylor & Francis Group, Philadelphia, PA.
Schüürmann, G. and Markert, B. (1997): *Ecotoxicology: Ecological Fundamentals*, *Chemical Exposure*, *and Biological Effects*, *Environmental Science and Technology: A Wiley-Interscience Series of Texts and Monographs*, Wiley, New York.
Streit, B. and Braunbeck, T. (1997): *Encyclopedic Dictionary of Ecotoxicology and Environmental Chemistry*, 2nd edn., Taylor & Francis Group, New York.
Suter, G. (2006): *Ecological Risk Assessment*, 2nd edn., CRC Press, Boca Raton, FL.
Suter, G., Efroymson, R., Sample, B., and Jones, D. (2000): *Ecological Risk Assessment for Contaminated Sites*, CRC Press, Boca Raton, FL.
U.S. Environmental Protection Agency (2000): *Ecological Risk Assessment: Federal Guidelines*, ABS Consulting, Houston, TX.
Walker, C. H., Hopkin, S. P., Sibley, R. M., and Peakall, D. B. (2012): *Principles of Ecotoxicology*, 4th edn., CRC Press, Boca Raton, FL.
Wright, D. A. and Welbourn, P. (2002): *Environmental Toxicology*, Cambridge University Press, Cambridge, U.K.
Yen, T. F. (2005): *Environmental Chemistry: Chemistry of Major Environmental Cycles*, World Scientific, Hackensack, NJ.
Zakrzewski, S. F. (2002): *Environmental Toxicology*, 3rd edn., Oxford University Press, Oxford, U.K.

Industrial Hygiene/Occupational Health

American Conference of Governmental Industrial Hygienists (ACGIH), Cincinnati, OH, http://www.acgih.org; numerous relevant publications, including their *TLVs and BEIs and Documentation for Deriving Them*.

American Industrial Hygiene Association (AIHA), Reston, VA, http://www.aiha.org; numerous relevant publications, including their *Emergency Response Planning Guidelines and Workplace Environmental Exposure Level Handbook*.

Berger, E. H., Ward, W. D., Royster, J. C., and Morrill, L. H. (1986): *Noise and Hearing Conservation Manual*, American Industrial Hygiene Association, Reston, VA.

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Journals and Newsletters

Great strides have been made in the early twenty-first century to provide more immediate and easy access to scientific journals. Journals covering many areas of toxicology and related disciplines that affect toxicological research are published by various groups, including commercial publishers, societies, and government agencies. Traditional print journals are giving way to electronic format, which is speeding the transfer of new research and findings to scientists around the world. Free online journals and publishing services go even further to share results with an even wider audience.

Scientific journals covering research and other topics in toxicology have been with us since at least the mid-nineteenth century. Among the successors to key early journals begun in the 1930s and 1940s are *Archives of Toxicology*, *Pharmacology and Toxicology*, *Toxicology and Applied Pharmacology*, and *Eksperimentalnaia i Klinicheskaia Farmakologiia*.

Today, dozens of journals focus on *Toxicology*, either broadly or on special subareas, such as *Biomarkers*, *Carcinogenesis*, and *Metabolism*. In addition, many others focus on applications of toxicology in drug development or in settings such as the ambient environment or the workplace. Other journals cover research and analysis that cross multiple disciplines and address issues—for example, in environmental or public health.

The most significant advancement in the last decade has been the proliferation of access via the Internet and other electronic media. Many journals now provide subscribers and others with early notification of publications (tables of contents). These services alert readers to the latest research quickly and can often be tailored to the user's particular interests. Some journals provide direct access to abstracts online for paid subscribers and the general public, and many now provide full-text online for subscribers or for single article purchase. Further innovations in access to journal literature are described later in this chapter.

Many journals now encourage authors to submit their articles electronically to facilitate faster peer review and acceptance. Journal peer reviews are often handled through e-mail or the Internet, and the use of these technologies has shortened review time considerably. Some journals release accepted papers in manuscript form almost immediately upon acceptance. These papers have not yet been copyedited or formatted for publication and are eventually replaced with the official publication version; however, they are considered published and are citable.

To keep current with available journals is difficult. Names change, as do publishers. New journals are born and others cease publication. The Internet is the easiest way to find a journal of interest, as most publishers have websites for their journals, where one can find information on contents, subscriptions, submissions, and sometimes access to abstracts and full text of articles. *New-Jour* is a comprehensive and free listing of electronic journals and newsletters on the Internet

and is updated frequently (http://gort.ucsd.edu/newjour/NewJourWel.html). In addition, one can access *PubList* (http://www.publist.com/), a free service that provides searches of a database of over 150,000 magazines, journals, newsletters, and other periodicals from around the world; *Ulrich's Periodicals Directory*, a bibliographic database with information on more than 260,000 print and electronic periodicals (http://www.bowker.com/brands/ulrichs.htm); and the U.S. Library of Congress, which has online catalogs of its extensive holdings, including journals (http://www.loc.gov/).

Table 13.1 provides some key toxicology journals, largely those in English. These journals cover toxicology and the related areas of occupational and environmental health, ecotoxicology, risk analysis, and medicine. Readers can easily locate current websites for these journals or their publishers via a standard web search engine, such as Google. It should be emphasized that this is a selective list of journals. Compared to journals, newsletters offer a generally less formal but sometimes more compelling means of keeping up-to-date in a subject. A few of the more prominent online toxicology newsletters are listed in Table 13.2.

Organizations

An extensive listing of toxicology-related organizations, as well as many other resources, appeared in Wexler et al.'s *Information Resources in Toxicology* (2009).[1] Many different types of organizations are associated with toxicology. These include professional societies, trade associations, government organizations, NGOs, centers or departments of universities, and companies. Key examples of these types of organizations are described later in this chapter, together with noteworthy publications and websites providing compilations of this type of information. The reader is urged to exercise caution when considering data and advice from the web, and should carefully consider the source. The authors of this chapter are not responsible for the information provided by the organizations noted, including the content of their sites. Furthermore, the authors do not necessarily endorse any products or services mentioned by the organizations.

Professional Societies

Professional societies associated with toxicology have been reviewed by Kehrer and Mirsalis[2] and Patterson et al.[3]

American College of Toxicology

The mission of the American College of Toxicology (ACT; http://www.actox.org) is to educate and lead professionals in industry, government, and related areas of toxicology by actively promoting the exchange of information and perspectives on the current status of safety assessment and the applications of new developments in toxicology. ACT maintains an outstanding collection of toxicology-related links in its website. The ACT newsletter is available on the website, as are announcements of upcoming meetings. ACT publishes the *International Journal of Toxicology*.

TABLE 13.1
Selected Toxicology Journals

Title
Acta Pharmacologica et Toxicologica
Adverse Drug Reaction Bulletin
Adverse Drug Reactions and Toxicological Reviews
American Journal of Industrial Medicine
Annals of Occupational Hygiene
Annals of the ICRP
Annual Review of Pharmacology and Toxicology
Aquatic Toxicology
Archives of Environmental Contamination and Toxicology
Archives of Environmental Health
Archives of Toxicology
Basis and Clinical Pharmacology and Toxicology
Biomarkers
Birth Defects Research
Bulletin of Environmental Contamination and Toxicology
Carcinogenesis
Cardiovascular Toxicology
Cell Biology and Toxicology
Chemical Research in Toxicology
Chemico Biological Interactions
Chemosphere
Clinical Toxicology
Comparative Biochemistry and Physiology. Part C: Pharmacology, Toxicology, and Endocrinology
Contact Dermatitis
Critical Reviews in Toxicology
Cutaneous and Ocular Toxicology
Dose–Response
Drug and Chemical Toxicology
Drugs
Ecotoxicology
Ecotoxicology Reviews
Environmental Health Perspectives
Environmental Research
Environmental Toxicology
Environmental Toxicology and Chemistry
Environmental Toxicology and Pharmacology
Environmental Toxicology and Water Quality
European Journal of Pharmacology
Experimental and Toxicologic Pathology
Expert Opinion on Drug Metabolism and Toxicology
Forensic Toxicology
Food and Chemical Toxicology
Free Radical Biology and Medicine
Human and Experimental Toxicology
Immunopharmacology and Immunotoxicology
Inhalation Toxicology
Integrated Environmental Assessment and Management
International Journal of Toxicology
Journal of Analytical Toxicology
Journal of Applied Toxicology
Journal of Biochemical Toxicology

(*continued*)

TABLE 13.1 (continued)
Selected Toxicology Journals

The Journal of Exposure Science and Environmental Epidemiology (JESEE), formerly the Journal of Exposure Analysis and Environmental Epidemiology
Journal of Immunotoxicology
The Journal of Occupational and Environmental Hygiene
Journal of Occupational and Environmental Medicine
Journal of Pharmacological and Toxicological Methods
Journal of Toxicological Sciences
Journal of Toxicology: Clinical Toxicology
Journal of Toxicology and Environmental Health
Molecular Carcinogenesis
Mutagenesis
Nanotoxicology
Natural Toxins
Neurotoxicity Research
NeuroToxicology
Neurotoxicology and Teratology
Particle and Fibre Toxicology
Pharmacology and Toxicology
Phytomedicine
Regulatory Toxicology and Pharmacology
Reproductive Toxicology
Risk Analysis
Science of the Total Environment
Teratogenesis, Carcinogenesis, Mutagenesis
Teratology
Toxicologic Pathology
Toxicological Reviews
Toxicological Sciences
Toxicology
Toxicology and Applied Pharmacology
Toxicology and Industrial Health
Toxicology In Vitro
Toxicology Letters
Toxicology Mechanisms and Methods
Toxicon
Xenobiotica

International Association of Forensic Toxicologists

The aims of International Association of Forensic Toxicologists (TIAFTnet; http://www.tiaft.org/) are to promote cooperation and coordination of efforts among members and to encourage research in forensic toxicology and related areas of analytical toxicology. The website contains information on systematic toxicological analysis (STA) and the STA Committee for the identification of toxic compounds and metabolites in biological samples.

International Society of Exposure Sciences

The International Society of Exposure Sciences (ISES; http://www.isesweb.org/), formerly the International Society of Exposure Analysis—ISEA, promotes "the use of exposure in the fields of public, occupational, and environmental health." The ISEA was established in 1989 to foster and advance the science of exposure analysis related to environmental contaminants, both for human populations and ecosystems. The membership promotes communication among all disciplines involved in exposure analysis, recommends exposure analysis approaches to address substantive or methodological concerns, and works to strengthen the impact of exposure assessment on environmental policy. ISES publishes *The Journal of Exposure Science and Environmental Epidemiology (JESEE).*

International Union of Toxicology

The International Union of Toxicology (IUTOX; http://www.iutox.org/) members include SOT and over other societies around the world. IUTOX now has over 50 national/regional society members representing over 20,000 toxicologists from industry, academia, and government as members. Its purpose is to foster international scientific cooperation among national and other groups of toxicologists and promote worldwide acquisition, dissemination, and utilization of toxicology knowledge. The IUTOX website provides links to the sites of all available member societies (http://www.iutox.org/members.asp).

Society for Risk Analysis

The Society for Risk Analysis (SRA; http://www.sra.org) is a multidisciplinary and international society addressing all

TABLE 13.2
Online Toxicology Newsletters

American Academy of Clinical Toxicology (AACT)	http://www.clintox.org/AACTionNews.cfm
Biological Effects of Low Level Exposures (BELLE)	http://www.belleonline.com/
The Hamner Institutes for Health Sciences Newsletter	http://www.thehamner.org/
Greenfacts.org	http://greenfacts.org/
National Institute of Occupational Safety and Health (NIOSH) E-News	http://www.cdc.gov/niosh/enews/
SETAC Globe and SETAC News	http://www.setac.org/globe/globe-archive.html
Society for Risk Analysis (SRA) Risk Newsletter	http://www.sra.org/newsletter.php
Society of Toxicology (SOT) Communiqué Newsletter	http://www.toxicology.org/ms/communique.asp
Trends in Risk Science and Application	http://www.gradientcorp.com/publications/trends.php

areas of risk analysis related to human health and the environment, including risk assessment, risk characterization, risk communication, risk management, and policy relating to risk. SRA publishes a peer-reviewed journal, *Risk Analysis*, which provides a focal point for new developments in risk analysis for scientists from a wide range of disciplines. The journal covers health risks; engineering, mathematical, and theoretical aspects of risks; and social and psychological aspects of risk such as risk perception, acceptability, economics, and ethics. The society's newsletter and annual meeting abstracts are available from its website.

Society of Environmental Toxicology and Chemistry

The Society of Environmental Toxicology and Chemistry (SETAC; http://www.setac.org/) is a worldwide professional society that promotes the advancement and application of scientific research related to contaminants and other stressors in the environment. SETAC publishes two journals: *Environmental Toxicology and Chemistry* and *Integrated Environmental Assessment and Management* and several newsletters. Its website provides access to education materials such as topical webinars, a career center, workshop reports, and technical issue papers.

Society of Forensic Toxicologists

The Society of Forensic Toxicologists (SOFT; http://www.soft-tox.org/) is a member organization of practicing forensic toxicologists and others organized to promote and develop forensic toxicology. Forensic toxicologists analyze biological fluids and tissues for drugs and/or poisons and interpret the analytical results in a judicial context. SOFT sponsors a certification program for its members and its website contains annual meeting information, education and training, research, employment opportunities, publications, and related links.

Society of Toxicology

The SOT (http://www.toxicology.org/) is perhaps the best-known professional society associated with toxicology and has a global, albeit mostly U.S., membership. SOT is an organization of scientists who practice toxicology in many areas. The society holds annual meetings, publishes a journal and a member newsletter, and sponsors continuing education courses. The society addresses toxicological issues through several of its specialty sections. It maintains an excellent collection of toxicology-related websites of various organizations, along with other sites of interest for toxicologists and for people considering a career in, or just seeking information about, toxicology (http://www.toxicology.org/AI/CRAD/careerguide.asp).

Trade Associations

Many trade associations are active in toxicology efforts and evaluations. Listed in the following are a number of broad chemical trade associations. There are many other more specialized groups that represent specific areas.

American Chemistry Council

The American Chemistry Council (ACC; http://www.americanchemistry.com/), formerly the Chemical Manufacturers' Association, is the voice of the U.S. chemical industry. The ACC represents the chemical industry on public policy issues; coordinates the industry's research and testing programs (e.g., the Long-Range Research Initiative [LRI]); and administers the industry's environmental, health, and safety performance improvement initiative, known as Responsible Care®. Members include corporations in the chemical and chemical-using (e.g., consumer product) industries.

British Industrial Biological Research Association

The members of British Industrial Biological Research Association (BIBRA; http://www.bibra-information.co.uk) include British chemical manufacturers. Its activities include the development of summaries (toxicology profiles) of the data for numerous chemicals.

European Chemical Industry Council

The European Chemical Industry Council (Cefic; http://www.cefic.org/) is both the forum and the voice of the European chemical industry. It represents, directly or indirectly, chemical companies that account for nearly a third of the world chemical production. It has numerous working groups related to chemical safety and the regulation of chemicals, and sponsors research related to toxicology and risk assessment. Cefic has developed guidance documents and tools to assist companies in implementing compliance with the European chemicals legislation, Registration, Evaluation, Authorization and Restriction of Chemical Substances (REACH), and makes these available free of charge on the website.

European Centre for Ecotoxicology and Toxicology of Chemicals

The focus of European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC; http://www.ecetoc.org/) includes the manufacture, processing, handling, and use of chemicals. ECETOC also cooperates with governmental agencies and other organizations concerned with the effects of chemicals on health and the environment.

International Council of Chemical Associations

The International Council of Chemical Associations (ICCA; http://www.icca-chem.org/) is the global voice of the chemical industry, representing chemical manufacturers and producers. It accounts for more than 75% of chemical manufacturing operations. ICCA promotes and coordinates Responsible Care® and other voluntary chemical industry initiatives and has a central role in the exchange of information within the international industry and in the development of position statements on matters of policy. It is also the main channel of communication between the industry and various international organizations that are concerned with health, environment, and trade-related issues.

Government Organizations

Government organizations serving as toxicology information resources are numerous and include federal, state/provisional, and local government agencies, as well as international bodies representing or aiding multiple countries. Relevant toxicological resources are widespread.[4]

Environment Canada and Health Canada

The Canadian Environmental Protection Act (CEPA) of 1999 requires the establishment of a Priority Substances List (PSL) to identify substances that are of priority for assessing whether environmental exposure to them poses a risk to the health of Canadians or to the environment. Over 60 priority substances have been evaluated, and some of the assessment documents containing tolerable intakes, tolerable concentrations, and tumorigenic doses and concentrations for these substances are available through their website (http://www.ec.gc.ca/lcpe-cepa/default.asp?lang=En&n=C6C230D5-1). Health Canada also maintains a Domestic Substances List (DSL), which contains over 23,000 chemical substances that are used, imported, or manufactured in Canada for commercial purposes at quantities greater than 100 kg per year (http://www.hc-sc.gc.ca/ewh-semt/contaminants/existsub/index-eng.php).

European Union, European Commission, Joint Research Centre, European Chemicals Agency

European Chemicals Agency (ECHA) is the driving force among European regulatory authorities in implementing the EU's REACH, which was implemented in 2007. REACH's primary aim is "to ensure a high level of protection of human health and the environment." REACH places the burden of proof on industry, which has to collect or generate the data necessary to ensure the safe use of chemicals. ECHA helps companies to comply with the legislation, advances the safe use of chemicals, provides information on chemicals, and addresses chemicals of concern. Chemical information is publicly available through ECHA's central database (http://www.echa.europa.eu/), which houses the International Uniform Chemical Information Database (IUCLID).

The European Commission's Joint Research Centre also includes the Institute for Health and Consumer Protection (IHCP). The mission of the IHCP is to "provide scientific and technical support in the areas of food, consumer products, chemicals and public health" (http://ihcp.jrc.ec.europa.eu/home). IHCP houses the European Union System for the Evaluation of Substances (EUSES), an integrated modeling system that uses a single framework for comparing the potential risks of different substances released to multiple environmental media (water, soil, and air) and multiple human exposure pathways (inhalation, ingestion, and dermal). EUSES can be used for indirect human exposures and for consumer product and worker exposures. IHCP maintains a number of databases, including the European Indoor Air Monitoring and Exposure Assessment Project (AIRMEX), European Chemical Substances Information System (ESIS), and the European Database of EXport and IMport of certain dangerous chemicals (EDEXIM). IHCP also houses the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM is "the reference centre, at an international level, for the development, scientific and regulatory acceptance of alternative testing methods aimed at replacing, reducing or refining the use of laboratory animals and to be applied in different fields in the biomedical sciences."

National Institute of Public Health and the Environment, the Netherlands

The knowledge and expertise of National Institute of Public Health and the Environment (RIVM; http://www.rivm.nl/en/) in the fields of health, nutrition, and environmental protection (including the conduct of research, monitoring, modeling, and risk assessment) are used primarily for advising the Dutch government. As an example of its efforts, RIVM develops human toxicological risk limits (maximum permissible risks or MPRs). Reports for a variety of chemicals based on chemical assessments are compiled in the framework of the Dutch governmental program on risks in relation to soil quality. These MPR values are published in RIVM reports, many of which can be downloaded from the Publications section of this site.

U.S. Centers for Disease Control and Prevention, Department of Health and Human Services

The Agency for Toxic Substances and Disease Registry (ATSDR; http://www.atsdr.cdc.gov) is an agency within the CDC. Its functions include public health assessments of waste sites, health consultations concerning specific hazardous substances, health surveillance and registries, response to emergency releases of hazardous substances, applied research in support of public health assessments, information development and dissemination, and education and training concerning hazardous substances. ATSDR develops toxicological profiles (http://www.atsdr.cdc.gov/toxprofiles/index.asp) for hazardous substances found at National Priority List sites and for the Department of Defense and the Department of Energy (DOE) for substances related to federal sites. Within these documents, ATSDR develops minimal risk values (MRLs; see http://www.atsdr.cdc.gov/mrls/index.asp). Also available is ATSDR's Tox-FAQs™ (http://www.atsdr.cdc.gov/toxfaqs/index.asp), a series of summaries about hazardous substances and their health effects. Information for this series is excerpted from the ATSDR Toxicological Profiles and Public Health Statements.

The NIOSH (http://www.cdc.gov/niosh/) is another part of the CDC and is the federal agency responsible for conducting research and making recommendations for the prevention of work-related injury and illness. Its objectives include the conduct of research to reduce work-related illnesses and injuries, the promotion of safe and healthy workplaces through interventions, recommendations and capacity building, and the enhancement of global workplace safety and health through international collaborations. The NIOSH website provides

access to information on numerous workplace health and safety topics, including chemicals, hazards and exposures, emergency preparedness and response, safety and prevention, and on emerging issues such as nanotechnology.

U.S. Consumer Product Safety Commission

The Consumer Product Safety Commission (CPSC; http://www.cpsc.gov/) protects the U.S. public against risk of injury or harm from consumer products. Among its efforts, the CPSC evaluates and develops standards and guidelines for safety issues and regulations for labeling and packaging. It has also performed exposure-related research as part of the development of risk assessments for exposures to various chemicals in consumer products.

U.S. Department of Energy

The DOE's Risk Assessment Information System (RAIS; http:/rais.ornl.gov/) is a website developed to provide a service-oriented environmental risk assessment expert system. RAIS provides tools for performing basic risk assessment activities, such as preliminary remediation goals, toxicity values and profiles (including the EPA's Integrated Risk Information System [IRIS] and HEAST), federal and state guidelines, human health risk models, and ecological benchmarks.

U.S. Environmental Protection Agency

The mission of the EPA, founded in 1970, is to protect human health and the environment. Several of its components are of particular relevance to toxicology. The National Center for Environmental Assessment (NCEA; http://www.epa.gov/ncea/), for example, serves as the EPA national resource center for the overall process of human health and ecological risk assessments, including the integration of hazard, dose–response, and exposure data and models to produce risk characterizations. The Office of Pollution Prevention and Toxics (OPPT; http://www.epa.gov/oppt/) promotes pollution prevention, safer chemicals, risk reduction, and public understanding of risks. Its Office of Research and Development (ORD; http://www.epa.gov/ord/) is the scientific research arm of EPA. ORD's research helps provide the solid underpinning of science and technology for EPA.

Numerous databases accessible through the EPA's website present a wealth of relevant and reliable data.[5] The IRIS (http://www.epa.gov/iris) is the EPA's consensus database of information on human health effects that may result from exposure to various chemicals found in the environment, including values such as oral reference doses (RfDs) and inhalation reference concentrations (RfCs) for noncarcinogenic health effects, as well as oral slope factors and oral and inhalation unit risks for carcinogenic effects. The Toxics Release Inventory (TRI; http://www.epa.gov/tri) is a database containing information focused on the estimated numbers of pounds of certain toxic chemicals released into the environment, augmented by source reduction and recycling data. Both IRIS and TRI are accessible via the EPA's website and the NLM's toxicology data network (TOXNET®) system (http://toxnet.nlm.nih.gov). Recent informatics efforts in genomics at EPA and National Institute of Environmental Health Sciences (NIEHS) are discussed further later in this chapter.

U.S. Food and Drug Administration

The FDA (http://www.fda.gov/) is an agency consisting of a number of centers within the Department of Health and Human Services with the mission of protecting the public health of Americans by helping safe and effective products reach the market in a timely way, monitoring products for continued safety after they are in use, and helping the public get accurate, science-based information. The Center for Biologics Evaluation and Research (CBER; http://www.fda.gov/BiologicsBloodVaccines/) regulates biological products for disease prevention and treatment. The Center for Devices and Radiological Health (CDRH; http://www.fda.gov/Radiation-EmittingProducts/) ensures that new medical devices are safe and effective before they are marketed; monitors devices throughout the product life cycle, including a nationwide postmarket surveillance system; and ensures that radiation-emitting products (e.g., microwave ovens, television sets, cell phones, and laser products) meet radiation safety standards. The Center for Drug Evaluation and Research (CDER; http://www.fda.gov/ForIndustry/FDAeSubmitter/ucm274477.htm) evaluates all new prescription and over-the-counter drugs before they are sold and serves as a consumer watchdog for marketed drugs to be sure that they continue to meet the highest standards. The Center for Food Safety and Applied Nutrition (CFSAN; http://www.fda.gov/Food/) is responsible for the safety of the entire food supply, except for meat, poultry, and some egg products that are regulated by the U.S. Department of Agriculture. In addition, CFSAN has developed rapid methods for the detection of microbial and viral food contaminants and works closely with public and private sector partners to operate systems for rapid identification and control of outbreaks of foodborne diseases. The Center for Veterinary Medicine (CVM; http://www.fda.gov/AnimalVeterinary/) helps ensure that animal feed products are safe and evaluates the safety and effectiveness of drugs used to treat companion animals (e.g., dogs, cats, and horses). The mission of the National Center for Toxicological Research (NCTR; http://www.fda.gov/AboutFDA/CentersOffices/OC/OfficeofScientificandMedicalPrograms/NCTR/) is to conduct peer-reviewed scientific research that supports and anticipates the FDA's current and future regulatory needs. This involves fundamental and applied research specifically designed to define biological mechanisms of action underlying the toxicity of products regulated by the FDA. This research is aimed at understanding critical biological events in the expression of toxicity and at developing methods to improve the assessment of human exposure, susceptibility, and risk.

U.S. National Institutes of Health

The major information component of the NIH is the NLM. The TOXNET system (http://toxnet.nlm.nih.gov) is a group of databases managed by the NLM's Toxicology

and Environmental Health Information Program (TEHIP), situated within its Specialized Information Services (SIS) Division. The data banks and bibliographic files are built, maintained, and funded by several federal agencies. Its databases are described later in this chapter. Furthermore, the SIS Division of NLM provides online toxicology training via its Toxicology Tutor I, II, and III (http://sis.nlm.nih.gov/enviro/toxtutor.html) and provides extensive information about resources related to toxicology and environmental health education (http://sis.nlm.nih.gov/enviro/envirohealthlinks.html), including academic program directories, continuing education and tutorials, distance learning, etc.

Another NIH institute particularly relevant to toxicology is the NIEHS (http://www.niehs.nih.gov). Its website links to resources from the National Toxicology Program (NTP). The NTP consists of the relevant toxicology activities of the NIEHS, NIOSH, and NCTR. The goals of the NTP are to provide toxicological evaluations on substances of public health concern; develop and validate test methods; develop approaches and generate data that strengthen the scientific basis for risk assessments; and communicate program plans and results to all stakeholders including governmental agencies, the medical and scientific communities, and the public. The NTP website also provides access to NTP testing information and study results, the NTP Report on Carcinogens (RoC), chemical health and safety information, special reports, and announcements, as well as links to the websites for the NTP Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Children's Environmental Health Network (CEHN).

Other Organizations

Readers should take note that, as with all the lists in this chapter, the compilation of organizations is highly selective with regard to a number of variables, including geography. There are important toxicology-related groups and activities (e.g., in Asia, Africa, and Latin America) that, because of space limitations, could not be included here.

University-Affiliated Organizations

Extension Toxicology Network

Extension Toxicology Network (EXTOXNET; http://ace.orst.edu/info/extoxnet) is an effort of the University of California at Davis, Oregon State University, Michigan State University, Cornell University, and the University of Idaho. EXTOXNET provides information on pesticides, discussion of concepts in toxicology and environmental chemistry, and fact sheets. This information has been developed by toxicologists and chemists within the Extension Service of these universities with the goal of developing unbiased information in a format understandable by the nonexpert.

Harvard Center for Risk Analysis

The Harvard Center for Risk Analysis (HCRA; http://www.hcra.harvard.edu) attempts to provide a big-picture overview of public health by comparing and ranking a wide range of hazards; analyzing the results of dealing with those hazards in various ways; and developing sound scientific data identifying policy choices that are most likely to achieve the greatest health, safety, and environmental benefits with the most efficient use of finite resources. The center's current research programs focus on the areas of risk characterization and prioritization, improving exposure assessment, understanding dose–response relationships, and valuing risk reductions. The center has a newsletter entitled *Risk in Perspective*, which discusses results of research projects.

National Pesticide Information Center

The National Pesticide Information Center (NPIC; http://npic.orst.edu), a cooperative agreement between Oregon State University and the U.S. EPA, provides objective, science-based information about pesticides and pesticide-related topics to enable people to make informed decisions about pesticides and their use. NPIC operates a toll-free, bilingual telephone service to the public and professionals in the United States, Puerto Rico and the Virgin Islands, and maintains an informational website. NPIC analyzes trends for pesticide misuse, labeling issues, and risks to humans, animals, and the environment, and reports its findings back to EPA.

The Johns Hopkins University

Altweb (http://altweb.jhsph.edu/) is composed of a diverse group of organizations and individuals, many of whom serve in the Altweb Project Team and are interested in alternatives to animal testing. A number of these organizations maintain their own websites that provide key links from and to Altweb. The website is management by The Johns Hopkins University Center for Alternatives to Animal Testing (CAAT). The intent of Altweb is to be the online clearinghouse for resources, information, and news about alternatives to animal testing and to serve as the most comprehensive resource on animal alternatives for scientists, educators, veterinarians, and individuals throughout the world. This effort is designed to bring together government agencies, the academic community, animal protection groups, and private industry to encourage the use of alternative methods.

International Organizations

International Agency for Research on Cancer

The International Agency for Research on Cancer (IARC; http://www.iarc.fr/) is part of the World Health Organization, and its mission is to coordinate and conduct research on the causes of human cancer and the mechanisms of carcinogenesis, as well as to develop scientific strategies for cancer control. IARC is involved in both epidemiological and laboratory research and disseminates information through publications, meetings, courses, and fellowships. Cancer databases and other resources at IARC include the Monographs Database (a complete list of agents, mixtures, and exposures, all evaluated with their classifications) and the Cancer Epidemiology Database among others.

International Consumer Products Health and Safety Organization

The International Consumer Products Health and Safety Organization (ICPHSO; http://www.icphso.org/) is dedicated to health and safety issues related to consumer products manufactured and marketed in the global marketplace. ICPHSO includes a broad range of health and safety professionals and interested consumers, and sponsors workshops to inform and educate manufacturers, importers, distributors, retailers, and others of their product safety responsibilities.

International Labour Organization

The International Labour Organization (ILO; http://www.ilo.org/), an agency of the United Nations (UN), is the international organization responsible for drawing up and overseeing international labor standards. Its main aims are to promote rights at work, encourage decent employment opportunities, enhance social protection, and strengthen dialog on work-related issues. Of particular interest to toxicologists is its International Occupational Safety and Health Information Centre (CIS), the knowledge management hub of its Programme on Safety and Health at Work and the Environment (SafeWork; http://www.ilo.org/safework/lang--en/index.htm). The CIS compiles occupational safety and health-related information and ensures worldwide access to it, creates and disseminates ILO publications (including the ILO Encyclopedia and related products), and maintains an international network of national and international occupational safety and health institutions.

International Programme on Chemical Safety

The International Programme on Chemical Safety (IPCS; http://www.who.int/ipcs/en/) works to establish the scientific basis for the sound management of chemicals, and to strengthen national capabilities and capacities for chemical safety. IPCS aims at promoting the development, harmonization, and use of generally acceptable, scientifically sound methodologies for the evaluation of risks to human health and the environment from exposure to chemicals. The results of such work enhance mutual acceptance of risk assessment products. It publishes a variety of high-quality, peer-reviewed monographs, such as the Environmental Health Criteria (EHC) documents. The IPCS and Organization for Economic Cooperation and Development (OECD) have developed a framework for cooperation in the field of risk assessment methodologies, which ensures complementarity, mutual support and mutual involvement in the projects conducted by each organization.

The IPCS Harmonization Project tries to harmonize approaches to the assessment of risk from exposure to chemicals by increasing understanding and developing basic principles and guidance on specific chemical risk assessment issues. Harmonization enables efficient use of resources and consistency among assessments.

IPCS INCHEM (http://www.inchem.org) provides rapid access to internationally peer-reviewed information on chemicals commonly used throughout the world, which may also occur as contaminants in the environment and food. It consolidates information from a number of intergovernmental organizations whose goal is to assist in the sound management of chemicals. Among the documents offered are the Concise International Chemical Assessment Documents (CICADs), the International Chemical Safety Cards, and Poisons Information Monographs.

Organization for Economic Cooperation and Development

The mission of the OECD, simply put, is to promote policies that will improve the economic and social well-being of people around the world. Its program on chemical safety and biosafety, falling under the OECD's environment initiatives, is to protect human health and the environment through improving chemical safety; to make chemical control policies more transparent and save resources for government and industry; and to prevent unnecessary distortions in the trade of chemicals and chemical products. It also addresses pesticides, biocides, chemical accidents, and biotechnology.

Strategic Approach to International Chemicals Management

The Strategic Approach to International Chemicals Management (SAICM; http://www.chem.unep.ch/saicm), adopted by the International Conference on Chemicals Management (ICCM) in 2006 in Dubai, is a policy framework to foster the sound management of chemicals. It supports the goal agreed upon at the 2002 Johannesburg World Summit on Sustainable Development of ensuring that, by the year 2020, chemicals are produced and used in ways that minimize significant adverse impacts on the environment and human health. ICCM2 was held in 2009 in Geneva and ICCM3 in 2012 in Nairobi. SAICM's Quick Start Programme (QSP) includes a voluntary, time-limited trust fund, administered by the UN Environment Programme, and multilateral, bilateral, and other forms of cooperation.

Nongovernmental Organizations

The Hamner Institute

The Hamner Institute for Health Sciences (http://www.thehamner.org/about-the-hamner/) is an independent, nonprofit organization whose vision is "to be a global leader in translational life sciences research, innovation, and training." The Hamner is located in Research Triangle Park, North Carolina, USA focusing on translational biomedical research using a collaborative and cross-disciplinary approach. The Hamner scientists partner with academic institutions, industries, and governments from around the world to perform innovative research and development in environmental health and chemical risk assessment as well as biopharmaceutical safety, oncology, and nanotoxicology.

Health Effects Institute

The Health Effects Institute (HEI; http://www.healtheffects.org/) is an independent, nonprofit corporation chartered in

1980 to provide high-quality, impartial, and relevant science on the health effects of pollutants from motor vehicles and from other sources in the environment. Primarily supported by the U.S. EPA and the motor vehicle industry, HEI has funded hundreds of studies and published over 100 research reports and several special reports that provide important research findings on the health effects of a variety of pollutants, including carbon monoxide, methanol and aldehydes, nitrogen oxides, diesel exhaust, ozone, particulate matter, and air toxics.

International Life Sciences Institute

The International Life Sciences Institute (ILSI; http://rsi.ilsi.org/) is a nonprofit, worldwide scientific research foundation seeking to improve the well-being of the general public through the pursuit of sound and balanced science. Its goal is to further the understanding of scientific issues relating to nutrition, food safety, toxicology, risk assessment, and the environment.

Toxicology Excellence for Risk Assessment

Toxicology Excellence for Risk Assessment (TERA; http://www.tera.org) is an independent nonprofit research and education organization dedicated to the best use of toxicity data in risk assessment. TERA develops risk values; improves methods for human health risk assessment through their research program; sponsors expert review of risk assessments, risk values, methods, and research through their independent peer review and peer consultation programs; provides technical support, training courses, and risk communication to diverse groups through their education program; and compiles and distributes peer-reviewed risk values to the international user community through the International Toxicity Estimates for Risk (ITER) database, described later in this chapter.

Databases and Websites

The Internet plays a vital role in the provision of information resources. We have access to sources, including full-text journal articles, online that in the past were only available by physically visiting a library. The following websites and databases are intended to serve as a selective list and do not approach the scope of a comprehensive compilation.

Aggregated Computational Toxicology Online Resource

(http://actor.epa.gov/actor/faces/ACToRHome.jsp)—Aggregated Computational Toxicology Online Resource (ACToR) is the U.S. EPA's online warehouse of publicly available chemical toxicity data. ACToR collects data from over 650 public sources on over 500,000 chemicals.[6] The database can be searched by chemical name, registry number, and chemical structure. Queries in the ACToR database also include other U.S. EPA databases such as ToxRefDB (animal toxicity testing results), DSSTox (public forum for chemical structure files), ToxCastDB (ToxCast chemical testing results), and ExpoCastDB (exposure data summaries) in the search strategy.

CAL/EPA–OEHHA Toxicity Criteria Database

(http://www.oehha.ca.gov/risk/chemicaldb)—This toxicity criteria database is maintained by the California Office of Environmental Health Hazard Assessment (OEHHA). The database provides California public health goals (PHGs), acute and chronic reference exposure levels (RELs), cancer classification, cancer potency values, and Proposition 65 No Significant Risk Levels (NSRLs) and Maximum Allowable Daily Level (MADL).

Canadian Centre for Occupational Health and Safety

(http://www.ccohs.ca/)—The Canadian Centre for Occupational Health and Safety (CCOHS) promotes a safe and healthy working environment by providing e-courses, publications, fact sheets, pocket guides, Workplace Hazardous Materials Information System (WHMIS) criteria, and web information service databases. The Web databases include Registry of Toxic Effects of Chemical Substances (RTECS®), MSDS plus CHEMINFO, CHEMpendium, and Occupational Safety and Health (OSH) references and legislation regulations. Some of the databases are restricted to subscribers.

Chemical Effects in Biological Systems

(http://www.niehs.nih.gov/research/resources/databases/cebs)—Chemical Effects in Biological Systems (CEBS) is an integrated public repository for toxicogenomics data, including the study design and timeline, clinical chemistry and histopathology findings, and microarray and proteomics data. CEBS contains data derived from studies of chemicals and of genetic alterations, and is compatible with clinical and environmental studies.[7]

ProQuest Dialog™

(http://www.dialog.com)—Dialog is a vast database collection in science, engineering, business, and intellectual property with full-text access to most of the search results. The database offers comprehensive, global coverage of biomedical research, chemicals, computer science, energy and environment, health industry, mechanical and civil engineering, medical practice, medical devices, pharmaceuticals, food science, software, therapy and treatment breakthroughs, drug interactions, and toxicology, as well as many subject areas. Dialog database content is available in a desktop version through Telnet and Windows® software or through several web browser formats. Dialog is available by subscription or by transactional fees with an access fee through the DialogWeb browser. The following are examples of a few of the databases that can be searched within Dialog:

- *BIOSIS Previews*® is a database that constitutes the major English-language service providing comprehensive worldwide coverage of research in the biological and biomedical sciences. This database includes journal articles, indexes, monographs, meetings, reviews, book chapters, and some patents and is also a key resource for bioengineering and biotechnology researchers.

- *EMBASE*® is bibliographic database that provides comprehensive coverage of the literature on human medicine and related disciplines. The database emphasizes the pharmacological effects of drugs and chemicals.
- *SciSearch*®, a cited reference science database, is an international, multidisciplinary index to the literature of science, technology, biomedicine, and related disciplines produced by Thomson Reuters. It contains all of the records published in the *Science Citation Index* (SCI®), plus additional records in engineering technology, physical sciences, agriculture, biology, environmental sciences, clinical medicine, and life sciences.
- *MEDLINE* is a bibliographic database produced by the U.S. NLM. This database covers the field of medicine including dentistry, veterinary medicine, and medical psychology. It also covers other related fields such as clinical and preclinical medicine, anatomy, pharmacology, toxicology, genetics, microbiology, pathology, environmental health, occupational medicine, psychology, biomedical technology, health planning and administration, and space life science.

eChemPortal

(http://www.echemportal.org/echemportal/index?pageID=0&request_locale=en)—The eChemPortal database is maintained and updated by the OECD. This free database is a global portal to information on chemical substances including physical–chemical properties, ecotoxicity, environmental fate and behavior, and toxicity. Users can search 20+ different databases by these properties or by chemical identity. eChemPortal evolved from the EXICHEM database, which is no longer updated.

European Chemical Substances Information System

(http://esis.jrc.ec.europa.eu/)—This database is a wealth of information for European chemical information. The following areas can be searched: European Inventory of Existing Commercial Substances (EINECS), which contains general chemical information such as CAS Registry Numbers, EINECS numbers, substance names, chemical formulas, structure, classification, and labeling (risk and safety phrases, danger, etc.); European List of Notified Chemical Substances (ELINCS); high production volume chemicals (HPVCs) and low production volume chemicals (LPVCs), including EU producer/importer lists; IUCLID Chemical Data Sheets (in Adobe and OECD format); classification and labeling in accordance with the Globally Harmonized System (CLP/GHS); priority lists; and risk assessment processes and tracking systems in relation to Council Regulation (EEC) 793/93, also known as Online EUropean Risk Assessment Tracking System (ORATS).

ExPub

(http://www.expub.com/Default.aspx)—ExPub consists of more than 130 databases geared toward environmental health and safety professionals and others who need to understand the human health hazards associated with chronic and acute exposures to chemicals. ExPub provides users with access to over 2 million documents covering over 400,000 chemicals. It contains a blend of licensed content, public domain content, and gray literature with links to full-text documents when available. ExPub also includes domestic and international regulatory lists. The ExPub database is only available by subscription from Expert Publishing.

Haz-Map®

(http://hazmap.nlm.nih.gov/)—This occupational toxicology database is designed primarily for health and safety professionals but also for consumers seeking information about the health effects of exposure to chemicals at work. It contains approximately 1000 chemicals or biological agents and links jobs and hazardous tasks with occupational diseases and their symptoms. This association indicates an increased risk for significant exposure and subsequent disease.

ILPI–MSDS (Finding Material Safety Data Sheets on the Internet)

(http://www.ilpi.com/msds/index.html)—This website is a comprehensive MSDS resource maintained by Interactive Learning Paradigms, Inc. (ILPI). The website includes everything from links to material safety data sheets to OSHA regulations and interpretations.

INCHEM

(http://www.inchem.org/)—INCHEM is a website with search capabilities to provide easy access to international chemical safety information from various organizations. The website is housed by the IPCS in cooperation with CCOHS. CICADs, EHC Monographs, IARC Summaries, Joint Expert Committee on Food Additives (JECFA)—Monographs and Evaluations, Joint Meeting on Pesticide Residues (JMPR), and Screening Information Data Set (SIDS) for HPVCs are all searchable from this website.

National Service Center for Environmental Publications

(http://www.epa.gov/ncepihom/index.htm)—The National Service Center for Environmental Publications (NSCEP) maintains and distributes EPA publications in hardcopy, CD-ROM, and other multimedia formats. The current publication inventory includes over 7,000 titles in hardcopy and 35,000 digital titles that are free to all requestors.

NIOSH Databases and Information Resources

(http://www.cdc.gov/niosh/database.html)—The NIOSH website contains a vast array of occupational health and safety resources. The most popular databases include the International Chemical Safety Cards (ICSC), NIOSH Pocket Guide to Chemical Hazards, Immediately Dangerous to Life and Health (IDLH) values, the Emergency Response Safety and Health Database (ERSH-DB), and NIOSHTIC-2 (a bibliographic database of occupational safety and health publications).

Pesticide Action Network Pesticide Database

(http://www.pesticideinfo.org)—The Pesticide Action Network (PAN) Pesticide Database is a project of Pesticide Action Network North America (PANNA) and has been supported by grants from the EPA and a number of foundations. This database is a diverse array of information on pesticides from many different sources that provides human toxicity (chronic and acute), ecotoxicity, and regulatory information for about 5400 pesticide active ingredients and their transformation products, as well as adjuvants and solvents used in pesticide products. References to data sources are provided.

Quertle

(http://www.quertle.info/)—Quertle is a relationship-driven biomedical searchable database. When using more than one search term, all the terms in the query must be found together in a meaningful way, not just scattered throughout the document. This database makes use of Quertle predefined Power Terms™ to search for categories of objects. Search results can be imported into reference management software.

Regional Screening Levels

(http://www.epa.gov/region09/waste/sfund/prg/index.html)—Regional Screening Levels (RSLs; formerly known as Preliminary Remediation Goals or PRGs) are tools for evaluating and cleaning up contaminated sites in the U.S. Superfund and the Resource Conservation and Recovery Act programs. They are risk-based concentrations derived by using risk assessment guidance from the EPA Superfund program, and are intended to assist risk assessors and others in initial screening-level evaluations of environmental measurements. The former PRGs (U.S. EPA Region 9) were harmonized with similar risk-based levels from U.S. EPA Regions 3 and 6 to create a single table of RSLs. The RSL tables can be downloaded in Excel® or PDF. RSLs are generic; they are calculated without site-specific information, however. The website has an online calculator to recalculate the RSL using site-specific data.

PubMed®

(http://pubmed.gov)—PubMed, a service of the NLM, includes over 15 million citations for biomedical articles back to the 1950s. These citations are from MEDLINE and additional life science journals. PubMed includes links to many sites providing full-text articles and other related resources. PubMed can be searched using medical subject heading (MeSH) terms, author names, title words, text words or phrases, journal names, or any combination of these. PubMed has the ability to instantly find related articles for any citation and search results can be imported into reference manager software.

Registry of Toxic Effects of Chemical Substances

(http://accelrys.com/products/databases/bioactivity/rtecs.html and http://ccinfoweb.ccohs.ca/rtecs/search.html)—The RTECS helps users find critical toxicological information by providing citations on over 169,000 chemical substances from more than 2,500 sources. The RTECS database covers several categories of toxicity data: acute toxicity, tumorigenicity, mutagenicity, skin and eye irritation, reproductive effects, and multiple dose effects. The RTECS® database was previously maintained by the NIOSH and is now licensed through Accelrys, Inc. The database is available from Accelrys as an ASCII text file for in-house use (does not include chemical structures) or through value-added resellers such as CCOHS, Chemical Abstracts Service, Dialog, and SiteHawk.

ScienceDirect

(http://www.sciencedirect.com/)—Since its launch in 1997, ScienceDirect has evolved from a web database of Elsevier journals to one of the world's largest providers of scientific, technical, and medical literature. Elsevier has digitized a remarkable amount of the pre-1995 content as possible, going back to the first volume/issue for many journals. Access to full-text articles is available to subscribers or on a pay-per-view basis, and some university libraries have electronic access. ScienceDirect is also available from SciVerse, a platform to search and access content from all Elsevier databases, including beta applications using one login.

Science.gov

(http://www.science.gov/index.html)—Science.gov is a search engine for government science information and research results. One-click search provides results from 50 databases and 200 million pages of government science information from 14 federal science agencies including but not limited to the Departments of Defense, Energy, Health and Human Services, the EPA, the National Aeronautics and Space Administration (NASA), and the National Science Foundation. Science.gov allows clustering or grouping of results by topic, year, or author, and includes Wikipedia topics and EurekAlert items related to the search. Search results can be imported into reference management software or e-mailed to colleagues.

Scirus

(http://www.scirus.com/srsapp/)—Scirus is a comprehensive science-specific Internet search engine. Driven by the latest search engine technology, Scirus searches over 460 million science-specific web pages.

Scopus

(http://www.scopus.com/)—Scopus is a navigation tool covering the world's largest collection of abstracts, references, and indexes of scientific, technical, and medical literature. Updated daily, it includes the abstracts and cited references of over 19,500 titles from more than 5,000 international publishers, and includes 4.6 million conference papers. Seamless links to full-text articles and other library resources make Scopus quick, easy, and comprehensive. Access is only available through subscribing libraries or institutions. Scopus is also available from SciVerse, a platform to search and access content from all Elsevier databases, including beta applications using one login.

Scorecard

(http://www.scorecard.org/)—Scorecard is an Internet service that provides information about chemical releases in the United States. It provides information about local air pollution, including interactive maps that can be accessed by Zip Code, information on toxic chemicals released by manufacturing facilities, and information about the health risks of air pollution. Scorecard ranks and compares pollution in areas across the United States and profiles 11,200 chemicals and shows locations in which they are used, as well as their hazards. Scorecard integrates over 400 scientific and governmental databases to generate its profiles. Environmental Defense transferred ownership of Scorecard to Green Media Toolshed in 2005.

Toxicology Data Network

(http://toxnet.nlm.nih.gov; as a link on http://sis.nlm.nih.gov)—TOXNET® is a group of databases covering toxicology, hazardous chemicals, environmental health, and related areas. It is managed by the TEHIP in the SIS Division of the NLM. Many TOXNET databases are available in mobile versions. TOXNET provides free access and easy searching of many databases, including:

- Carcinogenic Potency Database (CPDB)—CPDB reports standardized analyses of animal cancer tests on more than 1500 chemicals for the results of 6540 chronic, long-term animal cancer tests that have been conducted since the 1950s and reported in the general published literature or by the National Cancer Institute (NCI) and the NTP. The CPDB was developed at the University of California, Berkeley, and Lawrence Berkeley Laboratory.
- Chemical Carcinogenesis Research Information System (CCRIS)—A scientifically evaluated and fully referenced data bank developed by the NCI but no longer being updated, CCRIS contains some 9500 chemical records with carcinogenicity, mutagenicity, tumor promotion, and tumor inhibition test results. Data are derived from studies cited in primary journals, current awareness tools, NCI reports, and other special sources. Test results have been reviewed by experts in carcinogenesis and mutagenesis.
- ChemIDplus—A database providing access to structure and nomenclature authority databases used for the identification of chemical substances cited in NLM databases, ChemIDplus contains over 384,000 chemical records, of which over 300,000 include chemical structures. ChemIDplus is searchable by name, synonym, CAS Registry Number, molecular formula, classification code, locator code, and structure.
- Comparative Toxicogenomics Database (CTD)©—The CTD is a compilation of manually curated data describing chemical–gene/protein interactions and chemical–disease and gene–disease relationships. The results help elucidate the molecular mechanisms underlying variable susceptibility and environmentally influenced diseases. The CTD was developed by North Carolina State University (NCSU) scientists located at NCSU and Mount Desert Island Biological Laboratory.
- Developmental and Reproductive Toxicology/Environmental Teratology Information Center (DART®/ETIC)—A bibliographic database covering literature on reproductive and developmental toxicology. DART/ETIC is no longer funded by the government agencies that created it but is still searchable as a distinct entity as well as a part of TOXLINE®.
- GENE-TOX (Genetic Toxicology)—This legacy toxicology database was created by the EPA and contains genetic toxicology test results on over 3000 chemicals. Selected literature was reviewed by scientific experts for each of the test systems under evaluation.
- Hazardous Substances Data Bank (HSDB®)—This factual database focuses on the toxicology of over 5000 potentially hazardous chemicals, but provides information in the areas of emergency handling procedures, industrial hygiene, environmental fate, human exposure, detection methods, and regulatory requirements. The data are fully referenced and peer reviewed by a scientific review panel composed of expert scientists.
- IRIS; also at http://www.epa.gov/iris—This database is managed by the NCEA of the EPA and contains carcinogenic and noncarcinogenic health-risk information on over 550 chemicals. These chemical files contain descriptive and quantitative information about oral RfDs and inhalation RfCs for chronic noncarcinogenic health effects and hazard identification, as well as oral slope factors and oral and inhalation unit risks for carcinogenic effects. IRIS risk assessment data have been scientifically reviewed by EPA scientists and represent EPA consensus. IRIS is widely used in the EPA for risk-based decision making.
- ITER; also at http://www.tera.org/iter—This database contains human health risk values and cancer classifications for over 680 chemicals of environmental concern from multiple organizations worldwide in support of human health risk assessments. It is compiled by TERA and provides a comparison of international risk assessment information in a side-by-side tabular format. ITER explains differences in risk values derived by different organizations and contains links to the source documentation. It is the only database that includes risk information from independent parties whose risk values have undergone peer review through TERA's ITER Peer Review Program. TERA also helped develop a companion database called RiskIE, available through

the Alliance for Risk Assessment (http://www.allianceforrisk.org/RiskIE.htm), which contains notifications about a variety of human health risk assessment projects that are underway or recently completed.
- LactMed (Drugs and Lactation Database)—Containing over 500 records, LactMed is a peer-reviewed and fully referenced database of drugs, including complementary and alternative medicines, to which breastfeeding mothers may be exposed. Among the data included are maternal and infant levels of drugs, possible effects on breastfed infants and on lactation, and alternative drugs to consider. A mobile device application is now available for the iphone/ipod or android systems.
- TRI—This series of databases describes the releases of toxic chemicals into the environment annually from 1987 to the most recently compiled reporting year. TRI is mandated by the Emergency Planning and Community Right-to-Know Act and is based on data submitted to the EPA from industrial facilities throughout the United States. Information is included on over 600 chemicals and chemical categories. Pollution prevention data are also reported by each facility for each chemical.
- TOXLINE®—A bibliographic database providing comprehensive coverage of biochemical, pharmacological, physiological, and toxicological effects of drugs and other chemicals. Its subfiles include literature from PubMed/MEDLINE, DART, the International Labour Office (CIS) and more. TOXLINE contains over 4 million citations, almost all with abstracts and/or index terms and CAS Registry Numbers.

The TOXNET *Multi-Database* option allows for simultaneous searching of HSDB, IRIS, ITER, CCRIS, GENETOX, CTD, and LactMed.

Other Resources

The NLM's Environmental Health and Toxicology pages (http://sis.nlm.nih.gov/enviro.html) contain links to additional relevant databases in areas such as occupational safety and health, household products, and dietary supplements. LiverTox and PillBox are two additional databases relevant to toxicology. The former provides up-to-date, accurate, and easily accessed information on the diagnosis, cause, frequency, patterns, and management of liver injury attributable to prescription and nonprescription medications, herbals, and dietary supplements. PillBox enables rapid identification of unknown solid-dosage medications (tablets/capsules) based on physical characteristics and high-resolution images. ToxLearn is the successor to ToxTutor, an online tutorial that trains users in the basic principles of toxicology. The toxicology glossary of International Union of Pure and Applied Chemistry (IUPAC) is also made available by NLM, as are programs geared toward the public and schoolchildren (e.g., ToxTown®, ToxMystery).

Toxic Substance Control Act Test Submission Database

(http://www.syrres.com/esc/tscats.htm; Also available as a subfile in NLM's TOXLINE database)—Toxic Substance Control Act Test Submission (TSCATS) Database was developed by Syracuse Research Corporation (SRC) for the EPA in 1985. It is a central system for the collection, maintenance, and dissemination of information on unpublished technical reports submitted by industry to the EPA under the Toxic Substances Control Act (TSCA). Studies on over 8000 chemicals are categorized into three broad subject areas (health effects, environmental effects, and environmental fate).

Other Tools

- *ChemBioFinder* (http://www.chemfinder.com/chembiofinder/Forms/Home/ContentArea/Home.aspx) is a compilation of free and subscription databases for chemical information including chemical structures, physical properties, and hyperlinks.
- *Merck Index* (http://themerckindex.chemfinder.com/TheMerckIndex/Forms/Home/ContentArea/Home.aspx) is an encyclopedia of chemicals, drugs, and biologicals that contains more than 10,000 monographs.
- *General search engines* include http://www.altavista.com; http://www.aol.com; http://www.ask.com; http://www.bing.com; http://www.google.com; http://www.lycos.com; http://www.msn.com; and http://www.yahoo.com.
- *Meta-search engines* include http://www.dogpile.com; http://www.info.com; http://www.monstercrawler.com; http://www.webcrawler.com;andhttp://www.yippy.com.

CONDUCTING A LITERATURE SEARCH

An effective literature review is often necessary at the beginning of a research project or thesis. The literature review should convey the available knowledge and ideas that have been established on the topic including the strengths and weaknesses of the knowledge.[8] It should be more than a descriptive list of the available studies or a set of study summaries. One should begin the literature review with a literature search defined by a guiding concept (e.g., the research objective, problem, issue, or thesis topic). After selecting the topic, one must determine the appropriate keywords and databases to use in the search (see the Databases and Websites section). The keywords should be a diverse set of related terms; often a librarian can provide assistance. The search results should be reviewed to determine if the search were wide enough to capture all the relevant material but still narrow enough to exclude most irrelevant material, and the number of results are appropriate for the topic. If there is a problem with any of these areas (i.e., too little data for a common topic, too much data for an obscure topic), the keywords for the search should be revised and the search conducted again until the results are satisfactory. Once the literature search results are adequate, they should be reviewed to

identify relevant literature. All relevant literature should be retrieved, read, and analyzed; additional relevant literature may be identified through the citations of reviewed literature. Organize the literature into sections that present themes or trends rather than list a summary of each study. By organizing the literature, one can critically analyze the data to synthesize and evaluate it in relation to the guiding concept (e.g., the research objective, problem, issue, or thesis topic).

New Developments and Recent Issues

One might think there is no more room for new technologies to expand the scope and power of information organization and retrieval, but it would be a flawed assumption. Periodically, new ways of managing information overload are developed. Some, inevitably, fall by the wayside, but others offer us opportunities to reconsider the way we have been processing and displaying information all along.

Open Access Publishing

It used to be understood, and was rarely questioned, that the fruits of scientific research, in the form of original data and information, were for sale; however, as a crisis evolved in the pricing of serials, globally networked computers became commonplace, and users began particularly to question the ethics of requiring paid subscriptions to access to data generated by taxpayer dollars, a movement to open access to literature began to take hold, despite continued resistance by some publishers.[6] The 2003 Bethesda Statement on Open Access Publishing (http://www.earlham.edu/~peters/fos/bethesda.htm) has defined open access publishing and formulated the following two conditions that must be met for a publication to be considered open access:

1. The author(s) and copyright holder(s) grant(s) to all users a free, irrevocable, worldwide, perpetual right of access to, and a license to copy, use, distribute, transmit, and display the work publicly and to make and distribute derivative works, in any digital medium for any responsible purpose, subject to proper attribution of authorship, as well as the right to make small numbers of printed copies for their personal use.
2. A complete version of the work and all supplemental materials, including a copy of the permission as stated previously, in a suitable standard electronic format is deposited immediately upon initial publication in at least one online repository that is supported by an academic institution, scholarly society, government agency, or other well-established organization that seeks to enable open access, unrestricted distribution, interoperability, and long-term archiving (for the biomedical sciences, PubMed Central [PMC] is such a repository).

Note that (1) open access is a property of individual works, not necessarily journals or publishers and (2) community standards, rather than copyright law, will continue to provide the mechanism for enforcement of proper attribution and responsible use of the published work, as they do now.

BioMed Central

Covering over 236 journals as of May 2012, BioMed Central (http://www.biomedcentral.com/) serves as an independent publishing house supporting free, immediate, and permanent open access to peer-reviewed biomedical research. Some of the journals require a subscription to view additional content, such as reviews. Among the journals relevant to toxicology are *Environmental Health* (http://www.ehjournal.net/), *Particle and Fibre Toxicology* (http://www.particleandfibretoxicology.com/), and *Tobacco Induced Diseases* (http://www.tobaccoinduceddiseases.com).

Public Library of Science

The Public Library of Science (http://www.plos.org) is a nonprofit publisher and advocacy organization and another publication outlet for peer-reviewed open access technical literature. As of May 2012, they have published online and in print, PLoS series in biology, medicine, genetics, computational biology, pathogens, neglected tropical diseases, and *PLoS ONE*, which publishes across the full range of life and health sciences. PLos Currents, an online publication platform for new scientific research, includes sections in disasters, evidence on genomic tests, Huntington Disease, influenza, muscular dystrophy, and Tree of Life.

Open Access Journals (A Small Sampling)

Many important journals are increasingly *free*. See http://freemedicaljournals.com.

Environmental Health Perspectives (EHP; http://ehp03.niehs.nih.gov/home.action) is a highly regarded monthly journal of peer-reviewed research and news, published by the NIEHS and was one of the very first in toxicology to be made available free online.

The Open Toxicology Journal (http://www.benthamscience.com/open/totoxij/index.htm) publishes research articles, reviews, letters, and guest-edited single topic issues in all areas of cellular, molecular, and biochemical toxicology.

ISRN Toxicology (http://www.isrn.com/journals/toxicology/) is a peer-reviewed, open access journal that publishes original research articles, review articles, and clinical studies in all areas of toxicology.

NIH Policy

The U.S. NIH has been an innovator in the implementation of public access. Its policy is designed to accelerate the public's access to published articles resulting from NIH-funded research. It calls on scientists to submit final peer-reviewed journal manuscripts arising from research funded by NIH to PMC upon acceptance for publication. These papers are then accessible to the public on PMC no later than 12 months after publication. PMC, launched in 2000 (http://www.ncbi.nlm.nih.gov/pmc), is a free full-text archive of biomedical and life sciences journal literature at NIH's NLM. Toxicology is

firmly within its scope. In keeping with NLM's legislative mandate to collect and preserve the biomedical literature, PMC serves as a digital counterpart to NLM's extensive print journal collection. As of May 1, 2012, 2.4 million articles were archived in PMC and 1049 journals fully participated in the process.

Global Initiatives

Open access is, by no means, limited to the United States. The UK's Wellcome Trust, for example, is a major source of support for researchers in biomedical research and the medical humanities. They seek to maximize the distribution of papers by these researchers by providing free, online access and require that electronic copies be made available as soon as possible through PMC and UK PubMed Central (UKPMC) and require that it be within 6 months of the journal publisher's official date of final publication.

Mapping and Other Visualizations

The old saw, "A picture is worth a thousand words," still holds true today. Although certain pictures cannot capture the complexity that requires a verbal explanation and need for an additional *thousand words* to be fully understood, pictures, in addition to presenting a visual image of the thing itself, are useful in enhancing or crystallizing concepts that words and numbers alone cannot. TOXMAP, for example, is a tool developed by the NLM to go hand in hand with the EPA's TRI, a database accessible via the NLM's TOXNET system (http://toxnet.nlm.nih.gov). TOXMAP helps users create maps showing where chemicals are released into the air, water, and ground. It identifies the releasing facilities, color-codes release amounts for a single year, and provides multiple-year chemical release trends.

One area in which visual aids have found increasing use is in online toxicology education. Examples from the NLM include ToxMystery (http://toxmystery.nlm.nih.gov), an interactive tutorial that teaches users about common hazards found in the home, and ToxTown (http://toxtown.nlm.nih.gov), which teaches about hazards found in the larger community. Another is the NLM Toxicology Tutorials (http://sis.nlm.nih.gov/enviro/toxtutor.html), in which users can learn about basic toxicologic principles, toxicokinetics, and cellular toxicology. A final example is ToxLearn (http://toxlearn.nlm.gov), a joint project of the NLM's TEHIP and the U.S. SOT. ToxLearn offers a multi-modular toxicology tutorial that is enhanced by a variety of helpful visual graphics.

The area of chemical structure and activity has benefited from the use of visualizations as well. The PubChem database (http://pubchem.ncbi.nlm.nih.gov), from the National Center for Biotechnology Information (NCBI), is a prime example. PubChem provides information on the biological activities of small molecules, and is organized as three linked databases, the PubChem Substance, PubChem Compound, and PubChem BioAssay, which includes the NCBI's protein 3D structure resource.

Chemical regulation is another area in which visual graphics can benefit comprehension of the various reference values that have been developed by federal, state, and professional organizations. For example, the U.S. EPA has responded to a need by federal, state, and international agencies for graphical arrays that compare human inhalation health effect reference values for specific chemicals, allowing comparisons across exposure durations, populations, and intended use. The result is a document titled *Graphical Arrays of Chemical-Specific Health Effect Reference Values for Inhalation Exposures* (http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=211003#Download). This review document is organized in two major sections. Section 1 provides background information on the various reference value systems, purposes, and limitations of the derived health effect reference values, and additional chemical-specific information. Section 2 provides summaries on the available inhalation health effect reference values on a chemical-by-chemical basis, also providing the details of the derivation of these reference values. The key element of each summary is a graphical array that compares the available reference values for each chemical.

Although there are scattered image files of toxicological photomicrographs, poisonous plants and animals, intact organisms with toxic damage, etc., no consolidated online library of toxicological images exists yet, although this would be a very useful project. One already available website of images is Clinical Toxinology Resources (http://www.toxinology.com), which is a searchable database of over 6000 images designed to meet the needs of anyone seeking information on venomous and poisonous organisms and poisonous plants throughout the world. This website was developed by the Toxinology Department of the Women's and Children's Hospital, Adelaide, and the Department of Paediatrics at the University of Adelaide.

Computational Toxicology and Toxicoinformatics

The advent of the *omics* disciplines (genomics, proteomics, and metabolomics) has given a boost to toxicology, and they offer new and still evolving approaches to understand the risks posed by many chemicals to human health and the environment. These new biological disciplines, when in the service of toxicology, have been collectively referred to as *toxicogenomics*. Developing mathematical and computer models to predict toxic outcomes and better understand mechanisms of action is known as *computational toxicology* or, sometimes, *toxicoinformatics*.[9–11]

Several major efforts within U.S. government agencies have been advanced to utilize these technologies. The NIEHS developed and sponsors the Toxicogenomics Research Consortium Cooperative Research Program (www.niehs.nih.gov/research/supported/centers/trc/coop/), as well as Environmental Genomics, Comparative Genomics, and Human Metabolism Groups. The EPA has established a National Center for Computational Toxicology (http://www.epa.gov/comptox/), which is part of a broader Chemical Safety for Sustainability Research Program, and the FDA's NCTR now offers a whole suite of bioinformatics tools for the analysis and integration of

genomics, transcriptomics, proteomics, and metabolomics data (www.fda.gov/ScienceResearch/BioinformaticsTools/).

In addition to the NCTR's suite of bioinformatics tools, there are other tools being developed to make sense of the enormous amounts of toxicology data being generated. The EPA's Distributed Structure-Searchable Toxicity (DSSTox) Database Network (http://www.epa.gov/NHEERL/dsstox/), for example, provides a community forum for publishing standard format, structure-annotated chemical toxicity data files for open public access. One of its goals is to facilitate development of improved models for predicting toxicity based on chemical structure.

Another relevant and evolving database is the CEBS Knowledge Base (http://www.niehs.nih.gov/research/resources/databases/cebs/) from the NIEHS. The stated goals of the CEBS are to

- Create a reference toxicogenomic information system of studies on environmental chemicals/stressors and their effects.
- Develop relational and descriptive compendia on toxicologically important genes, groups of genes, SNPs, mutants, and their functional phenotypes that are relevant to human health and environmental disease.
- Create a toxicogenomics knowledge base to support hypothesis-driven research.

Socialization of Push Technology

In the late 1990s, a new concept of information exchange was introduced, *push* technology. Push technology is a style of Internet-based communication in which the consumer initiates a request for information that is set to be delivered directly as the information becomes available. The first application of this was with the Blackberry and the ability to access e-mail directly on a handheld device. Other sources of push technology followed with such applications as listserv, e-subscriptions to newsletter or journals. More recently, the term *push technology* has been merged with *social media*. This merge has created a vast array of technologies such as RSS feeds, Facebook, Twitter, Wikipedia, podcasts, webcasts, blogs, widgets, and mobile device applications (Apps). It is now possible to receive information anywhere in almost any format desired.

The majority of the newer technologies utilized in social media have been viewed as personal resources, but many organizations have utilized these technologies to better communicate with others outside their organization whether it be colleagues or the general public. Social allows for the bidirectional flow of information among potentially millions of people in real time. Until recently, scientific information was generally provided by a few acknowledged experts in the field, was unidirectional in nature and relatively static.

Today, many professional societies, such as SRA and SOT, are utilizing social media. The SRA recently added social media subcommittee to manage all of its social media outlets, including Twitter and LinkedIn. They are utilizing these venues to communicate news to its members. The SOT utilizes various e-newsletters and an RSS feed for communicating the latest society news and research.

Here are examples of organizations that provide useful toxicology and other health-related information via social media, along with URLs that connect to their social media outlets or specific mobile applications:

National Library of Medicine (NLM):
http://www.nlm.nih.gov/socialmedia/index.html
http://toxnet.nlm.nih.gov/pda/
http://druginfo.nlm.nih.gov/m.drugportal
http://hazmap.nlm.nih.gov/index.php

U.S. Environmental Protection Agency (EPA):
http://www.epa.gov/epahome/socialmedia.html
http://www.epa.gov/greenversations/
http://www.epa.gov/epahome/podcasts.htm
http://www.epa.gov/widgets/

National Institute for Occupational Safety and Health (NIOSH):
http://www.cdc.gov/niosh/programs/cid/media.html
http://www.cdc.gov/niosh/enews/default.html
http://blogs.cdc.gov/niosh-science-blog/

Centers for Disease Control and Prevention (CDC):
http://www.cdc.gov/SocialMedia/
http://www.cdc.gov/mobile/mobileapp.html
http://www2c.cdc.gov/podcasts/

Food and Drug Administration (FDA):
http://www.fda.gov/NewsEvents/InteractiveMedia/ucm200144.htm

Society for Risk Analysis (SRA):
http://www.linkedin.com/groups?home=&gid=4265467
http://www.flickr.com/photos/society_for_risk_analysis

Society of Toxicology (SOT):
http://www.toxicology.org/main/news_feed.asp

When utilizing information from social media venues, it must be evaluated to determine the quality of the information provided. Fortunately, for most science-based information utilizing data from well-known organizations, such as U.S. government agencies or well-known reputable private organizations. Certain social media venues, for example, those of U.S. government agencies, such as the CDC and EPA, are closely vetted for the accuracy of information presented. Others, such as Wikipedia, are self-regulated to some extent, inasmuch as contributors serve as *peer reviewers*, limiting the amount of erroneous information that is posted. However, most social media outlets do not monitor their sites to ensure that the information presented is accurate or complete. Therefore, it is important for those seeking toxicology and other science-based information to have the tools to evaluate each resource as regards the quality of information content.

International Policy and Information Coordination

In addition to SAICM, mentioned earlier, under international organizations, a number of major multinational and international conventions seek to address issues of chemical safety. These include the EU's REACH, the GHS of Classification and Labeling of Chemicals, the

Stockholm Convention on Persistent Organic Pollutants, the Rotterdam Convention on Prior Informed Consent, and the Basel Convention on the Transboundary Movements of Hazardous and Other Wastes. The inevitable forward march of globalization requires interaction between toxicologists at an international level. The IUTOX, the voice of toxicology on the global stage, has been aware of this for some time.[12] Its over 50 affiliated societies represent six continents and over 20,000 toxicologists.

The World Library of Toxicology, Chemical Safety, and Environmental Health (http://www.worldtox.org), initiated by the NLM, and hosted by Toxipedia, is being designed as a portal to sources of information from specific countries and international groups and is intended to foster cooperation and collaboration in research and other activities and to minimize duplication of effort. Country Correspondents utilize Wiki technology to build this network. Additional sponsors and organizations are being sought to enhance this important resource that is still in its infancy and has much potential.

CONCLUSION

Information and data generated by advances in the toxicological sciences, in line with that of other scientific disciplines, continue to grow at a phenomenal rate. The increasing complexity derives from the interdisciplinary nature of toxicology and the societal manifestations of the science. Fortunately, the advent of computers and development and expansion of the web have allowed unprecedented access to and utilization of the vast toxicological information base as it continues to expand. Moreover, innovation in technology and software applications have greatly expanded and diversified the ways in which toxicological information is presented, distributed, and shared.

QUESTIONS

13.1 Identify some of the key web-based databases in toxicology, environmental health, and risk assessment.

13.2 What organizations consider toxicology from a global perspective?

13.3 Discuss such issues as mapping and computational toxicology with regard to the future direction of toxicological information; and entertain pluses and minuses and possible outcomes for information controversies, such as open access publishing.

13.4 How has the development of mobile devices, new applications, and social media changed the way in which toxicological information is disseminated? What are some advantages as well as potential pitfalls to the way in which toxicological information is now shared?

13.5 What is the purpose of a literature review and what should be included in the write-up?

KEYWORDS

Information, Informatics, Databases, Web Resources, Computer Searching

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Section II

Agents

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14 Food Safety and Foodborne Toxicants

Claire L. Kruger, Chada S. Reddy, Dietrich B. Conze, and A. Wallace Hayes

CONTENTS

DEFINITION OF FOOD

Food is defined in section 201(f) of the Food, Drug, and Cosmetic (FD&C) Act as "(1) articles used for food or drink for man or other animals, (2) chewing gum, and (3) articles used for components of any such article." Examples of food include

- Dietary supplements and dietary ingredients
- Infant formula
- Beverages (including alcoholic beverages and bottled water)
- Fruits and vegetables
- Fish and seafood
- Dairy products and shell eggs
- Raw agricultural commodities for use as food or components of food
- Canned and frozen foods

- Bakery goods, snack food, and candy (including chewing gum)
- Live food animals
- Animal feeds and pet food

Food is presumed to be safe (sections 201(f) and 402(a)(1) of the FD&C Act). However, as with any substance, safety is not absolute and the regulatory paradigms, safety standard, risk assessment process, and ultimate responsibility and authority for ensuring the safety of foods and food ingredients have evolved over time.

The original 1906 Food and Drugs Act forbid the marketing of any food containing "any added poisonous or deleterious substance which may render it injurious to health." Premarket approval was not required; the Food and Drug Administration (FDA) had the burden of proving that a food was adulterated. The 1938 FD&C Act was a watershed in the U.S. food policy (http://www.fda.gov/AboutFDA/WhatWeDo/History/ProductRegulation/ucm132818.htm). It pioneered policies designed to protect the pocketbooks of consumers, and food standards were enacted to ensure the *value expected* by consumers. Criteria for adulteration, mislabeled, or harmful food were described. The 1938 Act eliminated the *distinctive name proviso* and required instead that the label of a food *bear its common or usual name*. Food is determined to be misbranded if it is represented as a standardized food unless it conforms to that standard. The law provides for three kinds of food standards: (1) standards (definitions) of identity, (2) standards of quality, and (3) standards regulating the fill of container. The FD&C Act requires manufacturers to prove the safety of any product that would be marketed over state lines.

As defined in the FD&C Act, a food is considered to be adulterated if it contains any poisonous or deleterious substance that may *render it injurious to health*. Adulteration is defined as a food that bears or contains any added poisonous or added deleterious substance (other than a substance that is a pesticide chemical residue in or on a raw agricultural commodity or processed food, a food additive, a color additive, or a new animal drug); or if it bears or contains a pesticide chemical residue, a food additive, or a new animal drug that is unsafe; or if it consists of or is contaminated by any other substance that makes it unfit for food or renders it injurious to health; or if its container is composed of any poisonous or deleterious substance that may render the contents injurious to health; or if it has been intentionally subjected to radiation, unless the use of the radiation conforms with regulation. The act distinguishes, however, between substances naturally present and substances that have been added to the food. If the substance is something that has not been added to the food, the food is not to be considered adulterated under this clause if the quantity of this substance does not *ordinarily render it injurious to health*.

STANDARDS OF SAFETY FOR FOOD ADDITIVES, COLOR ADDITIVES, GRAS INGREDIENTS, AND NEW DIETARY INGREDIENTS (DIETARY SUPPLEMENTS)

The regulation of a substance in the food supply depends upon the intended use and the claims made for the product (Figure 14.1). Food is consumed for taste, aroma, and nutritive value. A new product may be regulated as a food additive or generally recognized as safe (GRAS) ingredient if the intent is for it to become a component of or affect the characteristics of a food. A food additive that is capable of and is intended to impart color when added or applied to a food must be regulated as a color additive. If a dietary substance(s) is intended to be used by people to supplement the diet by increasing the total dietary intake, then the substance is regulated as a dietary ingredient. Importantly, the supplement in which the dietary ingredient is contained must not be represented for use as a conventional food or as a sole item of a meal or the diet. A product that makes a statement that claims to diagnose, mitigate, treat, cure, or prevent disease is a drug claim and thus would be regulated as a drug. Regulation of drugs will not be discussed in this chapter.

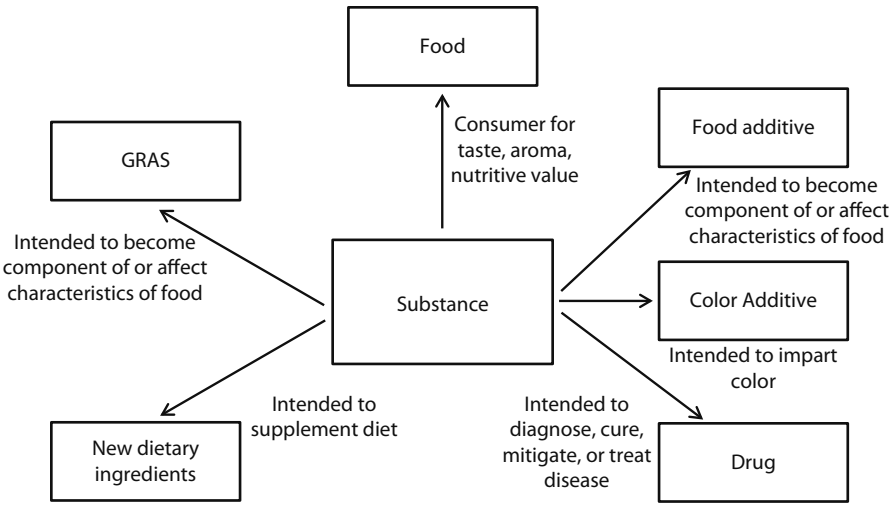


FIGURE 14.1 U.S. regulatory paths for new products.

The implications for the safety evaluation and risk assessment process needed to ensure compliance with applicable regulations for food and color additives, GRAS ingredients, and new dietary ingredients (NDIs) (dietary supplements) will be discussed further in this chapter. A decision tree (Figure 14.2) developed by the FDA utilizes information on intended use and existing authorizations to determine the regulatory path appropriate for a food ingredient (http://www.fda.gov/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/ucm228269.htm). It is the responsibility of the manufacturer of any food to ensure that all ingredients used are of food-grade purity and comply with specifications and limitations in all applicable authorizations. The overall regulatory status of a food is affected by the regulatory status of each individual food ingredient. To determine compliance, each authorization must consider three elements: identity of the substance, specifications including purity and physical properties, and limitations on the conditions of use.

Food Additives

Any substance that is reasonably expected to become a component of food is a food additive that is subject to premarket approval by the FDA, unless the substance is GRAS among experts qualified by scientific training and experience to evaluate its safety under the conditions of its intended use or meets one of the other exclusions from the food additive definition in section 201(s) of the Federal Food, Drug, and Cosmetic Act (FD&C). Any food additive that is intended to have a technical effect in the food is deemed unsafe unless it conforms either to the terms of a regulation prescribing its use or to an exemption for investigational use. Otherwise, in accordance with section 409 of the act, the substance is deemed an unsafe food additive. Any food that contains an unsafe food additive is adulterated under section 402(a)(2)(C) of the FD&C.

The Food Additives Amendment of 1958 requires premarket approval of new food additives by the FDA before they can be marketed, and the responsibility for proving their safety is placed on the petitioner. The safety standard is defined as reasonable certainty in the minds of competent scientists that a substance is not harmful under its intended conditions of use. Food additives are subject to the provisions of the Delaney clause. The Delaney clause was enacted in 1958 as part of the Food Additives Amendment. A food additive must be found *safe* before the FDA may approve its use, and this clause stipulates that the finding of safety may not be made for a food additive that has been shown to induce cancer in humans or in experimental animals. This is based on the judgment by the U.S. Congress that no food additive is likely to offer benefits sufficient to outweigh any risk of cancer.

The FDA's *Redbook II* provides guidelines for the safety testing of direct food additives. The extent of testing necessary for these additives is based on a concern level approach that takes into account the extent of exposure to the additive as well as the structural class of the compound. Safety evaluation for a direct food additive or color additive used in food involves assigning the additive to a concern level (i.e., low [I], intermediate [II], or high [III]) based on information on the additive's toxicological potential predicted from its chemical structure (i.e., low [A], intermediate [B], or high [C]) and an estimation of cumulative human exposure (Table 14.1) (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm054658.htm).

Frequently, exposure information has more weight than structure alert information in assigning additives to a concern level. However, if available, other information may be considered when setting the concern level for a food additive, and final safety decisions are made on a case-by-case basis.

The basic elements of a food additive petition (FAP) submitted to the FDA are the following (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm253328.htm):

- The identity and composition of the additive
- Proposed use
- Use level
- Data establishing the intended effect
- Quantitative detection methods in the food
- Estimated exposure from the proposed use (in food, drugs, cosmetics, or devices, as appropriate)
- Full reports of all safety studies
- Proposed tolerances (if needed)
- Environmental information (as required by the National Environmental Policy Act [NEPA], as revised [62 FR 40570; July 29, 1997])
- Ensure that consistent information is presented throughout all sections of the petition, including those pertaining to
  - Chemistry
  - Toxicology
  - Environmental science
  - Any other pertinent studies (e.g., microbiology)

An acceptable daily intake (ADI) is derived utilizing the results from appropriate toxicology studies, considering the significance of differences between treated and control groups with respect to dose-related trends, reproducibility, related findings, the magnitude and types of differences, and occurrence in both sexes. The ADI is compared to the estimated daily intake (EDI) (discussed later in this chapter). If the EDI is less than or approximates the ADI, the food additive is determined to be safe under the proposed conditions of use.

Color Additives

Any substance that is added to food and imparts color to the food is a color additive (see color additive definition in section 201(t) of the FD&C and 21 CFR 70.3(f) and the FDA's implementing regulations in 21 Code of Federal

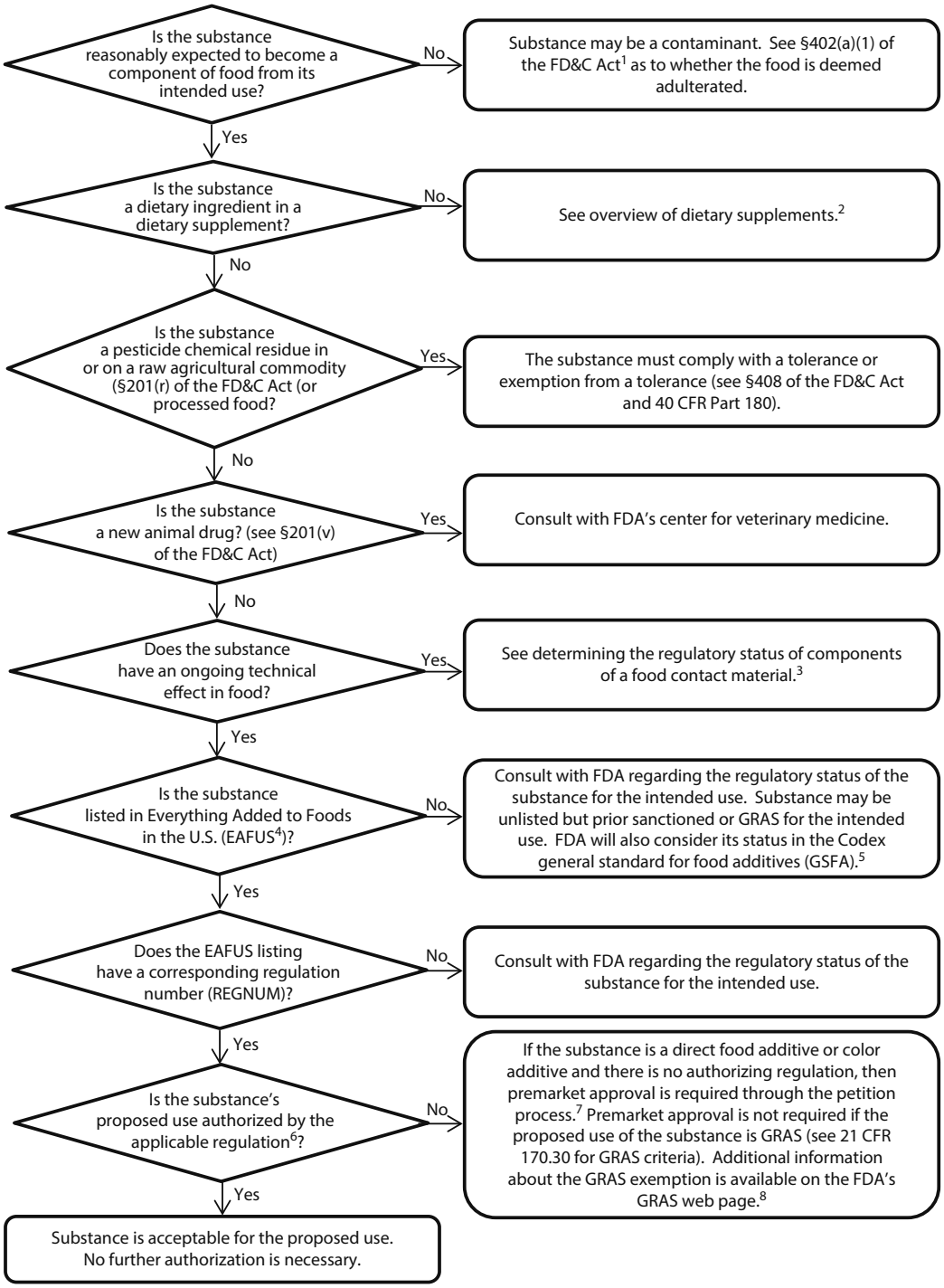


FIGURE 14.2 Food ingredient decision tree. [1]FD&C, U.S. Food and Drug Administration, Federal Food, Drug, and Cosmetic Act (FD&C Act), http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/default.htm; [2]U.S. Food and Drug Administration, Dietary Supplements, http://www.fda.gov/Food/DietarySupplements/default.htm; [3]U.S. Food and Drug Administration, Determining the Regulatory Status of Components of a Food Contact Material, http://www.fda.gov/Food/IngredientsPackagingLabeling/PackagingFCS/RegulatoryStatusFoodContactMaterial/ucm120771.htm; [4]U.S. Food and Drug Administration, Everything Added to Food in the United States (EAFUS), http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=eafusListing; [5]FAO/WHO Food Standards, Codex Alimentarius, Codex General Standard for Food Additives (GSFA) Online Database, http://www.codexalimentarius.net/gsfaonline/index.html;jsessionid=149CBF5BF97E536467770AEEBC15510D; [6]U.S. Food and Drug Administration, Code of Federal Regulations (CFR) Citations for Color Additives, Food Ingredients and Packaging, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm082463.htm; [7]U.S. Food and Drug Administration, Guidance for Industry: Questions and Answers About the Petition Process, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm253328.htm; and [8]U.S. Food and Drug Administration, Generally Recognized as Safe (GRAS), http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/default.htm.

TABLE 14.1
Recommended Toxicological Testing Summary Table for Additives Used in Food

Toxicity Tests[a]	Concern Level Low (I)	Level Intermediate (II)	Concern Level High (III)
Genetic toxicity tests[a]	X	X	X
Short-term toxicity tests with rodents[b]	X[c]	X[c,d]	X[c,d]
Subchronic toxicity studies with rodents[e]		X[c]	X[c,d]
Subchronic toxicity studies with nonrodents[e]		X[c]	X[c,d]
One-year toxicity studies with nonrodents[f]			X[c]
Chronic toxicity[g] or combined chronic toxicity/carcinogenicity studies with rodents			X[c]
Carcinogenicity studies with rodents[h]			X
Reproduction studies[i]		X[c]	X[c]
Developmental toxicity studies[j]		X[c,k]	X[c,k]
Metabolism and pharmacokinetic studies (available in PDF from 1993 *Draft Redbook II*)[l]		X[k]	X[k]
Human studies (available in PDF from 1993 *Draft Redbook II*)[m]			X[k]

[a] U.S. Food and Drug Administration, Redbook 2000: IV.C.1 Short-Term Tests for Genetic Toxicity, July 2007, Toxicological Principles for the Safety Assessment of Food Ingredients, Chapter IV.C.1. Short-Term Tests for Genetic Toxicity, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078321.htm.

[b] U.S. Food and Drug Administration, Redbook 2000: IV.C.3.a Short-Term Toxicity Studies with Rodents, November 2003, Toxicological Principles for the Safety Assessment of Food Ingredients, Chapter IV.C.3.a. Short-Term Toxicity Studies with Rodents, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078339.htm.

[c] Including screens for neurotoxicity[n] and immunotoxicity (available in PDF from 1993 *Draft Redbook II*).[o]

[d] If needed as preliminary to further study.

[e] U.S. Food and Drug Administration, Redbook 2000: IV.C.4.a Subchronic Toxicity Studies with Rodents, November 2003, Toxicological Principles for the Safety Assessment of Food Ingredients, Chapter IV.C.4.a. Subchronic Toxicity Studies with Rodents, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078345.htm.

[f] U.S. Food and Drug Administration, Redbook 2000: IV.C.5.b One-Year Toxicity Studies with Non-Rodents, November 2003, Toxicological Principles for the Safety Assessment of Food Ingredients, Chapter IV.C.5.b. One-Year Toxicity Studies with Non-Rodents, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078348.htm.

[g] U.S. Food and Drug Administration, Redbook 2000: IV.C.5.a Chronic Toxicity Studies with Rodents, July 2007, Toxicological Principles for the Safety Assessment of Food Ingredients, Chapter IV.C.5.a. Chronic Toxicity Studies with Rodents, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078349.htm.

[h] U.S. Food and Drug Administration, Redbook 2000: IV.C.6 Carcinogenicity Studies with Rodents, January 2006, Toxicological Principles for the Safety Assessment of Food Ingredients, Chapter IV.C.6. Carcinogenicity Studies with Rodents, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078388.htm.

[i] U.S. Food and Drug Administration, Redbook 2000: IV.C.9.a Guidelines for Reproduction Studies, July 2000, Toxicological Principles for the Safety Assessment of Food Ingredients, Chapter IV.C.9.a. Guidelines for Reproduction Studies, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078396.htm.

[j] U.S. Food and Drug Administration, Redbook 2000: IV.C.9.b Guidelines for Developmental Toxicity Studies, July 2000, Toxicological Principles for the Safety Assessment of Food Ingredients, Chapter IV.C.9.b. Guidelines for Developmental Toxicity Studies, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078399.htm.

[k] If indicated by available data or information.

[l] U.S. Food and Drug Administration, 1993 Draft Redbook II, Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food, Draft Guidance, Chapter V.B. Metabolism and Pharmacokinetic Studies, http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM078741.pdf.

[m] U.S. Food and Drug Administration, 1993 Draft Redbook II, Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food, Draft Guidance, Chapter VI.A. Clinical Evaluation of Foods and Food Additives, http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM078753.pdf.

[n] U.S. Food and Drug Administration, Redbook 2000: IV.C.10 Neurotoxicity Studies, July 2000, Toxicological Principles for the Safety Assessment of Food Ingredients, Chapter IV.C.10. Neurotoxicity Studies, http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078323.htm.

[o] U.S. Food and Drug Administration, 1993 Draft Redbook II, Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food, Draft Guidance, Chapter V.D. Immunotoxicity Studies, http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM078748.pdf.

Regulations (CFR) Part 70). Under section 201(t)(1) and 21 CFR 70.3(f), the term color additive means a material that is a dye, pigment, or other substance made by a process of synthesis or similar artifice, or extracted, isolated, or otherwise derived from a vegetable, animal, mineral, or other source, and that is capable (alone or through reaction with another substance) of imparting color when added or applied to a food, except that such term does not include any material that the secretary, by regulation, determines is used (or intended to be used) solely for a purpose or purposes other than coloring. Under 21 CFR 70.3(g), a material that otherwise meets the definition of color additive can be exempted from that definition on the basis that it is used or intended to be used solely for a purpose or purposes other than coloring, as long as the material is used in a way that any color imparted is clearly unimportant insofar as the appearance, value, marketability, or consumer acceptability is concerned. Any color additive in food is deemed unsafe unless its use is either permitted by regulation or exempted by regulation. Unlike the definition for food additive, there is no GRAS exemption for color additives. Any food that contains an unsafe color additive is adulterated under section 402(c) of the FD&C.

Following the passage of the Color Additive Amendment of 1960, 20 natural colors (comprising preparations such as dried algae meal, annatto extract, beet powder, grape skin extract, fruit juice, paprika, caramel, carrot oil, cochineal extract, ferrous gluconate, iron oxide, turmeric) were exempted from certification, whereas all the synthetic colors were required to be retested if questions regarding their safety arose. A provisional certification was given to those in use that required further testing. Currently, there are seven certified synthetic colors (FD&C colors blue no. 1, red no. 3, red no. 40, and yellow no. 5 are permanently listed, whereas FD&C blue no. 2, green no. 3, and yellow no. 6 are provisionally listed) with unlimited uses; one permanently listed color (citrus red no. 2) is used only for coloring skins of oranges at 2 ppm, and several colors including green 1, green 2, orange B, red 2, red 4, and violet 1 were delisted due to concerns of their carcinogenicity and other chronic toxic effects. A controversy linking food colors to allergies and hyperkinesis in children remains unresolved.

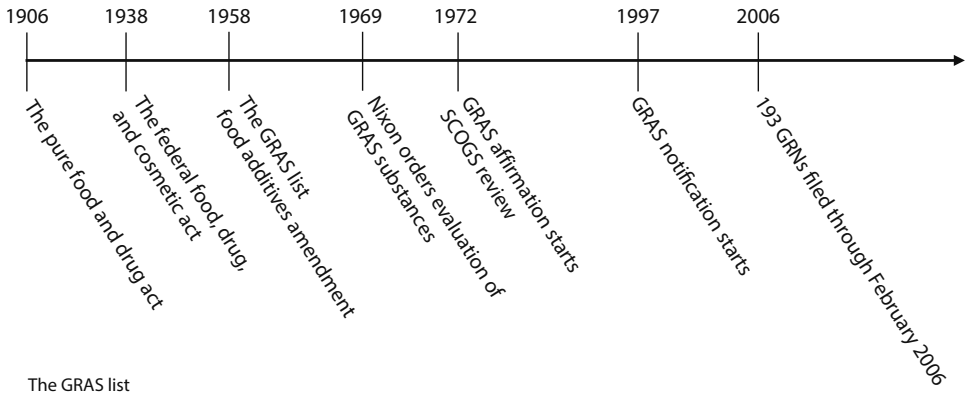
GRAS Ingredients

Congress has amended the FD&C Act many times; the most important of these amendments was the 1958 Food Additives Amendment. This amendment specifically regulates food additives and requires safety to be demonstrated prior to marketing. The critical standard of safety for approval of food additives is reasonable certainty of no harm. Congress did, however, in addition to giving FDA premarket approval of food additives, exclude a class of food ingredients in this amendment that are considered GRAS by qualified experts as having been adequately shown to be safe under the conditions of its intended use (21 CFR section 170.30). GRAS ingredients have no FDA premarket approval requirement nor is there a requirement for advance notice of marketing. The regulatory history of the GRAS process is illustrated in Figure 14.3.

- Under sections 201(s) and 409 of the act and the FDA's implementing regulations in 21 CFR 170.3 and 21 CFR 170.30, a determination of GRAS requires both technical evidence of safety and a basis to conclude that this evidence is generally known and accepted. The technical evidence of safety may be based on either scientific procedures or common use in food prior to January 1, 1958. Safety means that there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use [21 CFR section 170.3(i)]. Under 21 CFR 170.30(b), general recognition of safety through scientific procedures requires the same quantity and quality of scientific evidence as is required to obtain approval of the substance as a food additive and ordinarily is based upon published studies, which may be corroborated by unpublished studies and other data and information. Under 21 CFR 170.30(c) and 170.3(f), general recognition of safety through experience based on common use in foods requires a substantial history of consumption for food use by a significant number of consumers.

Therefore, the distinction between a GRAS substance and a food additive is that for a GRAS substance, there is common knowledge of safety within the expert community of the GRAS substance for its intended use. There is no distinction on the basis of the substance or the type of data and information necessary to support safety. For a determination of safety, this means that the scientific standard to which a GRAS substance is held is comparable to that of a food additive. GRAS is a rigorous process that relies on common knowledge and expert consensus about the safety of the substance for its intended use (Kruger et al., 2011).

As noted in the GRAS timeline (Figure 14.3), the GRAS status of a compound is established by recognition of safety among qualified experts, not premarket FDA approval. In 1972, the FDA conducted rulemaking to establish procedures used for a GRAS affirmation petition process for sponsors, at their discretion, to petition the FDA to review the GRAS status of substances. On April 17, 1997, however, the FDA released a proposed rule to replace the GRAS petition process with a GRAS notification procedure. Although the agency's 1997 policy with regard to GRAS notifications is embodied only in a proposed rule, the FDA has, in a de facto manner, already implemented the policy and will no longer accept GRAS affirmation petitions. Notification is not mandatory but is available to the sponsor of the product if it wishes to inform the FDA of its GRAS determination. The FDA does not make its own determination as to the GRAS status of a food ingredient,



The GRAS list
- 1958 food additives amendment: Congress recognized that many food substances would not require a formal premarket review by FDA to assure their safety
- Food additives excludes substances that are recognized, among qualified experts, as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food) to be safe under the conditions of their intended use.
- December 9, 1958: FDA published a list of GRAS substances and incorporated the list in Title 21 of the Code of Federal Regulations. The current list appears in 21 CFR Parts 182, 184, and 186.

Opinion letters
- Many manufacturers wrote to FDA and requested an opinion letter in which an FDA official would render an informal opinion on the GRAS status of use of the substance
- Revoked in 1970 (21 CFR 170.6; 35 FR 5810; April 9, 1970)

Comprehensive review
- October 30, 1969: President Nixon directed FDA to make a critical evaluation of the safety of GRAS food substances.
- March 28, 1972: Life Sciences Research Office (LSRO) of the Federation of American Societies for Experimental Biology (FASEB) began to summarize the available scientific literature and to recommend what restrictions, if any, on the use of the substances would be needed to ensure their safe use in food.

GRAS affirmation
- 1972: FDA conducted rulemaking to establish the procedures (21 CFR 170.35) that it would use to affirm the GRAS status of substances that were the subject of the GRAS review. That rulemaking included a mechanism (the GRAS affirmation petition process) whereby an individual could petition FDA to review the GRAS status of substances not being considered as part of the agency's GRAS review.
- 1973–1997: GRAS Affirmation Petition Process

GRAS notification
- April 17, 1997: FDA proposed to establish a anotification procedure whereby a person may inform FDA of a determination that the use of a substance is GRAS (62 FR 18938; April 17, 1997).
- Industry submits GRAS notice
- FDA is evaluating whether each submitted notice provides a sufficient basis for a GRAS determination and whether information in the notice or otherwise available to FDA raises issues that lead the agency to question whether use of the substance is GRAS

FIGURE 14.3 A GRAS timeline. (From Gaynor, P.M., Bonnette, R., Garcia, Jr. E., Kahl, L.S., Valerio, Jr. L.G., FDA's Approach to the GRAS Provision: A History of Processes, Excerpted from Poster Presention at the FDA Science Forum, April 2006, http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/ucm094040.htm.)

and consequently, new GRAS ingredients will no longer be listed in the CFR.

In general, the FDA's response to a notification has been in one of three categories:

- The agency does not question the basis for the notifier's GRAS determination.
- The agency concludes that the notice does not provide a sufficient basis for a GRAS determination (e.g., because the notice does not include appropriate data and information or because the available data and information raise questions about the safety of the notified substance).
- The response letter states that the agency has, at the notifier's request, ceased to evaluate the GRAS notice.

Safety Evaluation of a GRAS Ingredient

The following information is pivotal to the assessment of safety of a GRAS substance:

- *Description of the GRAS substance*
  - Included in this section is a review of the physical and chemical characteristics of the GRAS substance including chemical name(s) (and synonyms), CAS registry number(s), and chemical structure(s) and a description of final product characteristics including established food-grade specifications for the principal components, related substances, by-products, impurities and contaminants, and batch analysis results showing compliance with established food-grade specifications

- *Production Process*
  - Documentation of current good agricultural practice/current good manufacturing practice
  - A process flow diagram with detailed description for each step of the production process and operation parameters
  - A list of raw materials and processing aids with food-grade and regulatory compliance documentation
  - Critical control steps involved in the quality control process
  - Description of potential impurities to be carried over to the final product
- Documentation of stability and shelf life of the product
- *Historical Use, Regulatory Status, and Consumer Exposure*: A review of the history of use and/or natural occurrence of the ingredient in other foods, with a determination of the consequent intake or exposure estimate. An overview of current regulatory status if applicable. A description of the proposed use and use levels utilized to calculate an EDI of the GRAS substance.
- *Intended Effect*: Characterization of the intended function of the GRAS substance in the food.
- *Analytical Methodology*: Method for determining the quantity of the substance in or on food, and any substance formed in or on food, because of its use.
- *Review of Safety Data*: Evaluation of the actual use of the product and issues that may contribute to the safety of the product are included. An identification and critical review from the animal toxicology and clinical literature for safety information on primary components, related substances, secondary metabolites, impurities, and contaminants using relevant data for occurrence and/or levels present, estimated background intake, metabolic fate, toxicological activity, and pharmacological activity is included. Pivotal safety information must be published.
- *Safety Assessment and GRAS Determination*: Evaluation of the safety of consumption of the substance under its intended conditions of use including determination of an ADI for the substance as well as other components or contaminants (if present) and comparison of this ADI to the EDI of the substance from existing and proposed uses. As long as the EDI is less than (or approximates) the ADI, the substance can be considered safe under its intended conditions of use.

Examples of Approaches for GRAS Determination for Specific Types of Food Products

Foods Produced by Genetic Modification

Existing conventional food crops and the products made from them are recognized to be safe. Genetic modification, or the alteration in the genetic makeup of these crop plants for the purpose of developing new varieties, is traditionally done through conventional breeding and selection techniques. Genetic engineering, a more recent technique, is the process of removing a gene from one organism or plant and transferring it to a different organism or plant variety. The advantage is that this technique allows plant breeders to achieve, with great precision, desirable agronomic or quality improvements in food crops, such as resistance to pests and/or enhanced nutritional value (U.S. FDA, 1999). The new DNA introduced by genetic engineering produces a new protein and the safety of that protein is evaluated as part of the risk assessment process. The substances intentionally added to food via biotechnology to date have been well-characterized proteins, fats, and carbohydrates and are functionally very similar to other proteins, fats, and carbohydrates that are commonly and safely consumed in the diet and so will be presumptively GRAS. The safety of a genetically engineered food crop or a product made from that crop is evaluated by comparing the nutritional and toxicological equivalence of the product to its conventional counterpart. Guidance for safety testing of genetically engineered products to assure that no unintended changes in the composition of the food could adversely affect human health has been published by authoritative scientific and regulatory agencies (Codex Alimentarius Commission, 2003; EFSA, 2006a,b; FSANZ, 2007; Health Canada, 2006; ICMR, 2008; U.S. FDA, 1992). Any differences between the conventional and bioengineered product are identified and the safety of the introduced change is determined (U.S. FDA, 1992).

The FDA provided guidance on the information that should be included in the safety and nutritional assessment (http://www.fda.gov/NewsEvents/Testimony/ucm115032.htm). Some examples of this information would include the following:

- The name of the food and the crop from which it is derived
- The uses of the food, including both human food and animal feed uses
- The sources, identities, and functions of introduced genetic material
- The purpose or intended technical effect of the modification and its expected effect on the composition or characteristic properties of the food or feed
- The identity and function of any new products encoded by the introduced genetic material, including an estimate of its concentration
- Comparison of the composition or characteristics of the bioengineered food to that of food derived from the parental variety or other commonly consumed varieties with special emphasis on important nutrients, antinutrients, and toxicants that occur naturally in the food
- Information on whether the genetic modification altered the potential for the bioengineered food to induce an allergic response
- Other information relevant to the safety and nutritional assessment of the bioengineered food

If a bioengineered food included a new protein derived from an allergenic source and consumers would not expect it to be present based on the name of the food, the presence of that allergen must be disclosed on the label (U.S. FDA, 2001). All labeling requirements, including allergen labeling, that apply to conventional foods also apply to bioengineered foods. However, the FDA has not established mandatory labeling requirements to identify foods that have been derived from genetically engineered sources. The reason for this, as articulated in their policy (U.S. FDA, 1992), is that there is no basis to conclude that foods developed by genetic engineering present any different or greater safety concern than foods developed by traditional plant breeding. This is borne out by the conclusions of many risk assessments that have been conducted on bioengineered foods and found no evidence of harm, including allergic reactions (EFSA, 2003, 2004a,b, 2006a,b, 2007, 2008a,b, 2009a–d, 2010a,b, 2011a–e, 2012a–d). Furthermore, to date, there is no documented proof that any approved, commercially grown genetically engineered crop has caused allergic reactions because of the transgenically introduced protein (Goodman et al., 2008).

Food Ingredients Derived from Chemically Complex Extracts

Safe level of ingestion through scientific procedures can be derived by applying a UF to a no observed adverse effect level (NOAEL) derived from a well-conducted toxicology study to derive an ADI. However, when the level of addition of the ingredient to the feed in the animal study exceeds 5% (w/w), untoward physiological effects due to dietary imbalance alone may manifest in such studies (Hayes, 2008; Klaassen, 2008; Kruger and Mann, 2003; Office of Food Additive Safety, 2000). This limitation enters into consideration in the case of natural products, for example, crude extracts, which are composed of a mixture of tens or hundreds of compounds. Many of these compounds are present at such low concentrations that it is impracticable to concentrate the overall extract to such an extent that derivation of an ADI utilizing traditional UFs applied to NOAELs could be generated on each of the minor constituents. Additionally, these matrix molecules may exert an effect on the bioavailability of the active compounds that are present in the natural product (IFT, 2009). It is also important to note that the concept of the 100-fold UF is not appropriate for physiologically active substances. In these cases, the effects seen in animal studies may be due to the physiologic or pharmacologic activity of the active principle and not a classic toxicological response (Booth et al., 2012).

As discussed in a publication authored by members of the U.S. FDA and National Institute of Environmental Health Sciences (NIEHS), the safety assessment of botanical substances is complicated by various factors:

> Compositional diversity is a key factor because botanicals are complex mixtures, for which the identity of all the individual components is not known and the proportion of individual components varies with the source. There are various other factors, among which are the lack of standardization of the botanical (in terms of both materials and analytical methods), lack of identity of the active ingredients, and the use of different formulations of the botanical in the article of commerce when compared with the test substance. The paucity of data on the toxicology of whole extracts or the individual components of botanicals makes it very challenging to determine the safety of botanical substances for use in conventional food. As such, the review of botanical substances for safe use in conventional food must be approached with some skepticism, an open mind and utilization of the full arsenal of scientific tools available to assess the safety of such substances. There is no set formula for dealing with the safety evaluation of such materials or combination of materials. Each new submission must be dealt with on a case-by-case basis.
>
> **(Abdel-Rahman et al., 2011)**

Traditionally, safety determination of a complex natural product has relied on animal toxicology testing to compensate in many instances for the inability to assess safety due to poorly characterized extracts. An approach to determine the safety of natural products involves (1) review and analysis of the existing phytochemical and botanical literature, (2) establishing chemical composition of the raw material and the commercial product, (3) determination of health-based levels of exposure for the identified compounds or compound, and (4) utilization of published toxicology studies to establish safety of exposure to the extract through evaluation of the components/compound classes. A safety paradigm utilizing a thorough analytical elucidation of the composition of the complex natural product may allow a literature-based assessment of safety for the individual components/classes of compounds comprising the botanical extract.

Estimated Daily Intake

Estimates of dietary intake support the documentation of the safety of substances introduced into food either intentionally to accomplish a technical effect, adventitiously as a component of an added substance, or inadvertently through contamination resulting from processing or other sources.

The U.S. FDA's premarket approval processes for food and color additives require an estimate of the probable consumer intake of the additive to determine whether its use or presence in a food at a given concentration is safe. The key determinant in the safety evaluation of a substance found in or added to the diet is the relation of its probable human intake to the level at which adverse effects are observed in toxicological studies. Simply, *the dose makes the poison* (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm074725.htm).

In dietary intake assessments, the concentration of an ingredient or chemical constituent in food can be obtained from the intended use levels of the substance in target foods (typical, recommended, or maximum use level); the measured concentration in food as consumed, accounting for processing and storage losses of ingredient; the limit of detection (LOD)

or limit of quantification (LOQ) of the analytical method, as appropriate, if the concentration in the food is nondetectable or nonquantifiable at the LOD or LOQ; established limits for the substance (e.g., specifications in the CFR or the Food Chemical Codex [FCC]) for undesirable impurities and contaminants in food ingredients; or maximum levels for contaminants in foods adopted by a recognized standards-setting body, such as the Codex Alimentarius Commission.

The FDA typically uses the maximum intended use levels proposed to calculate a *worst case* level of intake. For estimating intake of constituents in food ingredients, specification limits (e.g., for appropriate heavy metals, such as lead, arsenic, and mercury) are used to estimate the potential intake of a constituent from the intended use of the ingredient.

There are a number of sources of data available for use in estimating intake of substances in the diet including the following:

- Food consumption surveys
- Food/ingredient disappearance figures
- Total diet study
- Body burden/excretion measurements: *biomarkers*

Each source has advantages and limitations. For a number of reasons, including cost and availability, breadth of data, and ease of data manipulation, the FDA relies primarily on data taken from food consumption surveys.

Food Consumption Surveys

Food consumption data may be collected at the national, household, or individual level. The FDA regularly uses food consumption surveys conducted nationwide at the individual level to estimate the dietary intake of substances. Consumption surveys at the level of the individual provide information on mean food intakes and the distribution of food intakes within subpopulations of individuals defined by demographic factors (e.g., age, gender) and health status (e.g., pregnancy, lactation). These surveys measure food intake by one or more methods, that is, food records or diaries, 24 h recalls, food frequency questionnaires (FFQ), and diet history. In the first two methods, participants record or recall (with a trained interviewer) the amounts and types of each food eaten during the day, both at home and away from home. For the food frequency methods, participants record or recall only the number of occasions each food was consumed over a specified period of time that may vary from 1 day to more than 1 year. These eating-occasion frequencies are multiplied by an appropriate food portion size (based on age–sex considerations) to obtain semiquantitative estimates of the daily food intake.

The U.S. Department of Agriculture (USDA) has collected national food consumption data for more than 70 years. Initially designed to help people achieve economical and nutritious diets, USDA's food consumption surveys gradually broadened in scope and purpose. Nationwide surveys were conducted in 1965–1966, 1977–1978, 1985–1986, 1987–1988, 1989–1991, 1994–1996, and 1998. The more recent surveys, called the Continuing Survey of Food Intakes by Individuals (CSFII) and conducted in 1989–1991, 1994–1996, and 1998, were combined with USDA's Diet and Health Knowledge Survey (DHKS), a telephone survey designed to measure knowledge and attitudes about diet and health. In 1971, the U.S. Department of Health and Human Services (HHS) added a nutrition component to its National Health Examination Survey (NHES), and the survey's name was changed to the National Health and Nutrition Examination Survey (NHANES). Between 1971 and 1994, three NHANES collected dietary data along with health data (1971–1975, 1976–1980, 1988–1994). In 1982–1984, Hispanic HANES was conducted to collect nutrition and health data on Mexican Americans, Cuban Americans, and Puerto Ricans. Since 1999, NHANES has been a continuous, annual survey program.

Continuing Survey of Food Intake by Individuals

The 1994–1996 and 1998 CSFII are the most recent in a series of USDA surveys; intake data from this survey have been released in two database forms: (1) food consumption expressed as food and nutrient intakes and (2) food consumption expressed in equivalent amounts of basic food commodities. The USDA's food coding database is linked to food commodities and nutrient values to convert the survey data (reported food consumption) into relevant outputs for the respective databases.

In each of the 3 survey years of the 1994–1996 CSFII, a nationally representative sample of approximately 5000 noninstitutionalized individuals residing in the United States provided, through in-person interviewing using a 1-day dietary intake questionnaire, food intakes on two nonconsecutive days (3–10 days apart) and health-related information. Additionally, there is a CSFII 1998 Supplemental Children's Survey with 2-day food and nutrient intake data for approximately 5300 children under the age of 10.

National Health and Nutrition Examination Survey

The National Center for Health Statistics' (NCHS) NHANES studies link information on food intake with respondent health information obtained by a physical examination, anthropometric measures, and laboratory analyses of various blood and urine parameters. Three NHANES studies were conducted between 1971 and 1994 (NHANES I–III). Information on vitamin, mineral, and dietary supplement use was collected in those surveys. The food intake data for NHANES were previously based on one 24 h recall that was supplemented with portion size information obtained using detailed measurement guides. NHANES sampled a larger number of individuals than CSFII (approximately 30,000 respondents in NHANES III) and included information on the monthly frequency of dietary supplement use by respondents. NHANES nutrient intake data have been used to a limited extent by the Office of Food Additive Safety (OFAS) but are a valuable source of information on the distribution of usual intakes of nutrients in the U.S. diet.

NHANES became a continuous program in 1999, with approximately 5000 individuals surveyed each year. NCHS released data sets to the public in 2-year cycles (NCHS, 1999–2000, 2001–2002). These dietary data are released in two files: a total nutrient intakes file and an individual food file (with detailed records of gram weights and nutrient values).

Integrated CSFII/NHANES

The CSFII and NHANES surveys were combined by Agricultural Research Service (ARS) and NCHS into a single, continuous, population-based national nutrition survey beginning in January 2002. While NHANES studies have historically included a 1-day recall, beginning in 2002, a second nonconsecutive 24 h recall was added.

The most recent CSFII/NHANES examination surveys for the years 2009–2010 are available for public use: http://wwwn.cdc.gov/nchs/nhanes/search/nhanes09_10.aspx. In 2009–2010, approximately 10,000 people across the United States completed the health examination component of the survey. Any combination of consecutive years of data collection is a nationally representative sample of the U.S. population. It is well established that the length of a dietary survey affects the estimated consumption of individual users and that short-term surveys, such as the typical 1-day dietary survey, overestimate consumption over longer time periods (Gregory et al., 1995). Because two 24 h dietary recalls administered on two nonconsecutive days (day 1 and day 2) are available from the NHANES (NCHS, 2009–2010) survey, these data can be used to generate estimates of intake.

The NHANES provides the most appropriate data for evaluating food-use and food consumption patterns in the United States, containing two years of data on individuals selected via stratified multistage probability sample of civilian noninstitutionalized population of the United States. NHANES (NCHS, 2009–2010) survey data were collected from individuals and households via 24 h dietary recalls administered on two nonconsecutive days (day 1 and day 2) throughout all four seasons of the year. Day 1 data were collected in person in the mobile examination center (MEC), and day 2 data were collected by telephone in the following 3–10 days, on different days of the week, to achieve the desired degree of statistical independence. The data were collected by first selecting primary sampling units (PSUs), which were counties throughout the United States. Small counties were combined to attain a minimum population size. These PSUs were segmented and households were chosen within each segment. One or more participants within a household were interviewed. Fifteen PSUs are visited each year. For the 2009–2010 NHANES, there were 13,272 persons selected; of these, 10,253 were considered respondents to the MEC examination and data collection; 9,754 of the MEC respondents provided complete dietary intakes for day 1, and of those providing the day 1 data, 8,405 provided complete dietary intakes for day 2.

In addition to collecting information on the types and quantities of foods being consumed, NHANES (NCHS, 2009–2010) collected socioeconomic, physiological, and demographic information from individual participants in the survey, such as sex, age, height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population. Among those who completed the food intake survey on both day 1 and day 2, 8301 respondents also provided physiological information including age, sex, and weight; of these, 7738 were 2 years and older.

Sample weights were incorporated with NHANES (NCHS, 2009–2010) to compensate for the potential underrepresentation of intakes from specific population groups as a result of sample variability due to survey design, differential nonresponse rates, or other factors, such as deficiencies in the sampling frame (CDC, 2006; USDA, 2012).

Statistical Methods

Consumption data from individual dietary records, detailing food items ingested by each survey participant, are used to generate estimates for the intake of an ingredient by the U.S. population. Estimates for the daily intake of ingredient represent projected 2-day averages for each individual from day 1 and day 2 of NHANES (NCHS, 2009–2010) data; these average amounts comprised the distribution from which mean and percentile intake estimates were produced. Mean and percentile estimates were generated incorporating sample weights in order to provide representative intakes for the entire U.S. population. *All-person* intake refers to the estimated intake averaged over all individuals surveyed, regardless of whether they consumed food products containing the ingredient, and therefore includes *zero* consumers (those who reported no intake of food products containing the ingredient during the two survey days). *All-user* intake refers to the estimated intake by those individuals consuming food products containing the ingredient, hence the *all-user* designation. Individuals are considered users if they consumed one or more food products containing the ingredient on either day 1 or day 2 of the survey.

Food Usage Data

The individual proposed food uses, default serving sizes, and the corresponding maximum use levels for specific foods as identified by food codes representative of each proposed use are chosen from the Food and Nutrition Database for Dietary Studies (FNDDS). In FNDDS, the primary (usually generic) description of a given food is assigned a unique eight-digit food code (CDC, 2006; USDA, 2012).

Food Survey Results

The estimated *all-person* and *all-user* total intakes of ingredient from all proposed food uses in the United States by population group is summarized to generate the EDI by gender and age group, as appropriate for comparison with the ADI to generate the safety assessment for the ingredient.

Dietary Supplements

The Dietary Supplement Health and Education Act of 1994 (DSHEA) created a new framework for the regulation of

dietary supplements. DSHEA signaled a major departure from the well-established *food* versus *drug* dichotomy that guided the FDA policy. The act reaffirmed the status of dietary supplements as foods. The legislation created a new category of food by specifically defining dietary supplements to include the following dietary ingredients: vitamins, minerals, herbs or other botanicals, amino acids, or other "dietary substance[s] for use by man to supplement the diet by increasing the total dietary intake." Moreover, concentrates, metabolites, constituents, extracts, or any combination of the dietary ingredients set forth earlier are also included in this definition. The second foundation of the dietary supplement definition addresses the form of the supplement. The product must be one "intended for ingestion in tablet, capsule, powder, softgel, gelcap or liquid form." A dietary supplement must not be "represented for use as a conventional food or as a sole item of a meal or the diet."

The DSHEA of 1994 (Public Law 103-417) defined the terms *dietary supplement* (section 201(ff) of the act [21 USC 321(ff)]) and *new dietary ingredient* (NDI) (section 413(c) of the act [21 USC 350b(c)]). A dietary supplement means a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: vitamin, mineral, herb or other botanical, and amino acid; a dietary substance for use by man to supplement the diet by increasing the total dietary intake; or a concentrate, metabolite, constituent, extract, or combination of any of the previously listed ingredients. An *NDI* is one that meets the aforementioned definition for a *dietary ingredient* and was not sold in the United States in a dietary supplement before October 15, 1994. Dietary supplements can be found in many forms such as tablets, capsules, softgels, gelcaps, liquids, or powders. They can also be in other forms, such as a bar; but if they are, information on their label must not represent the product as a conventional food or a sole item of a meal or diet. Whatever their form may be, DSHEA places dietary supplements in a special category under the general umbrella of *foods*, not drugs, and requires that every supplement be labeled a dietary supplement. An NDI is one that meets the aforementioned definition for a *dietary ingredient* and was not sold in the United States in a dietary supplement before October 15, 1994.

A key regulatory feature of this class of foods is that, unlike food additives, there is no requirement for premarket approval. New dietary supplements (one first marketed on or after October 15, 1994) are required only to provide advance notice of new ingredient marketing. For a dietary supplement, a premarket notification must include the "information, including any citation to published articles, which is the basis on which the manufacturer or distributor has concluded that a dietary supplement containing such dietary ingredient will reasonably be expected to be safe" under the conditions of use in the labeling.

As a food, dietary supplements are subject to the standard of adulterated food 21 CFR section 342(f) and are considered to be adulterated if either the dietary supplement or the dietary ingredient contained in the supplement presents a significant or unreasonable risk of illness or injury under conditions of use recommended or suggested in labeling, or if no conditions of use are suggested or recommended in the labeling, under ordinary conditions of use. Additionally, it is considered to be adulterated, if it is an NDI for which there is inadequate information to provide reasonable assurance that this ingredient does not present a significant or unreasonable risk of illness or injury. The United States bears the burden of proof to show that a dietary supplement is adulterated.

A dietary supplement is considered adulterated if it has been prepared, packed, or held under conditions that do not meet current good manufacturing practice regulations final ruling (https://www.federalregister.gov/articles/2007/06/25/07-3039/current-good-manufacturing-practice-in-manufacturing-packaging-labeling-or-holding-operations-for).

DSHEA also provided that a dietary supplement containing an NDI is adulterated unless it meets the requirements set forth in section 413 of the act, which requires premarket notification for certain NDIs. Under section 413(a) of the act, a dietary supplement that contains an NDI is deemed adulterated unless it meets one of two statutory requirements. One is that the dietary supplement contains only dietary ingredients that "have been present in the food supply as an article used for food in a form in which the food has not been chemically altered" (section 413(a)(1) of the act). The alternative requirement is (section 413(a)(2) of the act) that there be a history of use or other evidence of safety establishing that the dietary ingredient when used under the conditions recommended or suggested in the labeling of the dietary supplement will reasonably be expected to be safe. In addition, at least 75 days before being introduced or delivered for introduction into interstate commerce, the manufacturer or distributor must provide the FDA with information, including any citation to published articles, which is the basis on which the manufacturer or distributor has concluded that a dietary supplement containing such dietary ingredient will reasonably be expected to be safe. The FDA has issued a regulation section 190.6 (21 CFR 190.6) establishing the procedure by which a manufacturer or distributor of a dietary supplement that contains an NDI must submit the information required by section 413(a)(2) of the act.

A dietary supplement containing an NDI may be considered adulterated if there is inadequate information to provide reasonable assurance that the ingredient will not present a significant or unreasonable risk of illness or injury (http://www.fda.gov/Food/DietarySupplements/ucm109764.htm). It has been suggested that the wording of the statute has contributed to a lowering of the standard for safety compared with food additives or GRAS ingredients. Other provisions of the act that contribute to a sense that the assurance of safety is lowered are the following: (1) not requiring endorsement either by the agency or by experts qualified by scientific training and experience to evaluate safety; (2) by placing the burden of proof on the government to demonstrate a substance is unsafe; and (3) acceptance of *grandfathering* without stipulating consensus among experts or criteria for safety as the agency had already in place for GRAS substances (Burdock, 2000). The FDA has issued draft guidance to provide additional clarity on their current thinking and to assist industry in complying with DSHEA (Draft Guidance for Industry: Dietary Supplements: New Dietary Ingredient Notifications

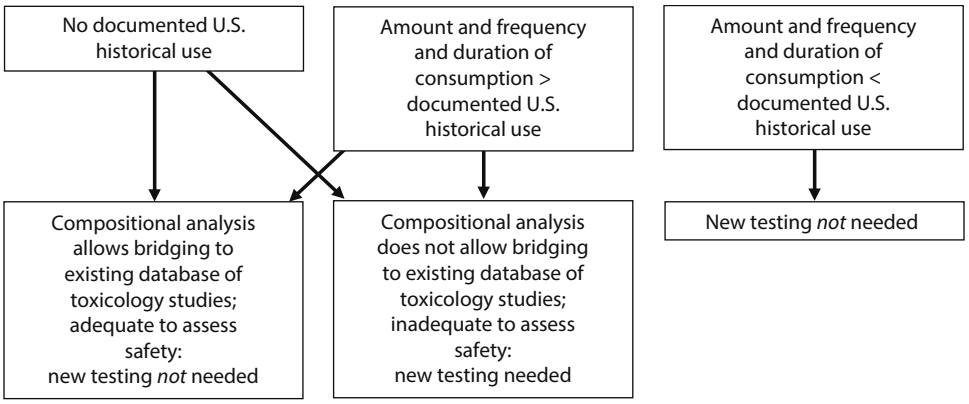


FIGURE 14.4 Decision tree approach for new toxicology testing NDI.

and Related Issues, July 2011). Pivotal to the application of the guidance as it pertains to the safety evaluation of an NDI, the FDA opined that

> the NDI safety standard is different than the standard for food additives, drugs, pesticides, and other FDA-regulated products. Recommendations in guidance documents that are tailored to the safety assessment needs of other FDA-regulated products may not always be appropriate for dietary ingredients and dietary supplements.

The FDA further indicated that

> You should use your own best judgment in compiling scientific evidence that provides a basis to conclude that the NDI that is the subject of your notification will reasonably be expected to be safe when used under the conditions recommended or suggested in the labeling of the dietary supplement described in the notification.

The guidance specifies that a change in the use of a dietary ingredient, by increasing the amount, frequency, or duration of intake compared to traditional use, triggers the classification as an NDI and, subsequently, the need for additional testing in order to complete the safety evaluation. The data needed may be derived from de novo toxicology testing, or as discussed in the section *Food Ingredients Derived from Chemically Complex Extracts*, data to evaluate safety may be available from the literature for the chemical classes that comprise the extract. A decision tree approach that summarizes the toxicology testing recommendations is found in Figure 14.4.

SUMMARY OF REGULATORY PATHS FOR GRAS SUBSTANCES, FOOD ADDITIVES, AND DIETARY SUPPLEMENTS

See Table 14.2.

FOOD CONTACT SUBSTANCES

In 1997, the Food and Drug Administration Modernization Act (FDAMA) amended the FD&C Act to include a notification process for food contact substances (FCSs). The act defined an FCS as "any substance intended for use as a component of materials used in manufacturing, packing, packaging, transporting, or holding food if such use is not intended to have a technical effect in such food" (U.S. FDA, 1998). Examples of FCSs include polymers (plastic packaging materials), pigments and antioxidants used in polymers, can coatings, adhesives, materials used during the manufacture of paper and paperboard, slimicides and biocides (antimicrobial agents), and sealants for lids and caps.

The term *safe*, as it refers to food additives and ingredients (including FCSs), is defined in 21 CFR 170.3(i) as a "reasonable certainty in the minds of competent scientists that a substance is not harmful under the intended conditions of use."

To obtain approval for a new FCS, a food contact notification (FCN) should be submitted to the OFAS at least 120 days prior to the marketing of the FCS. The level of data required to support the safety of an FCS depends on the EDI of the FCS and any impurities it may contain. The FCN should include detailed toxicological, chemical, and environmental information about the FCS and its impurities and should address the potential mutagenicity and carcinogenicity of the FCS and its impurities (www.cfsan.fda.gov/~dms/opa2pmnt.html).

For an FCS or impurity with an incremental dietary exposure ≤1.5 μg of the FCS per person per day (1.5 μg/p/day), toxicity studies are not ordinarily recommended. However, any available information on the mutagenic or carcinogenic potential of the FCS and its impurities should be provided in the notification. For an FCS or impurity with an incremental exposure between 1.5 and 150 μg/p/day, the FDA recommends a battery of short-term genetic toxicity tests: (1) a test for the induction of gene mutations in *Salmonella typhimurium* (the Ames test) and *Escherichia coli* and (2) a test for the induction of gene mutations or chromosome aberrations in mammalian cells in culture such as the mouse lymphoma assay.

For an FCS or impurity with an incremental exposure between 150 and 3000 μg/p/day, OFAS recommends other studies be conducted in addition to the previously mentioned genotoxicity assays. One such study is an in vivo chromosome aberration study, such as the micronucleus test in rodents. OFAS also recommends two subchronic toxicity studies, typically of 90-day duration, one in a rodent species

TABLE 14.2
Comparison of Regulatory Paths

Food Additive	GRAS	Dietary Supplement
FFDC 1938.	Exemption to food additives: Food Additives Amendment 1958. Notification process promulgated 1997.	DSHEA 1994. Draft Guidance for Industry 2011.
FAP	General recognition of safety by expert panel: GRAS dossier (self-GRAS or notification).	Pre-1994: no FDA notification. Post-1994: NDI notification to FDA.
Information and data may be unpublished.	Pivotal information and data must be published.	Information and data may be unpublished.
Assumes lifetime exposure.	Assumes lifetime exposure.	Duration and frequency of exposure dictated on label.
Cannot exclude subpopulations.	Cannot exclude subpopulations.	Can target and exclude subpopulations on the label.
EDI based on specific food uses and levels calculated using databases to derive mean and 90th percentile consumption.	EDI based on specific food uses and levels calculated using databases to derive mean and 90th percentile consumption.	EDI based on recommended use and levels as defined in the labeling.
Reasonable certainty of no harm *specific to use/intake* Delaney clause applies.	Reasonable certainty of no harm *specific to use/intake.*	Reasonably expected to be safe under the conditions of use defined in the labeling.
The FDA makes the determination of safety based on data provided by submitter.	General recognition of safety based on publicly available data and consensus of expert panel opinion.	Burden is on the submitter to establish safety for NDI under the conditions of use defined in the labeling.
FDA premarket approval required.	No FDA premarket approval.	No FDA premarket approval.
Published in 21 CFR.	Record of the voluntary notification and outcome on the FDA website.	Record of the mandatory premarket notification and outcome on the FDA website.

and one in a nonrodent species. These studies should provide adequate toxicological information to determine an ADI for the FCS and/or its constituents.

Depending on the results, these tests may indicate a need for further, more specialized toxicological testing to ensure consumer safety. Specialized testing may include evaluation of neurotoxicity (toxicity effects on the nervous system), immunotoxicity (toxicity effects on the immune system), teratogenicity (toxicity effects on embryos and fetuses), and reproductive toxicity (toxicity effects on the reproductive organs and functions). Depending on the results, the use of the chemical may be judged to be safe, additional tests may be requested to clarify questions raised by the current data, or OFAS may determine that a proposed exposure represents an unacceptable risk and object to the FCN.

For a dietary intake above 3000 μg/p/day, OFAS recommends that the notifier discuss the specific details of the submission with the FDA to determine if the submission of a FAP is more appropriate than submission of an FCN.

FOOD ALLERGY

Introduction

Food allergy, as defined by the World Health Organization in 2003, is the condition where an individual experiences an adverse immunological reaction to a particular type of food (Johansson et al., 2004). The eight most common foods that elicit allergic reactions are eggs, milk, fish, crustacean shellfish (e.g., crab, lobster, shrimp), tree nuts (e.g., almonds, walnuts, and pecans), peanuts, wheat, and soybeans, although reactions to many other foods can occur as well. Food-induced anaphylactic reactions are possible, but the more prevalent adverse immunological reactions include eczema, urticaria, angioedema, nausea, vomiting, diarrhea, rhinoconjunctivitis, and asthma. Recent estimates indicate that food allergies are on the rise, currently affecting approximately 5% of young children and 3%–4% of adults in westernized countries (Branum and Lukacs, 2009; Rona et al., 2007). Thus, there is increasing concern about the safety of food products both from the perspective of the consumer and the food manufacturer.

Currently, avoiding the eliciting allergen is the most successful means of preventing food allergic reactions. Although this strategy is manageable for consumers with appropriate labeling, it presents a major hurdle for the food industry because eliminating allergens from food is nearly impossible and product labeling can negatively impact product sales. To circumvent this, manufacturing facilities typically dedicate single production lines to manufacturing allergen-free products and/or clean their equipment meticulously between manufacturing runs of different foods. Nevertheless, the Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004 requires that food labels clearly identify the food source names of all ingredient that are or contain any protein derived from the eight most common food allergens.

It is now known that allergic individuals have *thresholds*, a dose at or below which an adverse effect is not seen in an experimental setting (Taylor et al., 2002), which indicates that manufactures may be able to allow minute amounts of allergic substances in food without posing a risk to the consumer.

Unfortunately, defining such thresholds has presented major challenges to physicians and food producers because the allergen sensitivities vary greatly and accumulating enough data to confidently identify the lowest tolerated dose of a particular allergen has been difficult. The concept of identifying safe levels of food allergens, however, is intriguing, and a considerable amount of resources is being invested in the development of validated diagnostic methods to determine the lowest tolerated doses and assessing the risks associated with the consumption of foods containing small amounts of allergenic substances.

The following sections review the immunopathophysiological mechanisms involved in food allergic reactions, tests used to diagnose food allergies, the concept of threshold doses, and allergenic determinants.

Oral Tolerance and Food Allergy

To understand food allergy, one must first appreciate the complexity of the immune system and its ability to respond to foreign substances (see Chapter 37). Briefly, the purpose of the immune system is to defend the host against invading pathogens and potentially dangerous substances. And to do so, it relies on a variety of cells and secreted factors to coordinate and execute the detection and eradication of the invading pathogen or dangerous substance. Implicit in this response, however, is its ability to discriminate foreign substances (nonself antigens) from those of the body (self antigens) and to respond to only nonself antigens (Janeway et al., 2005). The *absence* of self-reactivity is known as *natural* or *self*-tolerance. Importantly, if the immune system mistakenly reacts to self or becomes intolerant to self, an immune response develops, and a chronic inflammatory disease state, known as autoimmunity, results because the causative self antigen is in endless supply.

In the gastrointestinal (GI) tract, the concept of tolerance is vastly different. Every day, millions of foreign antigens are ingested, and moreover, the GI tract is home to a wide variety of commensal bacteria. So, why do the large majority of people not mount an immune response to food and commensal bacteria? Oral tolerance appears to be the answer. Unlike self-tolerance, oral tolerance is believed to be an active state of immune suppression whereby the immune response to the millions of foreign antigens ingested each day and normal gut flora is prevented (reviewed in Strobel and Mowat [1998]). The mechanisms that promote oral tolerance in humans are largely unknown, but studies in animal models suggest that they may involve a combination of neutralizing of foreign antigens with secreted IgA and inducing T-cell anergy, clonal deletion, and the suppressive activities of T regulatory cells.

Food allergies, like all allergies, result from an abnormal and misguided immune response to a substance that is otherwise harmless. Initially, the individual is exposed, reacts, and is asymptomatic, but when they reencounter the sensitizing antigen, the pathologies develop. Unfortunately, the reasons why one person is hypersensitive to the particular antigen as opposed to another are unclear, but it is currently thought to result from a failure to develop and/or the breakdown of oral tolerance. Furthermore, studies in animals suggest that genetics, age, dose and timing of antigen exposure, commensal gut flora, intestinal permeability, and properties of the antigen itself may contribute to the breakdown of oral tolerance (reviewed in Mayer et al. [2001]).

IgE- and Non-IgE-Mediated Hypersensitivity Reactions

Food allergies can be categorized into IgE-mediated and non-IgE-mediated reactions, which are not mutually exclusive. IgE-mediated reactions are true *allergic* responses, also known as type 1 hypersensitivity responses, that typically affect the cutaneous, respiratory, GI, and cardiovascular systems (Wang and Sampson, 2009). These involve IgE antibodies, which are produced by B cells, occur within 2 h of exposure, and can result in anaphylaxis. Mechanistically, they result when an antigen induces the abnormal skewing of CD4+ T cells to Th2 cells and activates B cells. Then, with the help of the abnormally skewed Th2 cell, the B cells undergo class-switching and produce antigen-specific IgE antibodies. The IgE antibodies bind Fc receptors expressed on the surface of mast cells and basophils, and when the antibodies bind the sensitizing antigen, the cells degranulate, releasing histamine and leukotrienes into the extracellular matrix. This leads to capillary venule dilation, endothelium activation, and increased vascular permeability, causing redness and swelling. If the antigen is systemic or rapidly absorbed, histamine and leukotriene release is widespread and can result in anaphylaxis and potentially death.

Non-IgE-mediated responses are less frequent and occur in the absence of detectable food-specific IgE antibodies in the serum or skin. Although the pathophysiological mechanisms that contribute to non-IgE-mediated responses are not well defined, they occur at least 2 h after exposure and appear to be the result of acute or chronic inflammation in the GI tract. From a more global immunological perspective, three types of non-IgE-mediated hypersensitivity reactions exist, that is, type 2 (cytotoxic responses), type 3 (immune complex reactions), and type 4 (delayed-type hypersensitivity [DTH] reactions), and all may contribute to the development of non-IgE-mediated food hypersensitivity responses. Cytotoxic reactions are triggered when antigen-specific IgM or IgG antibodies bind their cognate antigens bound to or found on the surface of cells. This activates a cascade of protein factors known as the complement cascade, which in turn releases the inflammatory mediator C5a, and results in the recognition and lysis of the antibody/complement-bound cell by macrophages. Immune complex reactions are similar, but are triggered when antibodies encounter soluble antigen. Aggregates of the antibody and antigen then form, are deposited in tissues, and cause complement activation and Fc receptor-mediated leukocyte activation. DTH reactions are unlike allergic, immune complex, and cytotoxic response, because they are antibody independent and are the product of an aberrant Th1 or cell-mediated immune response. DTH reactions are triggered when antigen-specific T cells reencounter the sensitizing antigen. The activated T cells in turn produce cytokines, which

promote inflammation. Examples of non-IgE-mediated food hypersensitivity reactions are food-protein-induced enterocolitis and proctocolitis, and examples of reactions that include both IgE- and non-IgE-mediated reactions are atopic dermatitis and eosinophilic gastroenteropathies (Wang and Sampson, 2009).

Diagnosing Food Allergy

Diagnosing food allergy presents a major challenge because it not only requires that the provoking allergen be identified, but also it also demands that the amount of the eliciting allergen and the type of response provoked by the allergen be determined. The primary tools used to diagnose food allergy are a detailed history including a diet record, physical examination, skin prick tests (SPTs), serum tests for food-specific IgE antibodies, trial elimination diets, and oral food challenges (OFCs) (reviewed in Chapman et al. [2006]).

The first steps to diagnosing food allergy are documenting a history of the patient's response and performing a physical exam. These analyses help identify whether or not food is the causative agent, the type of food that is causing the reaction, the amount of food needed to elicit the reaction, and whether or not the reaction is IgE-mediated and provide some insight into the type, timing, and duration of the clinical manifestations.

The next steps include SPTs, serum tests, trial elimination diets, and graded OFCs. SPTs detect the presence of allergen-specific IgE bound to mast cells and are performed by injecting minute amounts of potential food antigens into the epidermal layer of the skin and measuring the extent of redness and swelling, also known as the wheal-and-flare reaction that results from IgE cross-linking and mast cell degranulation. Wheals are generally considered to be significant when the diameter is 3 mm or greater than the wheal induced by the vehicle or negative control (Hill et al., 2004). Intracutaneous/intradermal testing (injecting a small amount of antigen in the dermis) is also performed; however, this is generally used as a follow-up if results from skin prick testing are negative and clinical suspicion still remains high (Carr and Saltoun, 2012). Serum IgE tests involve harvesting blood, collecting the serum, and analyzing it for the presence of antigen-specific IgE antibodies. Although both of these tests are very sensitive, they can produce false-positives, and moreover, their results do not always correlate with the pathophysiological responses to the suspected food. Furthermore, the quality of SPTs and serum IgE tests have been found to be dramatically affected by characteristics of the patient and the quality and characteristics of the reagents and techniques used to perform the assays (Guerin and Tioulong, 1979; Hurst et al., 2002). Thus, although positive results do not unambiguously rule in a particular food as a causative agent, negative results from SPTs and serum IgE test are very helpful in ruling out IgE-mediated responses.

Trial elimination diets involve removing suspected eliciting foods from the diet and monitoring the patient's pathophysiological response (Bock et al., 1988; Sicherer and Sampson, 1999). Because avoiding the eliciting allergen is the most effective way to prevent an allergic reaction, elimination diets may be an effective method for narrowing the search for the eliciting allergen. However, elimination of a potentially eliciting allergen may be difficult and the duration of the trial may not be long enough for the pathology to resolve. As a result, positive results cannot not be used to definitively diagnose food allergy unless other supporting data such as a clearly defined history, responses to SPTs, IgE serum tests, and graded food challenges are also known (Chapman et al., 2006).

OFCs are the most ideal tests for diagnosing food allergy because they directly replicate the route of exposure and define the lowest tolerated dose of the eliciting allergen. However, they can cause anaphylaxis, worsening of atopic dermatitis, and emotional distress. Therefore, OFCs should be performed in settings where medical assistance can be delivered immediately. Furthermore, if an individual presents with a clinical history that suggests a food allergy and has a positive SPT that indicates an IgE-mediated response, an OFC may not be necessary. However, if the history is unclear and the individual has positive SPT, then OFCs may be performed for diagnosis, and a previous episode of anaphylaxis to a food may exclude the use of an OFC (Peters et al., 2012). Graded OFCs are performed by gradually feeding increasing amounts of the suspected eliciting food to the patient and evaluating his or her response over the course of days to determine whether or not any component of the patient's response is non-IgE-mediated. They can also be performed as open, single-blinded, or double-blinded placebo-controlled challenges. The gold standard is the double-blind placebo-controlled food challenge (DBPCFC), which entails feeding test foods and placebos that have been randomly prepared by a third party to the patient (Bock et al., 1988; Nowak-Wegrzyn et al., 2009; Sicherer, 1999). Once the challenge is over, the contents of the test foods are disclosed, the contents are compared to the patient's response, and a diagnosis is made. Importantly, DBPCFC minimizes bias and subjective results, which are typically associated with open and single-blinded oral challenges. However, they do require a significant amount of planning, time, and resources and are primarily reserved for research studies and selected clinical cases.

Threshold Doses

Threshold as defined by the Oxford English Dictionary (9th edn.) is the limit below which a stimulus causes no reaction. In toxicology, it is defined as the dose at or below which a response is not seen in an experimental setting and falls between the NOAEL and lowest observed adverse effect level (LOAEL). For allergens, thresholds exist for both the sensitizing and eliciting phases of the reaction, but, because sensitization occurs asymptomatically, not much is known about the thresholds for the sensitizing phase of the response (Taylor et al., 2008). Thresholds are also specific to individuals, but with enough evidence, they can be determined for a population. For individuals, they are determined at an allergy

clinic by graded DBPCFCs. For populations, they are determined for a group of individuals with a specific type of food allergy, but as with any experimental data, the more individual thresholds obtained for a particular allergen, the greater confidence there is in establishing the *population* threshold. Lastly, thresholds vary from person to person and from allergen to allergen. For peanuts, for example, controlled clinical challenges have found that individual thresholds range from approximately 0.1 mg to 8 g of whole peanut (Taylor et al., 2009, 2010) and that peanut thresholds are, in general, much lower than those for soybeans (Ballmer-Weber et al., 2007; Taylor et al., 2009, 2010). The remainder of this section discusses the different types of thresholds and the approaches used for deriving population thresholds for the eliciting phase of the allergic reaction.

In 2006, the Center for Food Safety and Applied Nutrition at the FDA formed the Threshold Working Group to gather data on the exposure of allergic subjects to allergens and evaluate the possible approaches to establishing food allergen thresholds (The Threshold Working Group, 2008). They identified four general approaches, analytical methods based, safety assessment based, risk assessment based, and statutorily derived, that could be used to establish thresholds.

The analytical methods-based approach relies on the limits of detection for analytical methods used to verify compliance. So, in effect, they establish a *regulatory threshold*. For example, X food contains 10 ppm or greater of ingredient Y, based on the LOD for the method used to determine the concentration of Y. Importantly, this type of threshold is not correlated to biological effects, and thus, allergic reactions can still be provoked in sensitive populations even though the food contains allergenic substances at undetectable levels. Moreover, thresholds will be continually questioned with the development of more sensitive methods of detection.

The safety assessment-based approach relies on data from animal, human, and epidemiological studies that provide an exposure level for which there is no apparent or adverse effect or identify an LOAEL or NOAEL. Then, one or more uncertainty factors (UFs) are applied to account for interspecies and interindividual differences and other uncertainties in the data. The end result is an ADI or for an allergen, a threshold (Lehman and Fitzhugh, 1954). Crump (1984) introduced the benchmark dose (BMD)/margin of exposure (MoE) concept, which refined the classical safety assessment-based approach by fitting a curve to the data and then extrapolating to the dose that corresponds to a 10% response rate. Although both of these methods are routinely used for determining ADIs, it is important to note that they are *deterministic* in nature, relying on actual data points that do not account for the inherent variability in the population. As a result, they are limited to what is known and, because they are intended to ensure that even the most sensitive part of the population is protected under all conditions, generally overestimate health risk and can result in thresholds that are below those that could be reasonably obtained for food manufacturers (Spanjersberg et al., 2007). Furthermore, animal models do not accurately recapitulate allergic reactions in humans. The number and breadth of doses tested in both experimental and clinical study may not allow for an accurate determination of the threshold dose, and in the case of the BMD/MoE technique, the farther away one extrapolates from the actual data points, the greater the amount of the error.

The risk assessment-based approach relies on systematically examining scientific data of known or potential adverse health effects resulting from human exposure to a hazard. It involves identifying the hazard, establishing a dose–response relationship between a hazard's biological effects and the amount consumed, determining the nature and extent of an exposure, and integrating all of these components into an overall estimation of the potential risks to the population. Risk assessments can also be quantitative or qualitative: Quantitative risk assessments provide numerical estimates of the chance of illness or death after exposure to a specific hazard; qualitative risk assessments use verbal descriptors of the risk and uncertainties and often involve expert opinions. Both account for the cumulative probabilities of certain events happening and the uncertainties associated with those events; however, only the quantitative risk assessment-based approach provides insight into both the level of protection and the degree of uncertainty associated with an exposure level. Quantitative risk assessments also require that the entire dose–response curve be determined and validated mathematical procedures that account for population variability be applied. Currently, it is unclear whether or not enough DBPCFC data exist to develop dose–response curves for all the food allergens, yet probabilistic modeling appears to be the most effective mathematical method for quantifying thresholds (Kruizinga et al., 2008; Madsen et al., 2009; Spanjersberg et al., 2007; The Threshold Working Group, 2008).

The statutorily derived approach establishes a threshold by extrapolating from an exemption established by congress for another purpose. For example, FALCPA requires that food products containing an ingredient that contains protein derived from milk, fish, egg, crustacean shellfish, tree nuts, wheat, peanuts, and soybeans be labeled as containing food allergens except for highly refined oils. Thus, if consumption of another food containing levels of protein results in an exposure level that is equal to or less than the level in a typical serving of highly refined oil, it should not be associated with allergic reaction and a threshold can be established. Although this approach is derived from the law, it is not based on a rigorous and systematic evaluation of available scientific data. Furthermore, the data on the amount of protein in refined oils are lacking, and thresholds for all food allergens would be based primarily on the protein levels in highly refined soy and peanut oil.

In conclusion, the Threshold Working Group established that "the quantitative risk assessment approach provides the strongest, most transparent scientific analyses to establish thresholds for the major food allergens." However, because new data and diagnostic tools are being continuously produced, they recommend that thresholds established by any of the four approaches be periodically reevaluated.

Allergenic Determinants

Allergens, by definition, are antigens that are recognized by IgE antibodies and provoke IgE-mediated hypersensitivity responses (Aalberse, 2000). Not all allergens are created equal, however. Some are capable of inducing the primary immune response, which elicits the IgE antibodies that make people sensitive, whereas others only engage preformed IgE antibodies and provoke secondary/hypersensitivity responses. Those that induce the primary responses are considered strong immunogens and are called *complete* allergens. Those that induce only secondary reactions, likely due to their binding cross-reacting IgE antibodies, are weak immunogens and therefore called *incomplete* allergens. Examples of complete antigens include Ara h 2 of peanut and Bet v 1 of birch pollen (Burks et al., 1995; Ipsen and Lowenstein, 1983). An incomplete allergen is Mal d 1 from apples, which provokes IgE-mediated hypersensitivity responses in birch pollen-sensitized individuals because of the homology between Mal d 1 and Bet v 1 and cross-reacting anti-Bet v 1 antibodies (Bjorksten et al., 1980; Holm et al., 2001; Lahti et al., 1980).

Although there have been a few reports of allergic reactions to carbohydrates (Chiang et al., 2012; Commins et al., 2009; Franck et al., 2005), most allergens are proteins or glycoproteins, clustering in less than 2% of all sequence-based protein families (Radauer et al., 2008; Sicherer and Sampson, 1999). Importantly, allergens are heterogeneous, and although they are all capable of binding IgE antibodies, efforts in defining the structural and biochemical determinants that would make an antigen allergenic have been unfruitful.

COMMON FOODBORNE TOXICANTS

Pesticide Residues

A crop protection chemical or pesticide is any substance or mixture of substances intended to control or destroy pests including insecticides, herbicides, fungicides, rodenticides, repellents, and fumigants (http://www.epa.gov/opp00001/regulating/registering/).

In the European Union, a crop protection dossier must comply with the data requirements in Regulations EU 543/2011 and 544/2011 and be submitted to a member state who will act as the *rapporteur member state*. In the United States, pesticides must be registered or exempted by the Environmental Protection Agency's (EPA) Office of Pesticide Programs before they can be sold or distributed in the United States. Once registered, a pesticide may not legally be used unless the use is consistent with the approved directions for use on the label. As part of the registration process, the EPA examines the ingredients of a pesticide; the site or crop on which it is to be used; the amount, frequency, and timing of its use; and storage and disposal practices.

The EPA sets limits on how much of a pesticide residue (maximum residue limits (MRLs) or tolerances) can remain in or on each treated food commodity. Tolerances are set to protect the consumer from potential harmful concentrations of pesticides. The tolerance is the residue level that triggers enforcement actions; if residues are above that level, the commodity is subject to seizure. In setting the tolerance, the EPA must make a safety finding that the pesticide can be used with *reasonable certainty of no harm* to humans, the environment, and nontarget species. The tolerance applies to food imported into this country, as well as to food grown in the United States. In August 1996, the FD&C was amended to include the Food Quality Protection Act (FQPA). This act required the EPA to reassess all of the pesticide tolerances that were in place to ensure that they met current safety standards and were supported by up-to-date scientific data.

The USDA enforces tolerances established for meat, poultry, and some egg products, while the FDA enforces tolerances established for other foods in interstate commerce to ensure that these limits are not exceeded. The International Maximum Residue Limit Database contains MRLs or tolerances for U.S. specialty crops. This database is maintained by the Foreign Agricultural Service, Horticultural and Tropical Products Division of the USDA (http://www.fas.usda.gov/htp/MRL.asp). It can be searched by crop or pesticide and contains information for the United States and 70 other countries.

Heavy Metals

A heavy metal is a member of a loosely defined subset of elements that exhibit metallic properties. Many definitions have been proposed—some based on density, some on atomic number or atomic weight, and some on chemical properties or toxicity (see Chapter 17). Examples of toxic metals include arsenic, cadmium, chromium, lead, mercury, and thallium. Some trace elements (copper, selenium, zinc) are essential to maintain the metabolism of the human body; however, at higher concentrations, these trace elements can be toxic. Poisoning typically results from drinking-water contamination, high ambient air concentrations, or intake via the food chain. The potential danger of heavy metals is increased because these metals tend to bioaccumulate in the body. Refer to Chapter 17 for details regarding the toxicity of these materials.

For food-grade standards, foods and food ingredients and additives conform to specification limits for heavy metals that are promulgated by authoritative agencies such as Food Chemicals Codex (foodchemicalscodex.org/), Joint Food and Agricultural Organization (FAO)/WHO Expert Committee on Food Additives, European Food Safety Authority, and the U.S. FDA. Specifications for heavy metals are based on health-based standards (FCC, 2003). With the exception of methyl mercury in fish, the U.S. FDA has not set regulatory standards for the other heavy metals in foods.

Bacteria and Viruses

Bacterial Infections and Intoxications

Foods contaminated with microbial agents are a major source of human disease, estimated to afflict tens of millions of people and to cost 22 billion dollars annually in

the United States. With few exceptions, most of these outbreaks can be prevented by adequate washing and cooking along with proper cooling, storage, and reheating of cooked foods in clean containers. Bacterial foodborne diseases may result from the consumption, in food, either of bacteria (e.g., *Salmonella* sp. and *Clostridium perfringens*) that can cause disease by multiplying in the intestinal mucosa where they may elaborate toxins (enterotoxins) or from preformed microbial toxins (staphylococcal enterotoxins and botulinum toxins). In addition to these well-known etiologies, genetic changes in bacteria that increase virulence, changes in eating habits, altered food production and distribution systems, increased number of immunocompromised food consumers, and improved detection systems have led to identification of other pathogens such as *E. coli*, *Listeria* sp., and *Yersinia* sp. causing foodborne illness.

C. perfringens frequently causes foodborne illness. Due to the ubiquitous distribution of the organism in soil and in the GI tract of man and animals, prevention is difficult. The enterotoxin, released during sporulation in the large intestine, causes fluid accumulation in the intestines. Among the five distinct types of *C. perfringens* (type A–E), type A is almost always involved in foodborne gastroenteritis and associated signs in humans. The α-toxin produced by certain types of *C. perfringens* possesses lethal, necrotizing, and hemolytic activities. Only meat and fish products provide all the amino acids and growth factors required for growth of *C. perfringens*. Roast beef, beef stew, gravy, and meat pies for type A and pork, other meats, and fish for type C are frequently involved (Bryan, 1979). Typically, foods involved are cooked at or below 100°C for less than an hour and are subsequently kept warm or slowly cooled. Spores that survive the heat shock multiply faster than those not subjected to heat treatment and elaborate the enterotoxin in the gut once the contaminated food has been consumed. The enterotoxin appears to form ion-permeable channels in the cell membrane leading to movement of extracellular calcium and water into the cells resulting in cell death (Osuntokun, 1973). Entry of the toxin into blood stream will lead to release of potassium from hepatocyte, hyperkalemic cardiac failure, and eventually death (Sugimoto et al., 1996). Multiplication and toxin production can be inhibited by heating food to a proper temperature (165°F–212°F), by prompt and effective cooling, and by avoiding prolonged reheating before consumption.

Staphylococcus aureus is probably the leading cause of foodborne illness worldwide. The organisms are gram-positive, nonmotile, and non-spore-forming cocci and occur ubiquitously in the environment. Although man is the leading source of food contamination by way of nasal discharge and infected cuts and wounds, the organism can be present in milk derived from mastitic cows and meat derived from arthritic poultry (Miller et al., 1998). Baked ham, poultry, fish and shellfish, meat and potato salads, cream-filled bakery goods, and high-protein leftover foods are frequently involved in such intoxication (Bryan, 1979). Multiplication of *S. aureus* in raw food products can be inhibited by other spoilage organisms. As a result, mostly cooked products subsequently contaminated by infected handlers and stored at warm temperatures for several hours before consumption are capable of causing intoxication. The causative agent is one of more than six immunologically distinct heat-stable enterotoxic proteins (MW 26,000–34,000) whose secretion is regulated by chromosomes during growth (A, D, and E) or by plasmids (B and C). In addition, *S. aureus* also produces many other substances such as coagulase, DNase, hemolysins, lipases, fibrinolysin, and hyaluronidase that are toxic to one or more animal species. Although all strains of *S. aureus* are potentially pathogenic, enterotoxin production is closely related to the presence of coagulase and DNase. Signs and symptoms begin 1–6 h after consumption of contaminated food and include nausea, salivation, vomiting, retching, occasional diarrhea, abdominal cramps, sweating, dehydration, and weakness followed by recovery in 1–3 days. Severe cases may show fever, chills, drop in blood pressure, and prostration (Miller et al., 1998). Preventive measures effective against *S. aureus* food intoxication include education of food handlers regarding hygienic practices to reduce postcooking contamination of high-protein foods and eliminating prolonged storage of cooked foods at room temperature.

Botulism is a neurotoxic syndrome caused by consumption of improperly cooked and stored foods containing one of seven (A–G) heat-labile neurotoxins produced by *Clostridium botulinum*. It is a ubiquitous, anaerobic, gram-positive, and motile rod capable of forming heat-resistant spores. High moisture, a pH above 4.6, and prolonged anaerobic storage are required for sufficient toxin production (Miller et al., 1998). Common foods involved are home canned fruits and vegetables such as beans, corn, leafy vegetables, and especially peppers, all of which contain toxins A and B. Nonpoultry meats contain toxin B, whereas cheese and other dairy products contain toxin A. Toxin E is isolated mostly from fish products (Miller et al., 1998). Types C and D, causing botulism in animals and birds, do not affect humans. Outbreaks, however, are often from more unusual sources such as chili peppers, tomatoes, and improperly handled baked potatoes wrapped in aluminum foil. Although the FDA approved purified botulinum toxin type A for treatment of eye muscle disorders, cervical dystonia (a neurological movement disorder causing severe neck and shoulder contractions), and frown lines between the eyebrows, because of its fatal effects in aerosolized form and its use in weapons by rogue states, it also is considered a potential agent of bioterrorism.

Botulinum toxins are stable in the acid pH of the stomach where it is protected from the gastric juice and pepsin by a nontoxic component of the toxin molecule. Once in the duodenum, it is activated by trypsin with no change in molecular size and subsequently absorbed into lymphatics. The toxin irreversibly binds to the myoneural junction and acting as a Zn endopeptidase degrades peptides involved in the release of acetylcholine (ACh) thus inhibiting its release at the peripheral cholinergic nerve endings (Miller et al., 1998). Signs and symptoms of botulism usually appear 12–24 h (range:

2 h–6 days) following consumption of the toxin-containing food. Initial signs of nausea, vomiting, and diarrhea are followed by predominantly neurologic signs including headache, dizziness, blurred and/or double vision, loss of light reflex, weakness of facial muscles, and pharyngeal paralysis (difficulty in speech and swallowing). Fever is absent. Sensory reflexes and mental alertness are intact. Paralysis of the respiratory muscles leads to failure of respiration and death, usually in 3–10 days (Smith 1977). Foodborne botulism can be prevented by proper canning technique, boiling vegetables for at least 3 min before serving, and discarding all swollen and damaged canned products after boiling. Control of cases of botulism involves the use of monovalent (E), bivalent (A and B), or polyvalent (A, B, and E) antitoxin, recall of all involved commercial products, proper reporting, and epidemiologic investigation. Boiling for 3 min or heating at 80°C for 30 min destroys the preformed toxin, whereas the use of salt, the antimicrobial compound nisin, polyphosphates, smoke, spices, lactic acid, and nitrite can inhibit the growth of *C. botulinum* and thus prevent toxin formation (Miller et al., 1998). If the nitrite content of cured meats and fish as well as fermented sausages is reduced from current levels as a means of decreasing the level of carcinogenic dietary nitrosamines, it is conceivable that the incidence of botulism from the consumption of such foods will increase unless suitable replacements for the nitrite are found.

Bacillus cereus–associated foodborne disease outbreaks have occurred in Northern and Eastern Europe. A diarrheal illness involving a wide variety of meats and vegetables, various desserts, fish, pasta, milk, and ice cream (similar to that of *C. perfringens*) and a vomiting illness involving flour-based foods such as cereals and fried rice served in Chinese restaurants (similar to that of *S. aureus*) are both apparently caused by this organism (Miller et al., 1998). At least seven toxins including the heat-stable (121°C for 90 min) emetic toxin, cereulide, and the enterotoxins, hemolysin BL and its nonhemolytic homologue, contribute to the syndrome (Miller et al., 1998; Schoeni and Wong, 2005). Enterotoxin appears to disrupt cell membranes leading to increased permeability, whereas the mechanism of emetic toxin is unknown.

Salmonella sp. consists of over 2200 serotypes possessing somatic O, flagellar H, and capsular Vi antigens, of which 50 serotypes commonly occur. *Salmonella typhi, Salmonella paratyphi, and Salmonella sendai* are adapted to human hosts, which serve as sole carriers for those organisms. *S. typhimurium* and *Salmonella enteritidis* are the two most common disease-causing agents in the United States. Feces of infected humans, domestic and wild animals, and birds serve as sources of contamination in a variety of meat and milk products and more recently raw fruits and vegetables. Severe GI signs along with fever, septicemia, shock, and sequelae of embolism including pneumonia, meningitis, and abortion can occur following consumption of contaminated foods. Mortality is rare but occurs in the very young and the very old and in immunocompromised patients. Some individuals develop a chronic condition called Reiter's syndrome manifesting painful joints, irritated eyes, and painful urination. Enteritis can result both from bacterial multiplication within the mucosa and from enterotoxins secreted by some serotypes. Thorough cooking of meats; pasteurization of milk and dairy and egg products; prevention of cross-contamination between cooked and raw products; and finally testing, isolation, and treatment of carrier animals and food handling personnel are all extremely important in controlling the incidence of this most common foodborne disease (Ekperigen and Nagaraja, 1998). In this regard, evidence of emergence of antibiotic-resistant strains such as *S. typhimurium* DT104 in the United Kingdom and United States suggests that future research must be directed at understanding mechanisms of microbial adaptation to stresses if we are to better control such infections. Vaccination allows *Salmonella*-free birds; however, these birds should be housed in a *Salmonella*-free environment and fed *Salmonella*-free pelleted feed.

Shigella spp., especially *Shigella dysenteriae, Shigella flexneri, Shigella boydii*, and *Shigella sonnei*, cause an estimated 165 million cases of *Shigella* diarrhea (acute bacillary dysentery) annually, 99% of which occur in developing countries and 69% in children under 5 years of age resulting in 1.1 million deaths annually (Niyogi, 2005). Young children in day-care and custodial institutions are more susceptible. *Shigella enteritis* is characterized by fever, mucohemorrhagic diarrhea, abdominal cramps, and tenesmus. *Sh. flexneri* can lead to Reiter's syndrome (see *Salmonella*) and eventually to chronic arthritis. *Sh. dysenteriae* type 1 produces shiga toxin and can lead to life-threatening hemolytic uremic syndrome (HUS), the same complication that develops in some cases of infection with enterohemorrhagic *E. coli* (EHEC). Shigellosis is highly contagious producing a large number of secondary cases in each outbreak involving persons in contact with infected patients. Contamination of food by unhygienic food handlers and consumption of raw vegetables raised in contaminated soils are two main contributors to the incidence of *Shigella* diarrhea. Therapy with antibiotics and fluids and prevention through thorough hand washing are effective strategies against the spread of such infections.

Campylobacter jejuni and others in this genus (*Campylobacter sputorum*, *Campylobacter coli*, etc.) are the leading cause of bacterial diarrhea accounting for up to 2.4 million cases in the United States and up to 14% of diarrheal illness worldwide. Many aspects of the illness, including the symptoms that rarely require treatment, are similar to those of salmonellosis (Altekruse et al., 1998). Reactive arthritis, inflammation of urethra and conjunctiva, and Guillain–Barré syndrome (paralysis of limbs and weakness of respiratory muscles) have been described as sequelae in occasional cases (Altekruse et al., 1998).

E. coli, a close relative of the genus *Shigella*, has recently raised concern as a fatal foodborne disease agent. More than 160 serotypes (based on O, H, or capsular K antigen) of which 43 can induce gastroenteritis sometimes associated with life-threatening HUS in humans exist (Riemann and Oliver, 1998). In North America, HUS is the most common cause of acute kidney failure in children, who are

particularly prone to this complication. Pneumonia, meningitis, thrombotic and thrombocytopenic purpura, bladder and kidney infections, and septicemia may also result from *E. coli* infections. Based on virulence factors (which bestow the organism with the ability to attack, invade, and produce toxin in the host cells) located in the plasmids, five virotypes have been identified as pathogenic. These are enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (Eagg EC), enteropathogenic *E. coli* (EPEC), EHEC, and enteroinvasive *E. coli* (EIEC). Serogroup O 157:H7 belongs to the EHEC group. This organism produces a shiga-like toxin, which may be the most common serotype causing nausea, vomiting, watery, and/or bloody diarrhea. HUS is mainly traceable to consumption of contaminated beef products (Riemann and Oliver, 1998). Less frequently, unpasteurized milk and juices; ham, turkey, salami, and cheese sandwiches; dry fermented sausage; salad; and nonchlorinated water have been involved. The genome of *E. coli* O157:H7 is about 70% homologous with and 30% larger than that of the harmless serotype K12, suggesting that further study of differences between serotypes could lead to a better understanding of the virulence and pathogenicity and tissue predilection of *E. coli* O157:H7. Once in the intestines, *E. coli* produce shiga-like toxins SL1 and SL2 that act, similar to cholera toxin (CT) and ricin, by receptor binding and entry into a vesicular pathway, followed by release and translocation of the enzymatic A1 domain of the A subunit into the target cell cytosol, where covalent modification of intracellular targets leads to activation of adenylate cyclase and a sequence of events culminating in ion fluxes, secretion of serotonin and prostaglandins, and alteration in the expression of genes leading to intestinal cell death and life-threatening diarrheal disease (De Haan and Hirst, 2004; Riemann and Oliver, 1998).

Cronobacter sakazakii (*Enterobacter sakazakii*), found in human and animal gut and in the environment, has been implicated in outbreaks causing meningitis or enteritis, especially in infants where 20% to greater than 50% mortality has occurred. *Cr. sakazakii* has been detected in a variety of foods, but only powdered infant formula has been linked to disease outbreaks (Drudy et al., 2006). Survivors can have long-lasting neurological complications. The outcome in adults seems to be significantly milder.

The FAO/WHO Codex Alimentarius Commission sets international standards for food. Current codex microbiological specifications for powdered infant formula limit the amount of coliforms, which includes *Cr. sakazakii*. While this limit probably helps to prevent a number of outbreaks, it does not confer a sufficient level of safety as evidenced by outbreaks caused by powdered formula meeting the current specifications. Given new information on this emerging problem, the recent expert meeting recommended that codex revise the international standard to better address the microbiological risks of powdered infant formula, including establishing a microbial specification for *Cr. sakazakii* (www.codexalimentarius.org/input/download/standards/…/CXP_066e.pdf; http://www.who.int/foodsafety/publications/micro/mra10.pdf).

Vibrio cholerae, especially the serogroups O1 and O139, have been responsible for large epidemics of cholera worldwide. CT is structurally and mechanistically similar to the heat-labile *E. coli* enterotoxin (De Haan and Hirst, 2004). Although consumption of sewage-contaminated drinking water is the predominant source of major epidemics, foodborne vibriosis can result from the consumption of fecal contaminated foods such as vegetables, fish, and pork products. Consumption of raw vegetables fertilized with untreated sewage and of shellfish harvested from sewage-contaminated estuaries is also a common source. Symptoms of intoxication are severe diarrhea characterized by watery stool (often referred to as rice–water–stool), associated with muscle cramps, hypovolemia, hypotension, shock, and metabolic acidosis due to loss of bicarbonate and poor tissue perfusion. Therapy mainly involves oral or intravenous (in extremely severe cases) rehydration therapy.

Listeriosis, in addition to being transmitted by other routes, is an emerging foodborne disease resulting from the consumption of *Listeria monocytogenes*–contaminated soft cheeses, milk and other milk products, poultry, meat (especially deli meats and frankfurters), and other products (salads, coleslaw, etc.) derived from contaminated vegetables. Food products are contaminated by contact with soil, feces, discharges, and urine from infected animals and humans. The clinical foodborne disease, occurring mostly in pregnant women, neonates, and older and immunocompromised populations, is characterized by GI or flu-like symptoms within 12 h of exposure followed by bacteremia leading to abortions, stillbirths, or premature births in pregnant women; meningitis, respiratory distress, and skin nodules in the neonates; and meningitis-related signs in adults (Cooper and Walker, 1998). The disease can be treated with antibiotics and other supportive measures. Prevention involves improvement of sanitation of the environment and equipment and education to identify and avoid contaminated food products.

Farm-to-fork hygienic measures implemented in Europe seem to be paying off in that the incidence of salmonellosis and certain parasitic diseases shows a decreasing trend in parallel with those in animals (no authors, 2012). Increased use of x-ray irradiation and other microbial reduction strategies on perishable heat-sensitive products such as lettuce, spinach, tomatoes, and almonds is likely to contribute to this decline (Moosekian et al., 2012). Increased reports of antibiotic resistance of the foodborne microbes including *Ca. jejuni*, *Salmonella* spp., and verotoxigenic *E. coli* (VTEC) globally, however, and a lack of public health impact of a total European ban on nonmedical uses of antibiotics indicate that bacterial foodborne disease is a long-term human condition (Koluman and Dikisi, 2013). Impending climate change is likely to add to the burden of waterborne and foodborne disease in humans further.

Viral Foodborne Illnesses

In the United States, as in all industrialized countries, nearly every person will have viral gastroenteritis at least once with

610,000 hospitalizations and more than 4,000 deaths annually. The advent of polymerase chain reaction (PCR), microarray, and proteomic virus detection techniques is beginning to allow the realization that most of the foodborne diarrheal illnesses that failed detection in the past are likely of viral origin. Viruses can neither grow nor produce toxins in foods but can induce foodborne illness by their mere presence in fresh produce or in processed food contaminated by fecal material. As reviewed (Clark and McKendrick, 2004; Leach, 2004), noroviruses, hepatitis virus A and E, rotaviruses, and astroviruses are the major culprits contributing up to two-thirds of all foodborne microbial illnesses. Their highly infectious nature and survival in pH and temperature extremes renders prevention by education, hygiene, and immunization (such as for hepatitis A) especially important in the protection against these illnesses:

Norovirus, due to its ability to be shed by asymptomatic individuals and its environmental stability, is considered the etiological agent responsible for most cases of foodborne gastroenteritis in the United States and worldwide accounting from 60% to 93% of all viral gastroenteritis cases. Infections occur following ingestion of airborne or foodborne (shellfish, water) viral particles without breaks occurring mostly in hospitals, nursing homes, and cruise ships. Expression of carbohydrates belonging to the ABH histo-blood group antigens that allow intestinal cell attachment by noroviruses renders the individuals more susceptible to virus infection. Generally mild clinical features of acute infection include fever, nausea, vomiting, diarrhea, abdominal cramps, headache, and myalgia with more severe and sometimes a fatal course in patients with immunosuppression (cancer or posttransplantation chemotherapy) and a chronic course in normal but stressed individuals. Secondary attacks occur at a high rate, resulting in high rates of transmission and large outbreaks. The high level of norovirus genetic variability in response to changing environment will likely pose considerable challenges to disease control by vaccination akin to the situation with influenza virus.

Rotavirus is the foremost cause of severe gastroenteritis of young children under 5, resulting in over 2 million hospitalizations and up to 600,000 deaths per year worldwide. Fever, nausea, vomiting, diarrhea, abdominal cramps, headache, and myalgia are also common features with the severity increasing in immunocompromised individuals (e.g., HIV, solid organ transplantation, bone marrow transplantation). Rotavirus diarrhea results from a combination of cell-damage-induced malabsorptive, viral enterotoxic peptide–mediated secretory, and enteric nervous system–mediated hypermotility components. Rotavirus enters extraintestinal sites including the blood, central nervous system (CNS), liver, spleen, and kidney. Whether this explains the occasionally reported sudden death, convulsions, and biliary atresia in children remains an investigation.

Hepatitis A and E induce foodborne illness at a rate of 3.77 cases/100,000 population with young adult men (age 25–39 years) at highest risk. Hepatitis A is transmitted primarily by the fecal–oral route and through contaminated food or drinks, especially uncooked fruits and vegetables and shellfish collected from contaminated habitats. After ingestion and absorption, the virus replicates in the liver and is excreted in bile, reaching the highest concentrations in the stool within 2 weeks at which time the risk of transmission is highest. Twenty percent of children age <3 years and 75% of adolescents and adults show symptoms of fever, malaise, abdominal pain, and jaundice lasting for 2 months or longer culminating in fulminant liver failure with the highest case-fatality rates occurring in adults >50 years. No specific therapy is available. Good hand hygiene, effective public water sanitation, and food hygiene are important. Immunoglobulin (from pooled plasma) and two inactivated hepatitis A vaccines offer a high degree of short-term and long-term protection, respectively (Leach, 2004). These measures appear to be paying off as evidenced by steadily declining cases each year. Hepatitis E outbreaks are rare in the United States and are related to consumption of contaminated drinking water during travel to endemic areas such as South Asia and North Africa where mortality rate, especially in pregnant patients, can be high (15%–25%).

Other foodborne microbial agents that contribute significantly to gastroenteritis toll around the world with increasing severity and duration in children, elderly, and immunocompromised individuals include the following: *Yersinia* sp. resulting from consumption of improperly cooked chitterlings (porcine large intestines) by people of African origin during major holidays; *Cryptosporidium* sp. from contaminated water and unpasteurized apple cider; *Cyclospora* infection from contaminated water and fresh berries; *Brucella* sp. from unpasteurized milk and meats from infected cattle, sheep, goats, and their products; and *viruses* such as astroviruses, enteric adenovirus, severe-acute-respiratory-syndrome-inducing coronavirus (SARS-CoV), toroviruses, human parechovirus, picobirnaviruses, cytomegalovirus, and herpes simplex virus.

Mycotoxins

From the standpoint of human and animal health, toxigenic molds belonging to the genera *Aspergillus*, *Fusarium*, and *Penicillium* have received the most attention owing to their frequent occurrence in food and feed commodities (Reddy

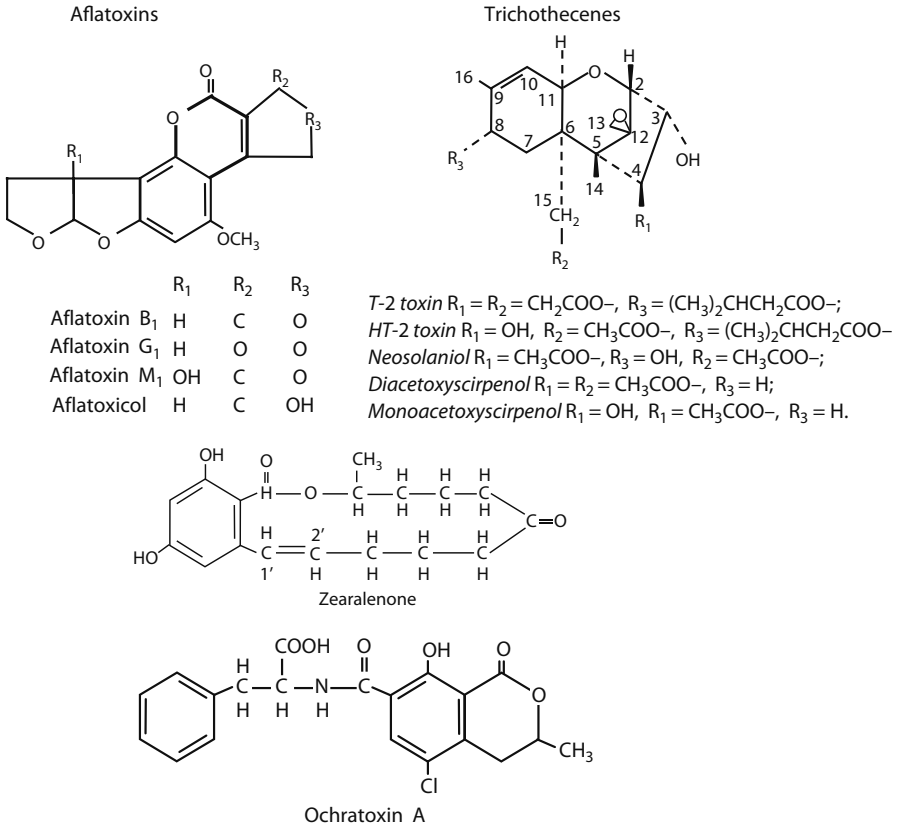


FIGURE 14.5 The structure of the mycotoxins, aflatoxins, trichothecenes, OTA, and zearalenone.

and Hayes, 2008). Unfavorable conditions such as drought and damage to seeds by insects or mechanical harvesting can enhance fungal toxin (mycotoxin) production during both growth and storage thus making mycotoxicoses a problem of both developing as well as developed countries. Although more than a hundred mycotoxins have been identified, the following discussion is limited to those with known public health significance. This subject has been recently reviewed (Bennett and Klich, 2003).

Aflatoxins are a group of highly substituted coumarins containing a fused dihydrofuran moiety (Figure 14.5) and are produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Four major aflatoxins designated B1, B2, G1, and G2 (based on blue or green fluorescence under ultraviolet [UV] light) are produced in varying quantities in a variety of produce including peanuts, various other nuts, cottonseed, corn, cereal grains, and figs that have not been adequately dried at harvest and stored at relatively high temperatures (Busby and Wogan, 1981a,b). Human exposure can also occur from consumption of products containing the aforementioned contaminated food materials as well as from tissues and milk (AFM_1, a hydroxylated metabolite) of food animals that have consumed mold-infested feed.

Aflatoxin B_1 (AFB_1), the most potent and the most commonly occurring aflatoxin, is acutely toxic (LD50 0.3–0.9 mg/kg) to all species of animals, birds, and fishes (Coulombe, 1991). The sensitivity of the animals varies based on the balance between metabolic activation (cytochrome P450) and protection (glutathione [GSH] synthesis) mechanisms with the mouse being very resistant to the carcinogenic effects of AFB_1. Effects of AFB_1 in animals are predominantly on liver and include death without signs, or signs of anorexia, depression, ataxia, dyspnea, anemia, and hemorrhages from body orifices acutely. In subchronic cases icterus, hypoprothrombinemia, hematomas, and gastroenteritis are common. Chronic aflatoxicosis, characterized by bile duct proliferation, periportal fibrosis, icterus, and cirrhosis of liver and associated with loss of weight and reduced resistance to disease (immune suppression), is more prevalent in domestic animals and is also likely to occur in humans (Osweiler et al., 1985). Prolonged exposure to low levels of AFB_1 in animals also leads to hepatoma, cholangiocarcinoma, or hepatocellular carcinoma, and other tumors (Busby and Wogan, 1981a,b). Based on a review of various epidemiological studies, the U.S. National Research Council (NRC, 1996) concluded that the risk of primary hepatocellular carcinoma from AFB_1 exposure may be one in 10,000 in the United States. However, in populations infected with hepatitis B, the risk may be 10–100 times higher. AFB_1 is mutagenic following metabolic activation in many systems including HeLa cells, *Bacillus subtilis, Neurospora crassa, and S. typhimurium* (Busby and Wogan, 1981a,b). AFB_1 is partly metabolized by the cytochrome P450 system in the liver into a variety of reactive products (e.g., AFB_1 8, 9-epoxide) and forms adducts with protein and DNA (Hsieh, 1986). The DNA lesions lead to inactivation of the tumor suppressor

gene ρ53 due to G > T transversions of codon 249 and can explain a high proportion of liver cancer in high-aflatoxin-exposure areas (Guerin and Tioulong, 1979). Such effect biomarkers as well as exposure biomarkers such as AFB_1–DNA or AFB_1–albumin adducts can be used to assess the effectiveness of preventive strategies (Ellenhorn and Barceloux, 1988). By way of protein adducts, AFB_1 inhibits many enzymes involved in DNA synthesis, including DNA-dependent RNA polymerase activity, messenger RNA synthesis, and protein synthesis (Hsieh, 1986), which may be related to several lesions and signs of aflatoxicosis including fatty liver (failure to mobilize fats from the liver), coagulopathy (inhibition of prothrombin synthesis), and reduced immune function.

Other less widespread human clinical syndromes in which aflatoxins have been implicated include childhood cirrhosis in India, possibly Reye's syndrome in many parts of the world, and acute hepatitis (aflatoxicosis) in India, Taiwan, and certain countries in Africa (Shank, 1981).

Widespread concern regarding the toxic effects of aflatoxins in humans and animals and the possible transfer of residues from animal tissues and milk to humans has led to regulatory actions governing the interstate as well as global transport and consumption of aflatoxin-contaminated food and feed commodities. Action levels for total aflatoxins in corn and other feed commodities used to feed mature nonlactating animals range from 100 to 300 ppb. For milk, the action level is 0.5 ppb. For other commodities destined for human consumption and interstate and potential global commerce, the action limit is 20 ppb (FDA Compliance Policy Guides 7106.10, 7120.26, 7126.23):

Ergot alkaloids cause a condition known as ergotism, which is now rare. In the midsixteenth century, the condition was first associated with the consumption of scabrous (ergotized) grain (rye, oats, wheat) subsequently found to be contaminated with *Claviceps purpurea*. Lysergic acid derivatives, the peptides and the amine alkaloids of ergot, were identified as the causative agents of the gangrenous and nervous forms of the disease. The gangrenous form, resulting from a predominance of alkaloids with α-adrenergic (ergotoxine) and vasopressor (ergotamine) action (Kunkel and Jallo, 1990), is typically manifested as prickly and intense heat and cold sensations in the limbs and swollen, inflamed, necrotic and gangrenous extremities that eventually sloughed off. Convulsive ergotism, characterized by CNS signs, numbness, cramps, severe convulsions, and death and abortions in animals, results from the antiserotonin or adrenergic effects in the CNS as well as the uterotonic effects of many of these alkaloids when combined (Kunkel and Jallo, 1990).

Fumonisins, produced by *Fusarium moniliforme Sheldon*, are common fungal contaminants of cereals, especially corn, around the world. Contamination of corn by *F. moniliforme* as well as its major metabolites, fumonisins B_1 and B_2, can induce one of several human and animal diseases among which are leukoencephalomalacia (LEM) in horses; pulmonary edema in swine; renal and hepatotoxicosis in horses, swine, and rats; and hepatocarcinogenic effect in rats (Dutton, 1996). Recent evidence suggests that FB_1 increases chromosomal aberrations in primary rat hepatocytes (Knasmuller et al., 1997) and developmental effects in the offspring secondary to hepatotoxicity in pregnant mice (Reddy et al., 1996). Consumption of high levels of fumonisins in homegrown corn has been associated with higher incidence of human esophageal cancer in certain regions of South Africa and China, northern Italy, and the United States (Charleston, SC). Fumonisins induce neural tube defects in animals, and their presence in corn products is potentially linked to a cluster of anencephaly and spina bifida cases in Texas (Bennett and Klich, 2003; Marasas, 1995). Although the mechanisms of toxic and carcinogenic effects are not clearly understood, inhibition of sphingolipid biosynthesis (Voss et al., 1995), enhancement of lipid peroxidation (Abado-Becognee et al., 1998), elevated secretion of tumor necrosis factor-alpha (Duffy et al., 1997), depletion of GSH levels (Kang and Alexander, 1996), elevated nitric oxide synthesis (Rotter and Oh, 1996), induction of protein kinase C translocation via its action on phorbol ester binding site (Yeung et al., 1996), and inhibition of protein serine/threonine phosphatases (Fukuda et al., 1996) are among the effects that can explain some or all of the effects of FB_1.

Ochratoxins, a group of seven isocoumarin derivatives linked with phenylalanine by an amide bond, are produced by *Aspergillus ochraceus* and *Penicillium verrucosum* (among others) in barley, corn, wheat, oats, rye, green coffee beans, peanuts, wine, cocoa, dried fruits, certain grape wines, and tissues (e.g., pork) and blood from contaminated animals (Bennett and Klich, 2003; Scudamore, 1998). In experimental animals, ochratoxin A (OTA) produces predominantly renal proximal tubular lesions and liver degeneration. The oral LD50 of OTA ranges between 0.2 mg/kg for the dog and 59 mg/kg in mice. Association between consumption of high-level OTA in the diet and nephropathy in humans and swine in the Balkan countries and swine in Denmark and the United States has been clearly established (Krogh et al., 1977; Lloyd et al., 1985). Signs include lassitude, fatigue, anorexia, abdominal (epigastric or diffuse) pain, and severe anemia followed by signs of renal damage. Reduced concentrating ability, reduced renal plasma flow, and decreased glomerular filtration occur sequentially accompanied by gross and microscopic renal changes including necrosis, fibrosis with some tubular regeneration, glomerular hyalinization, and interstitial sclerosis. Death results from uremia. Ochratoxins are

teratogens and probable (International Agency for Research on Cancer (IARC) class 2B) carcinogens (Scudamore, 1998) inducing hepatomas and renal adenomas secondary to genotoxic effects in mice (Kanisawa and Suzuki, 1978).

Relevant cellular effects that mediate the effects of OTA include alteration in enzymes involved in glucose metabolism, ATP synthesis, anion transport, lipid peroxidation, prostaglandins, and extracellular signal-regulated kinases (Creppy et al., 1995; Kuramochi et al., 1997; Meisner and Cimbala, 1985; Schramek et al., 1997).

Psoralens are furocoumarin compounds that have been used in repigmenting achromatic skin lesions in an acquired disease called vitiligo, in some suntan lotions, and in drugs used to treat psoriasis (Busby and Wogan, 1981a,b). Abuse of such compounds can result in dermatitis following exposure to the sun along with nausea, vomiting, vertigo, and mental excitation. A phototoxic dermatitis in celery pickers has also been linked to the presence of psoralens (8-methoxypsoralen, 5-methoxypsoralen, and trimethylpsoralen) in stalks infected with *Sclerotinia sclerotiorum* (pink rot), *Sclerotium rolfsii, Rhizoctonia solani*, or *Erwinia aroideae*, or in celery stalks soaked in 5% NaCl (NAS, 1977). Fig, parsley, parsnip, lime, and clove also contain psoralens. 8-Methoxypsoralen appears to undergo epoxidation of the furan ring similar to aflatoxins and may thus react with DNA in a similar fashion. Treatment with 8-methoxypsoralen and UV led to squamous cell carcinomas of the ear in mice (Busby and Wogan, 1981a,b).

Unlike other photosensitizing agents, psoralens seem to act by photoreacting with DNA and to a lesser extent with RNA. The mechanism of psoralen photosensitivity appears to involve intercalation and cross-linking of psoralen in the DNA, which occurs in three steps: (a) reversible intercalation of psoralen between two pyrimidines on opposing sides of the helix; (b) formation of a monoadduct with the 5,6 double bond of the pyrimidine following absorption of one quantum of UV light; and (c) cross-link formation by absorption of a second quantum of UV light and linking of the monoadduct to the 5,6 double bond of thymidine (Scott et al., 1976). In general, there is an excellent correlation between photoadduct formation and photosensitization of psoralens.

Trichothecenes are a group of 12,13-epoxy trichothecenes produced by Fusarium poae, Fusarium tricinctum, Fusarium graminearum, Fusarium nivale, Fusarium solani, Myrothecium roridum, and Stachybotrys atra, among others, in cereal grains including wheat. The group of macrocyclic trichothecenes including satratoxins, verrucarins, and roridins is produced mainly by Stachybotrys sp. in hay. Although more toxic, this group does not pose significant human health threat due to lack of prevalence. Group A trichothecenes (T-2 toxin, diacetoxyscirpenol) contain a side chain and are relatively less polar compared to group B (nivalenol, deoxynivalenol [DON], fusarenon). A two-volume treatise of trichothecene toxins and their role in human and animal health is available (Beasley, 1989).

Most trichothecenes of health significance are produced by *Fusarium* sp. Characteristic signs of alimentary toxic aleukia (ATA), caused by T-2 toxin and related trichothecenes, including radiometric damage such as irritation and necrosis of skin and mucous membranes, hemorrhage, destruction of thymus and bone marrow, and hematologic changes; nervous disturbances; necrotic angina; and shock are common to all toxic syndromes (Beasley, 1989). Feed refusal, vomiting, and immune suppression are common problems caused by DON-contaminated wheat and corn in farm animals, especially swine and dogs, and possibly humans (Osweiler et al., 1985; Pestka and Smolinski, 2005). Paradoxically, nivalenol and DON exposure for prolonged duration induced autoimmune-like effects similar to human IgA nephropathy (Rotter et al., 1996). Trichothecenes (T-2 toxin) can cause fetal death, abortions, and teratogenic effects (Beasley, 1989). Although several trichothecenes are genotoxic in bacterial, yeast, and cell culture systems (Knasmuller et al., 1997; Tsuda et al., 1998), they exhibit no initiator or promoter effect in whole animal systems (Lambert et al., 1995).

Metabolism of trichothecenes occurs rapidly through deacetylation and hydroxylation and subsequent glucuronidation in the liver and kidneys (Beasley, 1989; Rotter et al., 1996) thus posing little problem of residues in meats from contaminated animals. At the molecular level, DON and other trichothecenes disrupt normal cell function by inhibiting protein synthesis via binding to the ribosome and by activating critical cellular kinases involved in signal transduction related to proliferation, differentiation, and apoptosis (Pestka and Smolinski, 2005). In addition, they also effect serotonergic pathways in the brain and induce expression of a number of cytokines (Rotter et al., 1996). Recently, the European Commission Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established tolerable daily intakes of 1, 0.7, and 0.06 μg/kg body weight for DON, nivalenol, and the sum of T-2 and HT-2, respectively (Schlatter, 2004).

Zearalenone and zearalenol are nonsteroidal estrogenic contaminants (produced by *Fusarium roseum)* in grains such as corn, wheat, sorghum, barley, and oats. Zearalenone induces effects consistent with those produced by excessive steroidal estrogens, that is, anabolic and uterotropic activities and regulation of serum gonadotropins. Although

swine appear to be the most sensitive and exhibit signs of hyperestrogenic syndrome, that is, changes in serum luteinizing hormone, swollen and edematous vulva, hypertrophic myometrium, vaginal cornification and prolapse (in extreme cases), and infertility (Osweiler et al., 1985), human exposure to zearalenone and its metabolites by way of cereal products can also be significant. High frequency of premature menarche in Puerto Rico is suspected to be a result of high levels of zearalenone and similar compounds in the diet. Recommended safe daily human consumption of zearalenone appears to be 0.05 μg/kg body weight (Bennett and Klich, 2003).

Mode of action of zearalenone involves interaction with estrogen receptors, translocation of receptor–zearalenone complex to the nucleus, combination with chromatin receptors, selective RNA transcription leading to biochemical effects including increased water and lowered lipid content in muscle, and increased permeability of uterus to glucose, RNA, and protein precursors (Gentry, 1986). Available evidence indicates that rapid conversion of zearalenone and zearalanol to conjugated metabolites to be excreted in urine and feces makes consumption of meat and milk from animals receiving Ralgro an insignificant risk to humans.

Zearalenone is genotoxic in bacterial systems (Ghedira-Chekir et al., 1998), forms DNA adducts in female mouse tissues, and induces hepatocellular adenomas in female mice (Pfohl-Leszkowicz et al., 1995). Carcinogenic risk to humans and whether potentiative interaction exists between the adverse effects of zearalenone and those of dietary or endogenous estrogens as well as the xenoestrogens in the environment are unknown at the present time.

Other mycotoxins that have been identified either as contaminants in foods destined for human consumption or as metabolites of fungi isolated from human foods (Busby and Wogan, 1979) are summarized in Table 14.3. Although some of these have been associated with outbreaks of domestic animal diseases, the link between human consumption and disease is either emerging or nonexistent. Others have been shown to induce toxic and lethal effects in laboratory animals with no association between consumption of these toxins by animals or humans and a disease syndrome. Several of these, for example, cytochalasins and secalonic acid D (Reddy, 2005), have been used as research tools to expand our understanding of normal as well as abnormal cellular responses to xenobiotics.

Although it is difficult to assess the total significance of consumption of mycotoxins in human foods, it is easy to conceive that such a task requires extensive research into hundreds of known and potentially large number of as yet unknown mycotoxins. In spite of the vast number of toxic metabolites, reduction in mycotoxin levels in foods and feeds and the prevention of mycotoxicoses in humans and animals can be achieved for the most part by avoiding (1) stress in crops and (2) damage to seeds by pests and/or by mechanical harvesting. Rapid postharvest drying and avoiding conditions that promote mold growth during storage are equally important.

PREVENTION AND CONTROL OF MICROBIAL FOOD HAZARDS

The U.S. national animal health monitoring system has stepped up efforts to monitor food animal and poultry health on the farm and thus develop strategies to deal with potential increases in existing as well as emerging foodborne disease threats. The Food Safety Inspection Service (FSIS) of the USDA began implementing a hazard analysis and critical control point system (HACCP) for pathogen reduction in 1996 for all slaughter and processing operations. The HACCP directs each processing unit to (1) conduct a hazard analysis, (2) identify critical control points at which a safety hazard can be prevented, (3) establish limits at each point, (4) develop monitoring procedures and corrective action when limits are exceeded, and (5) implement record keeping that will allow subsequent verification by FSIS for compliance (Hogue et al., 1998). Data for *E. coli* and *Salmonella* burden of carcasses are used as evidence of fecal and enteric pathogen reduction. These two programs together with recent advances in the establishment of microbial genomic sequences and the development of PCR, DNA microarray, and proteomic methods will provide means for the rapid detection and identification of contaminating organisms and help minimize the incidence of foodborne disease from animal foods in the human populations. The recent approval by the FDA of low-dose irradiation of red meats to control pathogens coupled with previously approved irradiation of poultry for pathogen reduction; pork for control of trichinae; fruits, vegetables, and grains for insect control; and spices, seasonings, and dry enzymes used in food processing for microbial reduction (Andrews et al., 1998) should not only contribute to the reduction and potential prevention of foodborne disease caused by microbial pathogens but also help in increasing shelf life of such products without undesirable organoleptic, toxicological, or nutritional changes. Irradiation is yet to be approved for pathogen control of seafood products and is unsuitable for dairy products because of development of off-flavors and discoloration. In the final analysis, however, the keys to minimize the microbial foodborne illness is at the food preparer/consumer level in the form of hygienic processing, canning, and packaging; choosing reliable and clean food sources, water, and processing aids; cooking at the right temperature; avoiding cross-contamination (of cooked foods with raw); hygienic service (exclusion of infected food handlers from work); and/or prompt and appropriate storage and reheating. The Centers for Disease Control and Prevention (CDC) reports a significant decrease in foodborne disease burden in the last 10 years as a result of the existing regulatory and educational campaigns. It is hoped that the recently

TABLE 14.3
Miscellaneous Mycotoxins

Mycotoxin	Major Producing Organisms	Source of Fungi	Principal Toxic Effects
Alternariol and alternariol methyl ether	*Alternaria* sp.	Sorghum, peanuts, wheat	Highly teratogenic to mice; cytotoxic to HeLa cells; lethal to mice
Altenuene, altenuisol	*Alternaria* sp.	Peanuts	Cytotoxic to HeLa cells
Altertoxin I	*Alternaria* sp.	Sorghum, peanuts, wheat	Cytotoxic to HeLa cells; lethal to mice
Ascladiol	*Aspergillus clavatus*	Wheat flour	Lethal to mice
Austamide and congeners	*Aspergillus ustus*	Stored foodstuffs	Toxic to ducklings
Austadiol	*A. ustus*	Stored foodstuffs	Toxic to ducklings
Austin	*A. ustus*	Peas	Lethal to chicks
Austocystins	*A. ustus*	Stored foodstuffs	Toxic to ducklings; cytotoxic to monkey kidney epithelial cells
Chaetoglobosins	*Penicillium aurantiovirens, Chaetomium globosum*	Pecans	Toxic to chicks; cytotoxic to HeLa cells
Citreoviridin	*Penicillium citreoviride*	Rice	Neurotoxic, producing convulsions in mice
Citrinin	*Penicillium viridicatum, Penicillium citrinum*	Corn, barley	Nephrotoxic, swine
Cyclopiazonic acid	*Penicillium cyclopium*	Ground nuts, meat products	Nephrotoxic, enterotoxic
Cytochalasins	*A. clavatus* *Phoma* sp. *Phomopsis* sp. *Hormiscium* sp. *Metarhizium anisopliae*	Rice, potatoes, kodo millet, pecans, tomatoes	Cytotoxic to HeLa cells, teratogenic to mice and chickens
Diplodiatoxin	*Diplodia maydis*	Corn	Nephrotoxic and enterotoxic to cattle and sheep
Emodin	*Aspergillus wentii*	Chestnuts	Lethal to chicks
Fumigaclavines	*Aspergillus fumigatus*	Silage	Enterotoxic to chicks
Kojic acid	*A. flavus*	Squash, spices	Lethal to mice
Malformins	*Aspergillus niger*	Onions, rice	Lethal to rats
Maltoryzine	*Aspergillus oryzae*	Malted barley	Hepatotoxic and causes paralysis
Moniliformin	*F. moniliforme*	Corn	Cardiotoxic in rodents
Oosporein (chaetomidin)	*Chaetomium trilaterale*	Peanuts	Lethal to chicks
Paspalamines	*Claviceps paspali*	Dallisgrass	Neurotoxic to cattle and horses; causes paspalum staggers
Patulin	*Penicillium urticae*	Apple juice	Lethal to mice; mutagenic; teratogenic to chicks; pulmonary effects in dog; carcinogenic to rats
Penicillic acid	*Penicillium* sp.	Corn, dried beans	Lethal to mice; mutagenic; carcinogenic to rats
PR toxin	*Penicillium roqueforti*	Mixed grains	Hepatotoxic and nephrotoxic to rats; abortion in cattle
Roseotoxin B	*Trichothecium roseum*	Corn	Toxic to mice and ducklings
Rubratoxins	*Penicillium rubrum*	Corn	Causes hemorrhage in animals; hepatoxic to cattle
Secalonic acids	*Aspergillus aculeatus* *Penicillium oxalicum*	Rice, corn	Lethal, cardiotoxic, lung irritant, and teratogenic to mice
Slaframine	*Rhizoctonia leguminicola*	Red clover	Salivation and lacrimation in horses and cattle
Sporidesmins	*Pithomyces chartarum*	Pasture grasses	Hepatotoxic, causes photosensitization in ruminants
Sterigmatocystin	*A. flavus*	Mammals	Mutagen, carcinogen, and hepatotoxic to mammals
Tenuazonic acid	*Alternaria* sp.	Grains, nuts	Lethal to mice
Terphenyllins	*Aspergillus candidus*	Wheat flour	Hepatoxic to mice; cytotoxic to HeLa cells
Tremorgenic mycotoxins			
Fumitremorgens A and B	*A. fumigatus*	Rice	Neurotoxic (prolonged tremors and convulsions)
Paxilline	*Penicillium paxilli*	Pecans	Neurotoxic (prolonged tremors and convulsions)
Penitrems A, B, and C	*P. cyclopium*	Peanuts, meat products, cheese	Neurotoxic (prolonged tremors and convulsions) to cattle, sheep, dogs, and horses

(*continued*)

TABLE 14.3 (continued)
Miscellaneous Mycotoxins

Mycotoxin	Major Producing Organisms	Source of Fungi	Principal Toxic Effects
Tryptoquivalines	*A. clavatus*	Rice	Neurotoxic (prolonged tremors and convulsions)
Verruculogen (TR-1)	*Penicillium verruculosum*	Peanuts	Neurotoxic (prolonged tremors and convulsions)
Unidentified toxin(s)	*Aspergillus terreus* *Balansia epichloe* *Epichloe typhina* *F. tricinctum* and others	Fescue grass	Gangrene (fescue foot); summer slump syndrome; fat necrosis and agalactia in cattle
Xanthoascin	*A. candidus*	Wheat flour	Hepatotoxic and cardiotoxic to mice

Source: Condensed and modified from Busby, W.F. Jr. and Wogan, G.N., Foodborne mycotoxins and alimentary mycotoxicoses, in *Foodborne Infections and Intoxications*, 2nd edn., Riemann, H. and Bryan, F.L., eds., Academic Press, New York, pp. 519–610, 1979.

passed Food Safety Modernization Act of 2011, which imposes a broader range of food industry responsibilities for safer manufacture of food products and allows for a broader authority for federal enforcement, will make even greater strides in the prevention of such problems.

Potential person-to-person spread of microbial diseases via the medium of food and the modern day global nature of human travel and movement of food necessitates global harmonization of efforts in the prevention of the spread of foodborne disease agents. A recent collaborative effort between WHO, FAO, and the World Organisation for Animal Health resulted in agreement (1) to develop reporting and surveillance methods of the incidence of the diseases at the national and international level; (2) to develop international animal health standards for foodborne disease agents that do not cause clinical disease in animals; (3) to study farm ecology (environmental survival, multiplication, and spread and colonization in the animal) of foodborne pathogens; (4) to harmonize foodborne disease investigation and diagnostic methodology and quality control; and (5) to develop uniform application of risk-based farm-to-table approach when developing food safety standards (Magnuson et al., 2011).

NATURAL OCCURRING TOXINS IN THE FOOD SUPPLY

Mushroom Peptides

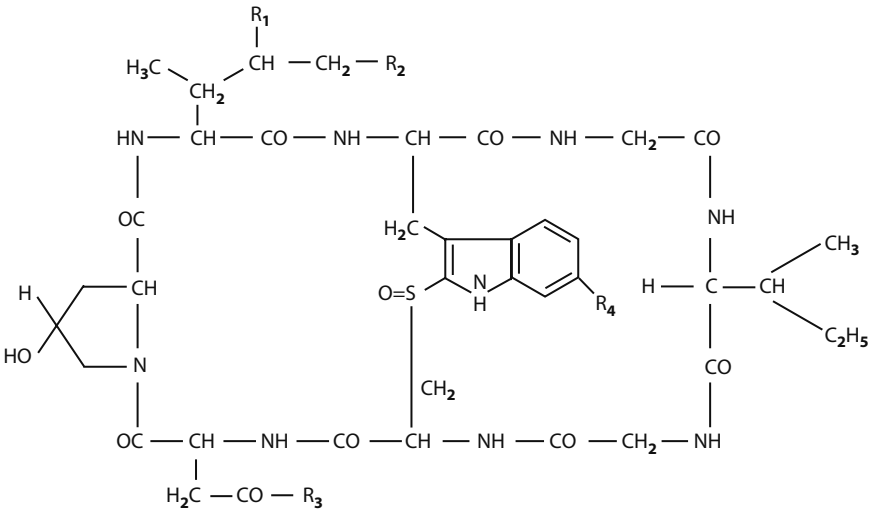
Cultivated mushrooms are, for the most part, safe. However, among the approximately 5000 species of mushrooms that exist in nature, at least 300 have been shown to be safely edible, while ingestion of up to 200 (generally collected from the wild) has been reported to be toxic and occasionally lethal (12 known) (Puschner and Wegenast, 2012) with 90% of the poisonings occurring in individuals under 19 years of age. One or more of the following classes of compound, cyclopeptide, orellanine, monomethylhydrazine, disulfiram-like, hallucinogenic indoles, muscarinic, isoxazole, GI-specific irritant toxins, are often involved in mushroom poisonings (Table 14.4).

Cyclopeptide toxicants are thermostable and are comprised of *amatoxin* (Figure 14.6), *phallotoxin*, *and verotoxin* groups, the latter two producing effects only at high doses. Approximately one-half of a mature cap of *Amanita verna* (destroying angel common in the United States) or *Amanita phalloides* (green death cap in Europe), containing amanitin, can be lethal in an adult (McPartland et al., 1997). Clinical effects that begin to appear after a 12 h latency period include epigastric tenderness, intense and cramping abdominal pain, nausea, vomiting, and severe secretory diarrhea (possibly bloody) and hepatomegaly with secondary acid–base disturbances, electrolyte abnormalities, hypoglycemia, dehydration, and hypotension. This is followed by elevation of liver enzymes (AST and ALT) and bilirubin, coagulopathy, hypoglycemia, acidosis, hepatic encephalopathy, hepatorenal syndrome, multiorgan failure (including pancreas, adrenal, and testes), disseminated intravascular coagulation, mesenteric thrombosis, convulsions, and death 6–16 days postingestion (Berger and Guss, 2005a). Fatalities are common (10%–30%) even following intensive symptomatic care, which includes fluid replacement, activated charcoal hemoperfusion, and forced diuresis. Penicillin therapy (by an unknown mechanism) and, in countries other than France and the United States, the use of silibinin (from the milk thistle plant, *Silybum marianum*) that prevents hepatocyte uptake of amatoxins have produced beneficial effects in direct relationship with the speed of onset of therapy (McPartland et al., 1997; Vetter, 1998). A return toward normal glucose, factor V, and fibrinogen is prognostic of recovery (McPartland et al., 1997) and may take several weeks to months. Amatoxins act by binding to and inhibiting RNA polymerase II and thus mRNA and protein synthesis leading to cell necrosis (Berger and Guss, 2005a; Vetter, 1998).

The other two groups of polypeptide toxins, the *phallotoxins and virotoxins*, are capable of causing toxic effects only at relatively high doses. The effects of phallotoxins include swelling of the liver due to engorgement of hepatic sinusoids with blood and depletion of blood in the peripheral circulation leading to shock. Reduction of cellular G-actin concentration by a combined effect of stimulated G-actin polymerization into F-actin and inhibition of F-actin depolymerization leading to a loss of membrane elasticity and thus to cell surface vesiculation leads to hepatocyte damage (Stob, 1983).

TABLE 14.4
Mushroom-Induced Syndromes

Syndrome	Mushroom Species	Toxic Compound(s)	Effects	Mechanism	Prevention/Treatment
Rapid onset:					
Gastrointestinal	*Chlorophyllum molybdites* *Entoloma lividum* *Omphalotus olearius* *Paxillus involutus* *Tricholoma pardinum*	Many unknown	Emesis, diarrhea	Unknown	Cooking/fluid replacement
Parasympathetic	*Inocyte* sp. *Clitocybe* sp. *Omphalotus illudens* *Amanita* sp.	Muscarine and related	Increased salivation, lacrimation, and urination; diarrhea; dyspnea; sweating; bradycardia; tremors, etc.	Parasympathetic stimulation	Avoid/atropine
CNS syndrome	*Psilocybe* sp. *Panaeolus* sp. *Copelandia* sp. *Gymnopilus* sp.	Psilocybin Psilocin	Hallucinations involving all sensations; hyperthermia, convulsions, coma, and death	Serotonin agonist	Avoid/diazepam and cooling
	Am. pantherina	Ibotenic acid, muscinol, stizolobic, and stizolobinic acid	Alternating depression and neuromuscular stimulation	Stimulation of bicuculline-reactive postsynaptic receptors	Avoid/diazepam and respiration
	Am. muscaria				
Alcohol sensitization	*Coprinus* sp. *Clitocybe clavipes* *Boletus luridus* *Verpa bohemica*	Coprine and others	Nausea, vomiting, headache, hypotension, tingling, palpitations, tachycardia, testicular damage, etc.	Inhibit acetaldehyde dehydrogenase	Avoid mushroom and alcohol/ supportive
Delayed onset:					
Headache	*G. esculenta* (false morel) *Gyromitra* sp.	Gyromitrin, monomethylhydrazine, etc.	Fatigue, head- and body ache, vomiting, liver damage, death, carcinogenic	Interfere with pyridoxine	Cook or dry, don't inhale vapors
Nephropathy	*Cortinarius* sp.	Orellanine Cortinarin	Polydypsia, oliguria, nausea, head and body aches, chills, etc. Renal tubular and liver necrosis, death	Membrane damage from oxygen-derived free radicals (similar to paraquat)	Hemodialysis
Carcinogenic	*Agaricus bisporus* (edible)	Agaritine, hydrazines	Lung tumors	Genotoxic	Cooking
Hepatotoxic	*Am. phalloides* (Europe) *Amanita virosa* (the United States) *Galerina* sp. *Lepiota* sp.	Amatoxins, phallotoxins, and virotoxins	Emesis and diarrhea, increase in serum enzymes, decrease in glucose and clotting factors, hepatic and renal damage, jaundice, coma, and death	1. Inhibit RNA polymerase 2. Enhance G-actin polymerization into F-actin 3. Inhibit F-actin depolymerization	1. Correct glucose and clotting effects 2. Decontaminate 3. Penicillin and silibinin 4. Supportive 5. Transplant liver



	R_1	R_2	R_3	R_4
α-Amanitin	OH	OH	NH_2	OH
β-Amanitin	OH	OH	OH	OH
γ-Amanitin	CH	H	NH_2	OH
ε-Amanitin	OH	H	OH	OH
Amanin	OH	OH	OH	H
Amanullin	H	H	NH_2	OH
Amaninamide	OH	OH	NH_2	H

FIGURE 14.6 The structures of amatoxins.

The following toxic syndromes produced by other (some nonpeptide) classes of mushroom toxins are summarized in Table 14.4 and discussed in detail (Berger and Guss, 2005b).

Certain degree of initial gastritis (vomiting) and/or enteritis (diarrhea) is a common feature of most mushroom poisonings. Delayed (for up to 3 weeks) acute *renal failure* (interstitial fibrosis and acute tubular necrosis manifesting as polyuria followed by oliguria) is caused by heat-stable bipyridyl orellanine, 2,2′ bipyridine-3,3′,4,4′-tetrol-1,1′-dioxide, or 3,3′,4,4′-tetrahydroxy-2,2′-bipyridine-N,N′-dioxide present in *Cortinarius orellanus, Cortinarius speciosissimus, Cortinarius splendens*, and *Cortinarius gentilis (in Europe) or Amanita smithiana* (*in the Pacific Northwest United States*). Toxicosis begins as weakness, lassitude, and headache and, in severe cases, hepatic failure developing over several days, resulting in hypoglycemia, delirium, and seizures progressing to coma and death, and is caused, especially in isoniazid-sensitive individuals, by a hydrolysis product (N-monomethylhydrazine) of the volatile nonpeptide toxin gyromitrin present in false morels represented by *Gyromitra* species, particularly *Gyromitra esculenta* in Europe. Renal failure can occur a few weeks after ingestion. Inhibition of gamma amino butyric acid (GABA) synthesis and/or induction of pyridoxine deficiency appears to be the contributory mechanism. *Am. smithiana* mushroom, easily mistaken for the matsutake or pine mushroom by overconfident collectors, poisoning presents within 6 h of ingestion with GI toxicity and develops delayed onset of renal insufficiency over the first 1–4 days. Mild elevation of hepatic transaminases peaks 24 h after the ingestion followed by renal injury by 3–5 days postingestion, earlier than with other orellanine-containing mushroom ingestion. Recovery can be expected with aggressive support requiring hemodialysis that may be discontinued after several weeks when creatinine returns to normal and urine output returns. This pattern of delayed-onset renal toxic mushroom ingestion is emerging among mushroom ingestions in Western North America (West et al., 2009).

A *disulfiram (Antabuse)-like* syndrome characterized by headache, paresthesias of the hands and feet, metallic taste, facial flushing, palpitations, tachycardia, orthostatic hypotension, chest pain, nausea, vomiting, and diaphoresis occurs if alcohol is consumed within 72 h after consumption of otherwise safe mushrooms, *Coprinus atramentarius* (common lawn mushroom) and *Clitocybe clavipes*. The syndrome lasts for up to 2 days and is induced by the amino acid coprine [N^5-(1-hydroxycyclopropyl)–L-glutamine]. 1-Aminocyclopropanol hydrochloride is a hydrolytic product of coprine that inhibits aldehyde dehydrogenase leading to accumulation of acetaldehyde from alcohol metabolism (Puschner and Wegenast, 2012).

A *cholinergic syndrome* characterized by salivation, lacrimation, abdominal pain, diarrhea, emesis, perspiration, and occasionally, miosis, rhinorrhea, flushed skin, bradycardia, and hypotension is induced by a heat-stable parasympathomimetic compound, muscarine, present in several lawn and park mushrooms of the genera *Inocybe* or *Clitocybe*. Muscarine activates ACh receptors on the heart, apocrine glands, and

smooth muscle. Nicotinic receptors are not affected unlike with insecticides that inhibit acetylcholinesterase. Because the toxin is not degraded by enzymes such as acetylcholinesterase, the binding to and stimulation of cholinergic receptors is indiscriminate (Puschner and Wegenast, 2012).

Psilocybin (O-phosphoryl-4-hydroxy-N,N-dimethyltryptamine), present in at least 75 mushrooms belonging to *Psilocybe* and *Panaeolus* and *Conocybe*, *Gymnopilus*, and *Stropharia* species, also called *funny* or *magic* mushrooms, alters brain catecholamine levels, especially serotonin, leading to lysergic acid diethylamide (LSD)-like signs including visual and auditory hallucinations, confusion, disorientation, inappropriate behavior, and mydriasis. Rarely, cardiotoxicity manifests as myocardial infarction and serious supraventricular tachycardia.

Beautifully colored mushrooms, *Amanita muscaria* and *Amanita pantherina*, contain isoxazoles (ibotenic acid and muscimol) whose thermostable metabolites stimulate N-methyl-D-aspartate (NMDA) and GABA receptors inducing a syndrome in which symptoms oscillate between various degrees of depression and hyperactivity associated with unrealistic and bizarre behavior.

Although *gastroenteritis* induced by most common mushrooms is mild, that induced by *Chlorophyllum molybdites* (found in lawns, fields, and open woods in southern and Midwestern United States) can be severe requiring immediate medical attention.

Other identified human conditions associated with mushroom production, commerce, and consumption are hypersensitivity to edible mushrooms in certain populations; hypersensitive allergic alveolitis and other pulmonary allergic changes in mushroom workers from spores of certain edible mushrooms (mushroom worker's lung); hemolytic reactions following consumption of mushrooms belonging to genera *Gyromitra*, *Boletus*, and *Paxillus*; and dermatitis (allergic) from contact with one or more species of the genera *Boletus*, *Lactarius*, *Calvaria*, and *Agaricus*. Treatment of such mushroom poisoning is mostly supportive.

Extracts and isolated metabolites from mushrooms can either enhance or suppress innate and acquired immunity leading to beneficial effects such as increased disease resistance, anticancer activity, suppression of autoimmune (T[H]1 type T-cell-mediated) and allergic (T[H]2 type T-cell-mediated) diseases. Mechanistically, low-molecular-weight (MW) metabolites affect apoptosis-, angiogenesis-, metastasis-, cell cycle-related signaling, and high-MW components (polysaccharides or polysaccharide–protein complexes) enhance innate and cell-mediated immune responses leading to altered mitogenic response, T-cell differentiation, and activation of immune effector cells such as lymphocytes, macrophages, and natural killer cells (Lull et al., 2005). Additional beneficial effects of mushrooms include inhibition of clotting and reductions in blood cholesterol and pressure (Berger and Guss, 2005b).

Toxicants in Food of Plant Origin

Foods of plant origin account for most (>70%) of the world's supply of protein. Although plants with obvious toxic effects have been excluded from human diet by trial and error, deleterious (toxic as well as antinutritive) effects from the following groups of compounds are deemed significant for human health.

Alkaloids are nitrogenous heterocyclic organic compounds that protect plants against herbivorous consumption and attack by insects, parasites, and competitors. Major alkaloid groups of concern from the standpoint of human consumption include pyrrolizidines, xanthines, and solanines. Others including piperidines from *Conium* and tobacco; quinolizidines from *Lupinus*. Indolizidines from *Astragalus*, *Swainsona*, and red clover are mainly consumed by grazing animals and can be potentially transferred to humans through milk.

Pyrrolizidine alkaloids (PAs) are a group of more than 300 plant metabolites, including the more toxic acyclic diesters and macrocyclic diesters such as retronecine, senecionine, and petasitenine, posing major threat to human and animal health by their ubiquitous presence in plants such as *Senecio*, *Crotalaria*, and *Heliotropium*. Human exposure and possible health effects result from the wide use of coltsfoot (*Tussilago*), comfrey (*Symphytum*), and petasites (*Petasites*) as herbal remedies, foods (salads), and tea; contamination of food grains with seeds from PA-containing plants; honey derived from pansy ragwort (*Senecio* sp.) and Patterson's curse (*Echium* sp.); and/or through milk from animals grazing alkaloid-containing plants mentioned earlier (Stegelmeier, 2011).

Highly reactive pyrrole derivatives of PA and/or their hydrolysis products formed by the action of mixed function oxidases are considered to be responsible for the toxic effects of PA. Many PAs and their pyrrole metabolites are bifunctional alkylating agents that cross-link to macromolecules including DNA, protein, amino acids, and GSH to produce adducts that inactivate these biomolecules. Typically, high mortality associated with endothelial proliferation and medial hypertrophy that causes occlusion of small branches of hepatic vein leading to liver dysfunction. Centrilobular hepatocyte necrosis and fibrosis (cirrhosis) with ascites/edema is more severe in children and experimental animals. Signs of liver damage include elevations of serum enzymes (AST, ALT, and others) and bilirubin, photosensitivity, and icterus (Stegelmeier, 2011). Simultaneous exposure of rats to bacterial endotoxin synergized the hepatotoxicity of monocrotaline (Yee et al., 2000). Occlusions in the renal and pulmonary arterioles can lead to renal disease with reduced urinary output, pulmonary hypertension, right ventricular hypertrophy, and ultimately to right heart congestive failure. PAs are developmentally toxic inducing lower jaw hypoplasia, musculoskeletal defects involving ribs, and general growth retardation in animals (Hirono, 1987) and hepatomegaly and ascites in humans (Rasenack et al., 2003).

PAs are mutagenic and are carcinogenic in experimental animals having been shown to induce one or more of the following types of cancer: leukemia, hepatic carcinoma, hemangioendothelial sarcoma in the liver, liver cell adenoma, cholangiosarcoma, astrocytoma, squamous cell carcinoma of

the skin, pulmonary adenoma, adenocarcinoma of the small intestines, adenomyoma of the ileum, and rhabdomyosarcoma in animals (Chen et al., 2010; NTP, 2011). Alterations in hepatocyte/endothelial vascular endothelial growth factor (VEGF), in KDR/flk-1 activation, and/or in other oncogenes and tumor suppressor genes such as *K-ras*, *beta-catenin*, and *p53* have been proposed as mechanisms leading to sustained endothelial cell proliferation and thus to hemangiosarcoma. Although no human data exist, riddelliine, a prototype genotoxic PA, is considered by the U.S. national toxicology programme (NTP) as a reasonably anticipated human carcinogen (NTP, 2011).

Solanum alkaloids including solanine, chaconine, and tomatine are found predominantly in potato, eggplant, and tomato (species of *Solanum* genus).

Potatoes, especially the sprouted, greened, blighted, injured, or spoiled, can contain greater than the USDA guideline of 20 mg/100 g of tuber. Exposure to light, immature tubers, wounding of potatoes, and stresses such as fungal attack can increase the content of *solanum alkaloids* severalfold (Beier, 1990) and may have contributed to intoxication in hundreds of cases of human poisonings (Morris and Lee, 1984). Signs of intoxication, some of which may be related to the irritant, estrogenic and cholinesterase-inhibiting activity of the alkaloids, in humans appear at >20 mg alkaloid/100 g of tuber and include headache, vomiting, diarrhea, neurological signs, debilitation, and even death. Recent studies suggest that these alkaloids may sensitize individuals to inflammatory bowel disease (Iablokov et al., 2010); may induce GI distress, dizziness, slurred speech, cranial nerve deficits, and ataxia (Smith et al., 2008); and may cause diabetes insipidus (Friedman et al., 2003). Prolonged exposure at lower doses of these alkaloids can cause increased liver to body weight ratios (Friedman et al., 2003) and antiandrogenic effects (Gupta and Dixit, 2002) in experimental animals. A combination of anticholinergic and antagonistic actions against TNFalpha-induced elevation of $[Ca^{2+}]i$ and plasminogen activator inhibitor likely accounts for these actions.

Exposure of pregnant rats to certain alkaloids including solanine, solasodine, choconine, and cytochalasins B, D, and E resulted in minor skeletal to major facial and CNS abnormalities in the offspring, whereas that of others such as tomatidine were without effects (Schardein, 1985). Baking, boiling, or microwaving does not destroy these alkaloids. Protection of tubers from sunlight, γ-irradiation, soaking in water under controlled conditions, dipping damaged potatoes in emulsified water, treating potatoes with sprout inhibitors during storage, waxing and heating, dipping in oils (corn, olive, or mineral), spraying tubers with lecithin (such as PAM), or simply spray rinsing tubers with an aqueous solution of an edible surfactant (Tween 85) appear to be some simple methods to prevent glycoalkaloid formation during storage (Sharma and Salunkhe, 1989).

The xanthine alkaloids, caffeine, theobromine, and theophylline, are found as major components of coffee (*Coffee arabica*), cocoa (*Theobroma cacao*), and tea (*Thea sinensis*), respectively. Caffeine, in addition, is added to many beverages, foods, and medications (Ellenhorn and Barceloux, 1988). Caffeine-related adverse effects begin when 0.5–1.0 g of caffeine (10 cups of coffee) is ingested by an adult with possible fatalities at 5 g in children and 5–10 g in adults (Daly, 1993; Ellenhorn and Barceloux, 1988). Caffeine and other methylxanthines enhance the release of catecholamines, inhibit phosphodiesterase leading to intracellular accumulation of cyclic AMP, block adenosine receptors, and cause increased release of Ca^{2+} from the terminal cisternae of the sarcoplasmic reticulum (Daly, 1993). Major effects of xanthines involve CNS stimulation (hyperesthesia to convulsions), emesis, cardiovascular effects (cardiac stimulation to arrhythmias), diuresis, and smooth muscle effects leading to decreased vascular resistance and bronchodilation (Daly, 1993). In addition, caffeine enhances gastric secretion of acid and pepsin. In habitual coffee drinkers, cessation of caffeine consumption often results in a withdrawal syndrome characterized by headache, fatigue, drowsiness, depression, difficulty concentrating, irritability, and lack of clarity in thinking among other symptoms (Juliano and Griffiths, 2004). Caffeine increases serum homocysteine, a risk factor for cardiovascular disease (Verhoef et al., 2002), and induces bone loss in postmenopausal women (Rapuri et al., 2001). Caffeine and theobromine are mutagenic in bacterial systems and can potentiate DNA damage caused by other genotoxicants but are neither directly carcinogenic in animals nor associated with human cancer (Ames, 1983; Daly, 1993). Caffeine actually appears to protect against certain cancers, type II diabetes, preeclampsia of pregnancy, and development of parkinsonism (Ascherio et al., 2001; Khoury et al., 2004; Salazar-Martinez et al., 2004). Caffeine is teratogenic in experimental animals causing mostly limb and facial defects (Schardein 1985). Although high caffeine consumption during pregnancy may increase the risk of spontaneous abortion and low birth-weight babies, no correlation exists between caffeine consumption and birth defects in humans (Khoury et al., 2004; Spencer et al., 1987). Greater danger, however, appears to be associated with combined consumption of caffeine with other vasoactive herbal ingredients and medications. Oral exposure to caffeine (30 mg/kg) along with ephedrine (25 mg/kg) in rats (1.4- and 12-fold, respectively, above average human exposure) resulted in death of rats within 4–5 h accompanied by massive interstitial hemorrhage and degeneration and necrosis of myofibers in the myocardium of the left ventricle and interventricular septum (Nyska et al., 2005). The Canadian government (Nawrot et al., 2003) issued guidelines to limit daily caffeine consumption to 400 mg (~eight cups of coffee) in healthy adults, 300 mg (~six cups of coffee) in women of reproductive age, and 2.5 mg/kg in children, as did the U.S. FDA for pregnant women (Daly, 1993).

Cyanogenic glycosides that release highly toxic hydrocyanic acid upon hydrolysis are derived not only from plants (more than 2000) but also from fungi, bacteria, and even members of the animal kingdom (Montgomery, 1980). Although cassava, sweet potatoes, yam, maize, millets, bamboo, sugarcane, peas, beans, almond kernel, lemon,

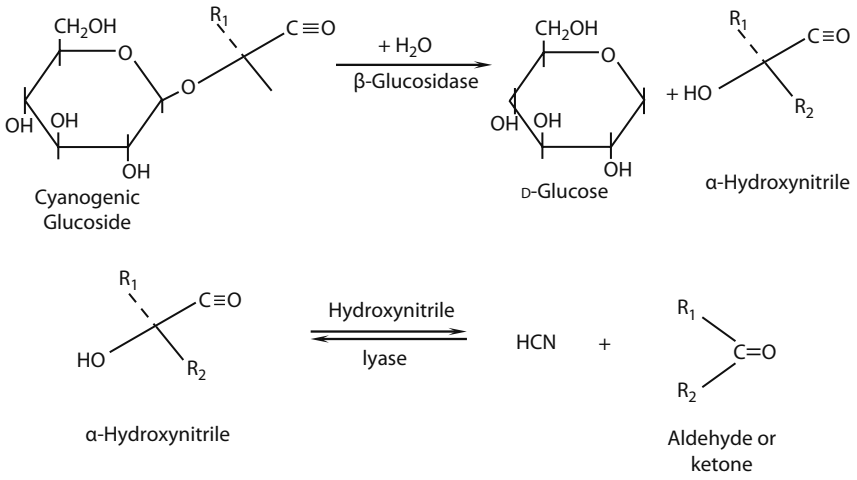


FIGURE 14.7 Enzymatic hydrolysis of cyanogenic glycosides.

lime, apple, pear, cherry, apricot, prune, and plum constitute sources for humans, poisonings are mainly associated with the consumption of improperly processed cassava in Africa, Asia, and Latin America (Barceloux, 2009). Among more than 20 glycosides identified, only four (i.e., prunasin, amygdalin, dhurrin, linamarin, and lotaustralin) appear to be of toxicologic importance (Barceloux, 2009). Cyanogenic lipids, although of unknown toxicological significance, are also present in plants and yield carbonyl compound and HCN upon hydrolysis (Deshpande and Sathe, 1991).

The hydrolysis of the glycoside is triggered by physical disruption (mastication, trampling, etc.) or stress (drought, cooking, frost, etc.) and is catalyzed by β-glucosidase and hydroxynitrile lyase, which are present within the plant or in bacteria in the GI tract of man and animals (Poulton, 1983). The scheme of breakdown leading to the formation of glucose and hydroxynitrile from the glycoside followed by breakdown of hydroxynitrile into carbonyl compounds and HCN is presented in Figure 14.7. Mitochondrial rhodanese catalyzes the conversion of HCN to thiocyanate in the presence of thiosulfate (Poulton, 1983).

Initially, the glycoside is hydrolyzed by a β-glucosidase releasing glucose and α-hydroxynitrile. The hydroxynitrile dissociates either enzymatically or nonenzymatically to yield HCN and the corresponding aldehyde or ketone.

Animals have often been acutely poisoned by young sorghum and arrow grass. Young bamboo shoots and tea made from peach leaves are examples of dietary sources of HCN poisoning in children. The minimal lethal dose of HCN in man and animals is 0.5–3.5 and 2–10 mg/kg, respectively. The acute effects of HCN result from its affinity toward metalloporphyrin-containing enzymes, more specifically cytochrome oxidase. Cyanide concentration of only 33 μM can completely block electron transfer through the mitochondrial electron transport chain and thus prevent O_2 utilization (Poulton, 1983). Death results from generalized cytotoxic anoxia. Signs of acute cyanide poisoning in humans are hyperventilation, headache, nausea and vomiting, generalized weakness, coma, and death due to respiratory depression and failure. Treatment of acute cyanide intoxication involves, in addition to artificial respiration, the conversion of hemoglobin in the blood to methemoglobin with nitrites (sodium or amyl). Methemoglobin competes with cytochrome oxidase for HCN and forms cyanmethemoglobin. Coadministration of sodium thiosulfate will convert free cyanide present in the blood to thiocyanate, which is eliminated in the urine. As free cyanide in the blood decreases, additional cyanide dissociates from the cyanmethemoglobin and is subsequently eliminated (Chen and Rose, 1952).

Tropical ataxic neuropathy (TAN), characterized by myelopathy, bilateral optical atrophy, deafness, and polyneuropathy (konzo), is an irreversible upper motoneuron paralytic disease (Banea-Muyambu et al., 1997) of women and children. Goiter, epigastric burning pain, dizziness, and abdominal distension/vomiting (Abuye et al., 1998) have been linked to longer-term consumption of cassava diets in Africa and other tropical countries (Osuntokun, 1973). These diets were also poor in protein and sulfur-containing amino acids that can detoxify HCN to thiocyanoalanine and subsequently to inert 2-amino-4-thiazolidine carboxylic acid (Poulton, 1983). Although the chronic effects of cyanogen exposure were earlier thought to be due to thiocyanates, recent evidence suggests that both linamarin and cyanide directly interact with methionine and cysteine making them unavailable for GSH synthesis and thus lead to GSH depletion leading to konzo in humans and animals (Banea-Muyambu et al., 1997; Nunn et al., 2011; Soto-Blanco et al., 2002). The recent introduction of a transgenic cassava (Biocassava+) with improved nutritional quality and longer shelf life, reduced glycoside content, and resistance to viral diseases is expected to reduce the incidence of konzo (Sayre et al., 2011).

Cycads, the palmlike plants adapted for adverse climatic conditions of the tropical and subtropical areas of the world, are still used (seeds and stem) as a source of starch in Guam, Kenya, Amami Oshima, Miyako Island, and southern Japan by small groups of people (Matsumoto, 1983). Adverse effects result from incomplete extraction of toxicants, including cycasin and β-N-methylamino-L-alanine (BMAA), during

preparation of the flour. Several neurologic conditions including a paralytic disease (amyotrophic lateral sclerosis [ALS]) and Parkinsonism–dementia (PD) have been reported among the native Chamorro in Guam resulting from the consumption of cycad. Gait disturbances, motor weakness, and paralysis have also been reported in cattle grazing on cycads. Parkinsonian features and degenerative changes in CNS motor neurons in monkeys and, very importantly, Alzheimer's dementia (AD) in human appear to be related to cycad toxicants, especially BMAA (Matsumoto, 1983; Murch et al., 2004; Spencer et al., 1987). In this regard, it is important to note that BMAA has been shown to be produced by potentially all Cyanobacteria (Cox et al., 2005) in many parts of the ecosystem including the root tissues of cycad trees. BMAA also accumulates in cycad seeds and is biomagnified by seed-eating flying foxes that, in turn, are consumed by the Chamorros.

BMAA has been found in the brains of Chamorros that died of neurodegenerative ALS/PD syndrome and in Canadians with AD (Murch et al., 2004), suggesting the possible involvement of Cyanobacteria as an etiological factor for AD. However, the inability to reproduce these findings (Montine et al., 2005) and the failure to produce lesions of AD in experimental animals fed with BMAA question this hypothesis. Mechanistically, attenuation of the cycad-induced neurotoxic syndrome by AP7 and MK801, two selective antagonists of the NMDA receptor and its associated ion channel, suggests a role of the excitatory neurotransmitters in the causation of ALS–PD, other motor-system diseases (Huntington's chorea, Parkinson's disease, and olivopontocerebellar atrophy), and possibly Alzheimer's disease (Spencer et al., 1987). Other effects of cycasin, its aglycone, or cycad flour include hepatic necrosis; subserosal hemorrhages; accumulation of yellow fluid in serosal cavities; benign and malignant tumors in the liver, kidney, lungs, and GI tract (mainly colon); neuroteratologic effects in offspring; death in experimental animals; and mutagenic effects in a variety of in vitro and in vivo systems.

Interestingly, cycasin is neither toxic nor carcinogenic when given parenterally to conventional rats or when given either orally or parenterally to germ-free rats, suggesting that the intestinal flora mediates cycasin toxicity. Bacterial β-glucosidase hydrolyses cycasin to the active carcinogen, methylazoxymethanol (MAM), which produces hepatomas in rats. MAM spontaneously breaks down to methyldiazonium hydroxide, which methylates hepatic DNA, RNA, and some enzymes (Matsumoto, 1983). Certain cycad glycosides inhibit aromatase and may be useful in the treatment of estrogen-dependent cancer (Kowalska et al., 1995).

Enzyme inhibitors present in plant and animal foods can inhibit the action of proteases, amylases, and lipases. However, only the inhibitors of proteases pose some hazard to human health. Although most of protease inhibitors (PIs) are active against serine and cysteine proteases (serpins and cystatins), PIs active against aspartic proteases and carboxypeptidases have also been identified. Pancreatic lipase inhibitors from a variety of plants are being exploited for their antiobesity effects, whereas the PIs appear to have anticancer effects (Clemente et al., 2011; de la Garza et al., 2011).

Kunitz inhibitor and the lower-MW Bowman–Birk inhibitor are the major PIs found in soybeans. They are acid and heat labile and capable of inhibiting trypsin- and chymotrypsin-like proteases involved in carcinogenesis. The fact that significant amounts of these inhibitors reach the large intestines may explain their protective effect against colorectal cancers (Clemente et al., 2011; Kassell, 1970). Egg white, milk, beans, peas, cereal grains, alfalfa, sunflower, and potatoes also have been shown to contain one or more PIs (Janssen, 1997).

The potential adverse effects of PIs include hypertrophy, adenomas and nodular hyperplasia of pancreas, growth depression, and allergic reactions in atopic children (Friedman and Brandon, 2001; Liener, 1980). Pancreatic hypertrophy is likely from constant pancreatic hypersecretion necessitated by release of a humoral agent cholecystokinin pancreozymin in the upper small intestine in response to a deficit in free digestive enzymes (Gallaher and Schneeman, 1984). Although any single source such as soybeans is unlikely to be consumed by humans in quantities of toxicological significance, consumption of multiple sources of PIs may increase the risk of pancreatic hypertrophy and cancer. Ironically, soybean and other trypsin inhibitors are gaining attention for their preventive and inhibitory effects on initiation, promotion, as well as metastasis of cancer induced by many agents in multiple tissues (DeClerk and Inven, 1994; Friedman and Brandon, 2001). Recent studies suggest that Bowman–Birk inhibitor–induced specific proteasomal inhibition leads to alterations in cell cycle proteins and to cell cycle arrest and thus accounts for anticancer effects of these agents (Chen et al., 2005). The contribution of other mechanisms such as inhibition of enzymes involved in oxygen-free radical formation and induction of amino acid deprivation in cancer cells by protease inhibition is yet to be investigated.

Estrogens in plants (phytoestrogens) include flavonoids, isoflavonoids, chalcons, coumestans, stilbenes, lignans, ginsenosides, and other saponins, as well as the recently discovered tetrahydrofurandiols (Lorand et al., 2010). Hundreds of species of plants contain plant sterols and estrogenic isoflavonoids (e.g., genistein, glycetein, daidzein) and/or their glycosides (genistin, glycetin, daidzin), coumestans (e.g., coumestrol, 4-O-methylcoumestrol), and lignans (Adams, 1989; Stob, 1983). Phytoestrogens, although capable of causing infertility in animals grazing heavily on estrogen(coumestan)-containing forages (subterranean clover, alfalfa), have not been proven to cause human health problems. The fungal estrogens, zearalenone and zearalenol, are two major resorcylic acid lactone estrogens produced in corn in response to infection by toxigenic strains of the fungus *F. roseum* and are discussed in the section dealing with the mycotoxins.

Xenoestrogens including dichloro diphenyl trichloroethane (DDT) and its metabolites, bisphenols, alkylphenols, dichlorophenols, methoxychlor, chlordecone, polychlorinated biphenyls (PCBs), and dioxins act via estrogen receptors alpha and beta, nuclear or membrane-bound receptors, receptor-independent mechanisms, or by interference with

the production and metabolism of ovarian estrogens. A mass balance of dietary levels of industrial and natural estrogens, coupled with their estimated estrogenic potencies, indicates that the dietary contribution of estrogenic industrial compounds is 0.0000025% of the daily intake of estrogenic flavonoids in the diet (Safe, 1995). These xenoestrogens are not covered in this chapter.

The average human adult is exposed to 102 μg dietary estrogenic equivalents (reflecting both potency and exposure) daily compared to 3.35 mg/day from estrogen replacement therapy and/or 16.7 mg/day from oral contraceptives (Safe, 1995). The exposure of consumers, especially infants, to high soy content is likely to be significant. Human infants can be exposed to 4 mg/kg body weight or more of isoflavones from soy-based formulas (Aldridge and Tahourdin, 1998). A recent study (Behr et al., 2011), however, estimated the dietary intake of phytoestrogens to be lower than previously estimated (27.5 and 34.0 ng EEQ/day for adults and 1.46 ng EEQ/day for infants). Although phytoestrogens bind to multiple steroid (estrogen, progesterone, and androgen) receptors, they are considered to be selective estrogen receptor β-modulators (SERM). Due to their lower potency (500–10,000 times) compared to estradiol, phytoestrogens can actually impede the action of endogenous estrogen and at higher doses can induce antigonadotropic effects at the hypothalamic, pituitary, and gonadal levels in both sexes (Aldridge and Tahourdin, 1998). Their multiple inputs into cellular signaling begin processes that eventually integrate at the level of mitogen-activated protein kinase activities to coordinately regulate broad cellular destinies, such as proliferation, apoptosis, or differentiation, which may account for their typical nonmonotonic dose–response behavior (Watson et al., 2010).

Effects of phytoestrogens reported in feed animals include infertility in sheep fed with subterranean clover and cattle consuming alfalfa (Altekruse et al., 1998). Feminization of males following developmental exposure has also been reported (Clarkson, 1995). Recent reports of detrimental effects of high levels of phytoestrogens in soy-based products on learning, memory, and anxiety behaviors in male, but not female, rats (Lephart et al., 2004) suggest the need for caution in soy-formula feeding of male infants. Although phytoestrogens appear to be noncarcinogenic when given orally (Verdeal et al., 1980), some (genistein, coumestrol, quercetin, zearalenone, resveratrol, and some metabolites of daidzein) are genotoxic and all exhibit proapoptotic effects in vitro (Bartholomew and Ryan, 1980; Stopper et al., 2005). In humans, reversible changes in menstrual cycle and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) surges in premenopausal women appear to result from soy consumption, but no developmental or infertility problems were noted in populations consuming large quantities of phytoestrogen (Aldridge and Tahourdin, 1998). Phytoestrogens may actually exert antioxidant activity and may protect humans against coronary heart disease; cancer of the breast, prostate, and colon; obesity; and postmenopausal osteoporosis (Adlercreutz, 1995; Beck et al., 2005; Lephart et al., 2004). The results of a study of 939 postmenopausal women participating in the Framingham Offspring Study revealed that a higher intake of phytoestrogens was associated with a favorable metabolic cardiovascular risk profile (de Kleijn et al., 2002).

Phytosterols (campesterol and sitosterol and their 5α-saturated stanols) are normal dietary components (200–300 mg/day) that chemically resemble and thus potentially interfere with absorption of dietary cholesterol and together reportedly decrease the incidence of coronary heart disease by 20%–25% (Gylling and Miettinen, 2005). Paradoxically, high levels of plasma phytosterols alone may be associated with increased coincidence of coronary heart disease. Because plant stanol esters reduce absorption and serum concentrations of both cholesterol and plant sterols, beneficial supplementation of human diets over the long term may be better accomplished with the use of plant stanols alone.

Glucosinolates (GS) are a group of more than 100 flavor-imparting thioglucoside compounds found at up to 60 mg/g in crucifers such as broccoli, cabbage, Brussels sprouts, cauliflower, calabrese, turnip, radish, horseradish, mustard, and rapeseed and related plants. Common names of some important GS include sinigrin, progoitrin, epiprogoitrin, glucobrassicin, neoglucobrassicin, and glucoraphanin. The parent GS as well as their products of plant and human digestive tract bacterial myrosinase (thioglucosidase) hydrolysis (isothiocyanates, nitriles, oxazolidinethione [OZT], thiocyanate ions, and more notably sulforaphene) have been reported to produce biological effects (Verkerk et al., 1998).

Although evidence is lacking in humans, thiocyanate ion inhibits the uptake of iodine by the thyroid leading to iodine-reversible hyperplasia and hypertrophy of the thyroid (cabbage and legume goiter) and growth suppression in animals. OZT also inhibits thyroxin synthesis and induces goiter (brassica seed goiter) in rats by inhibiting the incorporation of iodine into precursors of thyroxin (Matsumoto et al., 1968). This condition is not reversible by iodine supplementation. In addition to goiter, epiprogoitrin and progoitrin also induce liver and kidney enlargement and death at 2.6% in the diet via their nitrile metabolites (Van Etten and Tookey, 1983). Bile duct hyperplasia, hepatocyte necrosis, and megalocytosis of renal tubular epithelium were also seen in these animals (Tookey et al., 1980).

Isothiocyanates are embryocidal and cause fetal weight reduction (Beier, 1990). Isothiocyanates and certain GS (e.g., sinigrin) are mutagenic in the Ames assay, and certain desulfo precursors of GS may be carcinogenic, whereas thiocyanates are not (Beier, 1990; Weil et al., 2004). Moderate intake of cruciferous vegetables in man and animals, however, may exert anticarcinogenic effects attributable to the formation of isothiocyanates (at least 7), indoles, indole-3-carbinol, 3-indoleacetonitrile, and 3,3′-diindolylmethane (Verkerk et al., 1998). The chemoprotective effects of GS appear to result from a variety of distinct but interconnected signaling pathways involving detoxification, inflammation, apoptosis, and cell cycle and epigenetic regulation, among others (Navarro et al., 2011). A recent qualitative comparison of the

benefit and risk of broccoli consumption shows that the benefit from intake in modest quantities and in processed form outweighs potential risks (Latte et al., 2011).

Lectins (phytohemagglutinins) are high-MW (100,000–150,000) heat-labile proteins, lipoproteins, or glycoproteins (up to >10% of total seed protein) detected in over 800 edible plant species of which 600 belong to the Leguminosae (beans, peas) and in animals such as sponges, crustaceans, mollusks, fish blood, amphibian eggs, and even mammalian tissue. They possess the ability to agglutinate erythrocytes with known carbohydrate specificity since they have at least one noncatalytic domain that binds reversibly to specific monosaccharides or oligosaccharides. Animal lectins are usually present in low levels compared with the yields of plant lectins such as legume lectins and manifest a diversity of beneficial activities including antibacterial, antinematode, antitumor, immunomodulatory, antifungal, HIV-1 reverse transcriptase inhibitory, and anti-insect activities (Deshpande and Sathe, 1991; Lam and Ng, 2011).

Interactions of animal lectins such as annexin and galectin with animal cell proteins such as bcl2 and synexin and subsequent triggering of signaling cascades bestow them with an ability to regulate various endogenous functions involving glycoprotein and cell (normal and tumor) recognition, adhesion, and clearance; signal transduction; extracellular glycoprotein trafficking; and mitogenesis, apoptosis, and immune function (Mody et al., 1995; Van Damme et al., 2004).

Upon ingestion, plant lectins such as *concanavalin A* and *phytohemagglutinins* survive digestion by the GI enzymes and bind to membrane glycosyl groups of the cells lining the digestive tract. This leads to nonspecific inhibition of digestion and active and passive absorption of many nutrients (amino acids, fats, vitamins, minerals, thyroxin, etc.) across the intestinal mucosa, alters the bacterial flora, modulates the immune status of the digestive tract, damages the luminal membranes of the epithelium, and induces necrosis of intestinal epithelial cells (King et al., 1980; Vasconcelos and Oliveira, 2004). These effects account for the immediate effects including nausea, vomiting, and diarrhea as well as growth suppression and possibly goiter after long-term oral exposure to high levels (Janssen et al., 1997; Vandenborre et al., 2011). Mortality following acute systemic lectin exposure is associated with damage to the liver (Ikeguonu and Bassir, 1977) and other organs. The most toxic lectin, ricin from castor bean (lethal dose in humans of 1–10 μg/kg body weight), can cause severe intestinal epithelial cell necrosis and death from multiorgan damage (Van Genderen, 1997). Recent evidence suggests that lipid peroxidation mediated by reactive oxygen species may be involved in ricin-induced thyroid damage (Sandani et al., 1997). The lectin portion (B chain) of the ricin dipeptide binds the galactosyl residues on the surface of intestinal epithelial cells and facilitates the intracellular uptake of the enzymatic (RNA-specific N-glycosidase) A chain via clathrin-dependent as well as clathrin-independent endocytosis. The A chain then enters the golgi and ER, inhibits protein synthesis, and causes cell death (Sandvig and Van Deurs, 1997; Van Genderen, 1997). Less toxic lectins may act by the same mechanism to stimulate protein synthesis, mitogen activation, and immune stimulation.

Although ricin has been suggested as a potential warfare/terror agent via contamination of water/food, a substantial mass of the pure powder is required. In addition, the potential for success is low due to variables such as reticulation management, chlorination, mixing, and bacterial and UV light degradation. Injection is not a realistic option for large populations. Dermal absorption of ricin has not been demonstrated. Ricin, administered by inhalation, can be lethal from progressive and diffuse pulmonary edema with associated inflammation and necrosis of the alveolar pneumocytes; however, the technical and logistical skills required to achieve the ideal aerodynamic equivalent diameter for entry into the lungs are generally beyond the ability of most terrorists. These challenges make ricin generally unsuitable as an agent of bioterrorism (Schep et al., 2009).

Although *lipids* are essential for normal development, growth, and cellular function, their overconsumption has been associated with weight gain, obesity, cardiovascular disease, and metabolic syndrome, which, in addition to the aforementioned, is characterized by increased propensity for type 2 (insulin-resistant) diabetes. Factors such as a departure from established food use patterns, the use of new lipids in the human diet, or inborn errors of metabolism (due to polymorphism) act in concert with lipid overconsumption to induce hyperlipidemia and cardiovascular disease including hypertension and obesity. Recent evidence implicates a positive feedback circuit between high dietary fat and increase in brain galanin (GAL), a feeding stimulant peptide, in the onset of human overeating syndrome and obesity (Leibowitz, 2005). Mechanistically, circulating lipids interact directly (as fatty acids) or indirectly (via biosynthetic intermediates such as prostaglandins and leukotrienes or via the interaction of lipids and their derivatives such as diacylglycerol) with a variety of signal transduction pathways and transcription factors such as peroxisome-proliferator-activated receptor, liver X receptor, hepatocyte nuclear factor 4, carbohydrate-response-element-binding protein, farnesoid X receptor, sterol-regulatory-element-binding protein (SREBP) (Roche, 2004). These interactions lead to alterations in the expression of genes (such as adipocytokines) that mediate cellular responses involved in inflammation, a prerequisite to insulin resistance and diabetes. Paradoxically, dietary supplementation with monounsaturated fatty acids (oleic and omega- or alpha-3 fatty acids) and conjugated linoleic acids can prevent and/or reverse the onset of insulin resistance/metabolic syndrome by altering membrane fluidity and signaling and reducing adipose tissue TNFα and subsequent alterations in SREBP (Riccardi et al., 2004; Roche, 2004).

Erucic acid (*cis*-13-docosanoic acid) is predominantly a component of rape- (*Brassica napus* and *Brassica campestris*) and mustard (*Brassica hirta* and *Brassica juncea*) seeds. Canada, Argentina, Mexico, China, India, Pakistan, Japan, and several European countries are the major producers and users of these oils. Growth suppression, myocardial fatty infiltration, mononuclear cell infiltration, and fibrosis

were observed in weanling rats fed with erucic acid at levels supplying greater than 20% of the dietary calories. In addition, ducklings showed hydropericardium and cirrhosis, and guinea pigs developed splenomegaly and hemolytic anemia (Mattson, 1973). Organ-specific inhibition of glutamate oxidation and adenosine triphosphate (ATP) synthesis in cardiac mitochondria (Houtsmuller et al., 1970) could be mechanistically involved in the pathogenesis of these lesions. In humans, however, although the long-term use of Lorenzo's oil (oleic acid and erucic acid) in the treatment of adrenoleukodystrophy or adrenomyeloneuropathy leads to thrombocytopenia and lymphopenia (Unkrig et al., 1994), adverse effects from dietary consumption of erucic acid have not been reported.

Refsum disease is a genetic peroxisomal fatty acid oxidase and catalase deficiency resulting in an inability of the affected individuals to convert phytanic acid (3, 7, 11, 15-tetramethylhexadecanoic acid, a product of chlorophyll metabolism in the rumen) from dairy products and ruminant fats to α-hydroxyphytanic acid in preparation for further oxidation. This results in accumulation of lipids containing phytanic acid in many tissues and a disorder characterized by poor physical and mental growth, blindness, deafness, and other neurologic signs (Chow et al., 1992). Elimination of dairy and ruminant fats from the diet of these individuals results in partial remission.

Cyclopropene fatty acids such as sterculic acid (C19) and malvalic acid (C18) are natural components of oils from plants of the order Malvales, most important of which are cotton and kapok seeds. Cyclopropene fatty acids have been incriminated in the pink discoloration of egg whites and reduced egg production in cottonseed-fed laying hens, growth suppression and impaired female reproduction in rats, and increased saturated fatty acids (possibly cause atherosclerosis) in the tissues of pigs and other animals (Mattson, 1973). Cyclopropene fatty acids are carcinogens and markedly increased the carcinogenicity of aflatoxin in trout (Ames, 1983).

Increase in consumption of polyunsaturated fatty acids in the diet in order to lower blood cholesterol, although beneficial in decreasing the incidence of coronary disease, has raised concern for adverse effects such as increasing the total triglyceride levels and induction of vitamin E deficiency (Mattson, 1973; Riccardi et al., 2004). A study (Carrol, 1982) demonstrated a strong correlation between dietary fat and age-adjusted mortality rate from breast and intestinal cancer. Pancreatic cancer was found to be enhanced by a diet containing 20% corn oil but not by one containing 18% hydrogenated coconut oil and 2% corn oil (Roebuck et al., 1981). Unsaturated fatty acids are easily oxidized during cooking to a variety of mutagens, enals and other aldehydes, and alkoxy and hydroperoxy radicals (Ames, 1983). Lipid oxidation products alter signal transduction pathways (Suzuki et al., 1997) and thus enhance cell proliferation and potentially promote carcinogenesis. Lipid-induced inhibition of immune responses and enhanced formation of some of the known tumor promoters such as prostaglandins and bile acids also have been reported (Carrol, 1982). Interestingly, another study (Hayatsu et al., 1981) showed that oleic and linoleic acids may, in fact, be antimutagenic. The overall effect of dietary fats may depend on the ratio of beneficial fatty acids to those of the causative fatty acids for each effect.

Until a clear understanding of the role of dietary fat in human disease is obtained, prevention of weight gain, obesity, type 2 diabetes, and cardiovascular disease appears to be best achieved by a diet low in fat and sugars and high in fiber and protein.

Oxalates are present in large quantities (0.2%–2.0% on a fresh weight basis) in certain plants including spinach, rhubarb, beet leaves, tea, and cocoa. Cattle and sheep have been poisoned following ingestion of the toxic plants *Halogeton* and *Sarcobatus* (grease wood). Toxic signs result from binding of the oxalic acid to serum calcium leading to hypocalcemia, coagulation defects, and tetany. Degeneration and necrosis of kidneys and vasculature from Ca^{++} oxalate deposition may result in severe cases. However, a subpopulation of urinary stone-forming patients are *hyperabsorbers* of oxalates, absorbing more than the normal 3%–8% of the 150–250 mg/day of dietary oxalate intake, and may benefit from reduction of dietary oxalate intake (Massey, 2003). The possibility that antibiotic therapy–induced loss/reduction of the intestinal oxalate metabolizer, *Oxalobacter formigenes*, may contribute to elevated body and urinary oxalate, increasing the risk of recurrent calcium oxalate kidney stone formation (Stewart et al., 2004). Either directly or via their increased production of reactive oxygen species, oxalates alter lipid signaling pathways leading to changes in renal membrane characteristics and damage that promotes oxalate crystal enucleation and growth into stones (Scheid et al., 2004). Chronic oxalate consumption interferes with absorption of calcium, iron, magnesium, and copper and inhibits succinate dehydrogenase and carbohydrate metabolism (Osweiler et al., 1985). Approximately 2.5 kg of tomato or 0.5 kg of spinach leaves need to be consumed to approach a lethal dose (5 g or more) of oxalates.

Phytic acid, the hexaphosphoric ester of myo-inositol (IP6), is present at high levels (up to 1.5 g%) in the bran and germ of wheat with lesser amounts in other cereals, nuts, seeds, spices, and legumes (EFSA, 2011a). Phytates bind di- and trivalent metals in the following order: $Cu^{++} > Zn^{++} > Co^{++} > Mn^{++} > Fe^{+++} > Ca^{++}$, causing mineral deficiencies (especially of Ca^{++} and Fe^{+++}) in people in developing countries that are heavily reliant on cereals as the exclusive source of protein. Inclusion of phytase, an enzyme that releases phosphate from plant phytic acid, in animal feeds ensures phosphate utilization and reduces environmental phosphate pollution from animal production. By altering inositol second messenger pathways, phytates (especially in combination with inositol) inhibit cell cycle, increase malignant cell differentiation and reversion to normal cells, and are anticarcinogenic in animals and human beings against prostate and possibly breast, colon, liver, leukemia, prostate, sarcomas, and skin cancer (Singh and Agarwal, 2005). Supplementation with minerals and vitamin D can antagonize most effects of oxalates and phytates (Janssen et al., 1997).

Plant phenolics comprise a group of several thousand substituted phenolic compounds occurring in trace amounts as esters or glycoconjugates and play essential roles in plant growth and development, defense, symbiosis, pollen development and male fertility, polar auxin transport, protection against UV radiation, and cell cycle regulation. They are widespread constituents of fruits, vegetables, cereals, dry legumes, chocolate, and beverages such as tea, coffee, or wine. Acute human and animal poisonings are mostly caused by phenolics that are either uncommon or those present as contaminants in human food and include coumarins, aflatoxins, and gossypol. Phenolics common in human foods belong to three general classes, that is, nonflavonoids (gallic, syringic, caffeic, and other acids); flavonoids (flavones such as tangeritin; flavonols such as kaempferol and quercetin; isoflavones such as coumestrol, aurones, chalcones; and anthocyanin pigments); and polyphenols (tannins and lignin). Common human foods rich in flavonoids include coffee, chocolates, tea extract, green and black tea, pomegranate juice, grape juice/extracts, virgin olive oil, red wine, and soy proteins.

Polyphenols are widely distributed and present in relatively larger amounts in cereals, millets, legumes, and fruits. Deleterious effects of long-term exposure of both hydrolyzable (polyphenolic acid) and condensed (polyflavonoid) tannins include reductions in the digestibility of foods and feeds, protein utilization and body weight gain, damage to and sloughing of the mucosal lining of the GI tract, and cancer of the mouth and esophagus (Hirono, 1987; Reed, 1995). In contrast to mild acute effects in humans, livestock losses can exceed $10 million annually, attributable to the toxic effects of hydrolyzable oak tannins consumed when other forages are unavailable (Singleton and Kratzer, 1973). Epidemiological correlation exists between high consumption of condensed tannins (sorghums and dark beer prepared from them, tea, red wines, and areca nuts) and high rates of oral and esophageal cancer (Deshpande and Sathe, 1991). Parenteral exposure to tannins reportedly has led to high incidence of liver and other tumors in rodents (Hirono, 1987). On the other hand, negative association between tea drinking and stomach cancer (Stocks, 1970) and coffee consumption and kidney cancer (Jacobsen and Bjelke, 1982) also exists. Polyphenols, however, are not directly damaging to the DNA (Cheeke, 1989), and experimental evidence of anticarcinogenic effects of penta-O-gallyl-beta-D-glucose and epigallocatechin gallate, two green tea tannins (Fujiki et al., 1992), exists.

Flavonoids and nonflavonoids exert no less than 40 different physiologic and pharmacologic actions accounting for their therapeutic and extensive health food use. These include antiandrogenic, anticoagulant, antihistaminic, antihypercholesterolemic, anti-inflammatory, antinutritional (inhibit protein digestibility and nonheme iron absorption leading to iron deficiency), antioxidant, antiproliferative, antipruritic, antipyretic, antirheumatic, antiseptic, antithrombogenic, antithyroid, antitumor, apoptotic, estrogenic, and vasoactive effects. In addition, polyphenols alter the bioavailability and thus the biologic effects of certain drugs including benzodiazepines, terfenidine, and cyclosporine. Many if not all of these actions are based on their UV-absorbing, chelating, oxidative phosphorylation uncoupling and/or oxidant/antioxidant properties. In addition, induction of P450-mediated enzymes and alteration of other enzymes (phospholipases, ATPases, cycloxygenases, lipoxygenases, protein kinases) plus effects on oncogenes and other signaling components critical for cell survival and proliferation (Cheeke, 1989; Formica and Regelson, 1995) contribute to an array of opposing effects in many systems. Although human consumption of flavonoids can be greater than 1 g/day (Janssen et al., 1997), the toxicological implications of exposure to flavonoids and other simple phenolics arise from lifetime and not acute exposure. High intake of flavonoid supplements in humans and/or experimental feeding of high dietary levels have caused acute renal failure possibly due to hemolysis and liver failure as well as contact dermatitis and anemia (Lin et al., 1997; Mennen et al., 2005).

Endocrine disruption involving inhibition of thyroid peroxidase and thyroid hormone (TH) biosynthesis by flavonoids can lead to increased thyroid weight (goiter) and decreased plasma levels of THs. These alterations are of particular concern for babies exposed to high doses of isoflavones in soy-based infant formula. The estrogenic (at moderate doses) and antiandrogenic (at higher levels) activities of the flavonoids are of limited concern in adults at normal dietary intake (0.2–5 mg/day from Western diet and 20–120 mg/day of isoflavones from Asian diet). However, their antiluteinizing hormone effect at levels present in soy-based infant formula can have adverse effects on sexual maturation of male infants, who normally exhibit luteinizing hormone secretion between birth and 6 months of age.

Many flavonoids including quercetin, kaempferol, myricetin, hesperetin, naringenin, wogonin, and norwogonin as well as their glycosides were mutagenic in bacterial and/or mammalian systems (Hirono, 1987; MacGregor, 1984). Although polyphenols such as caffeic acid, quercetin, and green tea catechins are known to induce tumors in the forestomach, colon, kidney, and highly oxidative tissues in rats and mice, reports of their carcinogenic effects in humans are scanty, likely due to the efficient repair of quercetin quinone methide–DNA adducts in humans (Barotto et al., 1998; Dunnick and Hailey, 1992; Mennen et al., 2005). Preponderance of evidence from animal and in vitro systems, however, points to the preventive effects of flavonoids/polyphenols against cardiovascular diseases, cancers, neurodegenerative diseases, diabetes, and osteoporosis (Scalbert et al., 2005). Clinical studies on biomarkers of oxidative stress, cardiovascular disease risk factors, and tumor or bone resorption biomarkers, however, have often led to contradictory results, likely due to differences in types and levels of phenolics consumed. For example, phenolics are known to exert antioxidant effects at low doses and prooxidant effect at higher doses (Yamanaka et al., 1997). A recent study associated increasing intake of flavones and flavonols, but not other flavonoids, with decreased incidence of breast cancer (Bosetti et al., 2005). Anticarcinogenic

effects of phenolics in animals appear to involve both initiation and progression phases of cancer and a combination of mechanisms including inhibition of metabolic enzymes leading to reduced levels of reactive intermediates, induction of detoxifying enzymes such as GSH-s-transferase, reduced formation of oxidation products, alteration of the activity of protein kinases and oncogenes that stimulate cell proliferation, increase apoptosis, reduced expression of matrix metalloproteinases involved in metastasis, and inhibition of angiogenesis (Balasubramanian and Govindaswamy, 1996; Cheeke, 1989; Hirono, 1987; Kanadaswami et al., 2005; Lin and Ho, 1994; Williamson et al., 1998). Protective effects of flavonoids on the cardiovascular system have been shown more consistently in both animals and human beings. The mechanisms for such effects appear to involve reduction of low-density lipoprotein oxidation (antiatherogenic effect) and platelet aggregation (antithrombotic), vasodilation, relaxation of cardiovascular smooth muscle and their anti-inflammatory and antihypercholesterolemic (decrease in LDL and/or increase in HDL) effects among others (Formica and Regelson, 1995; Howard and Kritchevsky, 1996; Manach et al., 2005).

Gossypol (1,1,6,6,7,7-hexahydroxy-5,5-diisopropyl-3,3-dimethyl [2,2-binaphthalene]-8,8-dicarboxaldehyde), a yellow phenolic pigment in cottonseed, can bind to proteins and minerals and reduce the biological availability of iron and lysine (Janssen, 1997). Similar to other phenolics, free gossypol (>60 ppm) inhibits oxidative phosphorylation and causes a myriad of other effects leading to acute toxicity in animals on a high cottonseed diet. In general, higher doses cause cardiac failure associated with liver and lung (pulmonary edema) damage, whereas chronic exposure leads to general malnutrition and reproductive effects (Cheeke, 1989). Signs of gossypol toxicity include loss of appetite and body weight; rough hair coat; edematous fluid in body cavities, lungs, and pericardium giving rise to gasping; hemorrhagic degenerative changes in liver; and necrosis of cardiac myocytes (Zelski et al., 1995). Changes in plasma K^{++} (increase in calves and decrease in humans) may be responsible for gossypol toxicity. Olive discoloration of yolk and decreased egg hatchability occur in poultry (Cheeke, 1989). Male antifertility effects of gossypol in mammals are only partially reversible and include reduced sperm production as well as motility during the late stages of spermatogenesis likely caused by mitochondrial damage (Randel et al., 1992) or inhibition of protein kinases (Teng, 1995). Gossypol is not mutagenic in the Ames test (Cheeke, 1989) but appears to induce genetic damage (dominant lethal mutations) in rats and may be both an initiator and promoter of carcinogenesis (Ames, 1983). In rat lymphocytes, gossypol induced DNA breaks secondary to cytotoxicity (Quintana et al., 2000).

Gossypol and polyphenol (tannin) toxicity can be prevented by the addition of iron, supplemental protein, vitamins E and K, and alkalinizing agents such as sodium hydroxide. In addition, nonionic detergents such as Tween 80, methyl donors such as choline and methionine, and dehulling and peeling of grains and fruits have also been shown to counteract the toxic effects of tannins (Deshpande, 2002; Singleton and Kratzer, 1973). Although having the potential to eliminate gossypol toxicity, a glandless (gossypol-free) variety of cottonseed appears to be more susceptible to insect attack and has yet to gain popularity.

Proteins, peptides, and amino acids, in an average American diet, should supply about 15% of total calories. Long-term consumption of high level of protein, especially animal derived and in amounts that supply >45% of daily caloric needs, has been associated with weakness, nausea, diarrhea, diabetes, renal glomerular sclerosis, Crohn's disease, and osteoporosis (Barzel and Massey, 1998; Cordain et al., 2000; Shoda et al., 1996; Wolever et al., 1997). A study (Huang et al., 2005) showed that soybean proteins, extracted with 20% ethanol, can inhibit TH receptor (TR) binding to TR element in TH-regulated genes and hypothesized that soy protein rather than other components may account for its hypocholesterolemic and hypolipidemic and thus cardioprotective effects of soybeans. Feeding of high level of soy protein, however, markedly increased pancreatic weights and reduced spleen weights in both male and female rats possibly due to the presence of active residual trypsin inhibitors (known to induce hypertrophy and hyperplasia of the acinar cells) and soy fiber, respectively. Products such as D-amino acids and lysinoalanine formed during alkaline/heat treatment of proteins such as casein, lactalbumin, soy protein isolate, or wheat proteins can reduce the digestibility of other dietary proteins (Gilani et al., 2005).

Protein toxicants such as the allergen hemagglutinins (lectins) and enzyme inhibitors have been discussed elsewhere in this chapter. Toxic peptides from mushrooms are discussed in the succeeding text.

Certain microbial protein toxins are discussed in subsequent sections. Adverse effects from amino acids (Garlick, 2004) appear to be restricted to very high parenteral doses and/or diets with low protein levels. An increase in the consumption of amino acid dietary supplements, flavorings (glutamate as monosodium glutamate [MSG] and aspartate and phenylalanine in aspartame), health promoters, performance enhancers, and behavior modifiers calls for increased vigilance for potential adverse effects associated with such uses. Some examples include hyperlipidemia, hypercholesterolemia, enlarged liver and reduced plasma copper (all reversed by copper supplementation in rats), and increases in urinary zinc, headache, weakness, drowsiness, nausea, anorexia, painful eyes, changed visual acuity, mental confusion, poor memory, and depression in overweight human subjects given 24–64 g/day of histidine. Excessive intake of methionine induces hyperhomocystinemia with or without cardiac disease in both rats and human subjects, among other effects, from excess methionine. Ocular lesions/visual disturbances secondary to accumulation of tyrosine crystals and behavioral/perceptual and performance/intellectual deficits in neonates and animals appear following in utero and/or neonatal exposure to high-tyrosine diets/formulas. Therapeutic use of greater than 10 times the required dose of amino acids, when given on an empty stomach, can lead to adverse effects including gastric distress (essential amino acids); nausea,

febrile reaction, and/or headaches (methionine, isoleucine, and threonine); and disorientation (methionine and tryptophan) in mental patients treated with monoamine oxidase (MAO) inhibitors (Harper, 1973).

Hypoglycin A (β-methylene cyclopropyl alanine) and its γ-glutamyl conjugate, hypoglycin B, are components of the fruit of the plant, *Blighia sapida* (ackee in Jamaica and is in Nigeria). Consumption of this fruit in the unripened stage has been associated with hypoglycemia resulting from inhibition of gluconeogenesis involving inhibition of fatty acyl-CoA dehydrogenases and thus β-oxidation of fatty acids by cyclopropylacetyl CoA (a metabolite of hypoglycin A). Signs of intoxication include vomiting, convulsions, hypothermia, coma, and even death. Pretreatment with clofibrate (stimulator of peroxisomal fatty acid oxidases) prevented many but not all signs, lesions, and biochemical effects (Van Hoff et al., 1985).

Koa haole (*Leucaena leucocephala*), a legume found in Hawaii, and other legume species belonging to Mimosidae family have potentially high nutritive value for animals and humans (NAS, 1997). However, use of these legumes is precluded in ruminants by the goitrogenic effect of the metabolite (3,4-dihydroxypyridine) of an unusual amino acid, mimosine [3-N-(3-hydroxypyridone-4)-2-aminopropionic acid], present in this plant. Mimosine also causes reversible destruction of hair follicle matrix (loss of hair), reduced bone strength and mineral composition in poultry, and growth depression in both ruminants and nonruminants. The ability of mimosine to chelate Zn and Mg, reduce plasma thyroid and other hormone levels (Puchala et al., 1996), and inhibit a large number of enzymes leading to DNA synthesis inhibition and cell cycle arrest (Kalejta and Hamlin, 1997; Liener, 1980) explains many of the effects.

Djenkolic acid, which is an amino acid that is structurally similar to cystine, is present in the djenkol bean (*Pithecellobium lobatum*) in Sumatra and Java. It can neither substitute for cystine nor can it be totally metabolized but can crystallize in the kidney causing hematuria and crystalluria (Liener, 1980).

Favism, a hemolytic disease (accompanied by jaundice and hemoglobinuria) in persons genetically deficient in glucose-6-phosphate dehydrogenase (G6PD) and thus in NADPH and reduced GSH content, results from the consumption of the amino acid 3,4-dihydroxyphenylalanine and the pyrimidine aglycones (divicine and isouramil) of glycosides, vicine and convicine, in broad beans (*Vicia faba*) mainly in the Mediterranean region and in the Middle East (Chevion et al., 1983).

The etiology of the neurologic disease characterized by posterior sensory ataxia in cattle consuming cycads may be an amino acid, BMAA. Certain seleno-amino acids such as methylselenocysteine, selenocystathionine, selenocysteine, and selenomethionine in plants that grow on high Se soils (Liener, 1980), when incorporated into structural animal proteins, may produce defective hair and hooves that are eventually lost during longer-term exposure in livestock. In human beings, a syndrome characterized by abdominal distress, nausea, vomiting, diarrhea, and loss of scalp and body hair had been reported following consumption of coco de momo (*Lecythis ollaria*) nuts containing high levels of selenocystathionine (Aronow and Kerdel-Vegas, 1965).

The amino acids L-2,4-diaminobutyric acid (DABA), 3-N-oxalyl-L-2,3-diaminopropionic acid (ODAP), 3-cyanoalanine, 4-glutamylcyanoalanine, and related homologues, present in seeds of several species of Lathyrus and *Vicia sativa* in the Indian subcontinent, have been implicated in the pathogenesis of neurolathyrism, a syndrome characterized by muscular rigidity, weakness, paralysis of leg muscles, and death following long-term high-level consumption of *Lathyrus sativus* seeds (Van Genderen, 1997). The mechanism of action appears to involve irreversible binding of ODAP to the glutamate receptor and enhanced release/reduced reuptake of glutamine at relevant nerve terminals leading to vascular degeneration and necrosis of neurons (Padmanaban, 1980). In certain individuals, amino acids such as β-aminopropionitrile and the dipeptide (N-γ-glutamyl) aminopropionitrile as well as certain urides, hydrazides and hydrazines, from the green parts of *Lathyrus* and other plants lead to osteolathyrism characterized by bone deformities and reduction in the tensile strength of the aorta (Haque et al., 1997) resulting from the irreversible inhibition of lysyl oxidase and interference with cross-linking of collagen (Wilmarth and Froines, 1992). More recent observational and experimental findings relating to the vascular and systemic effects of molecular pathways implicated in the phenomenon of animal and human lathyrism suggest that vascular adhesion factor seems to be involved in the molecular and developmental pathways of the genetically triggered thoracic aortic diseases and thus could be a potential therapeutic target for these conditions (Sherif, 2010).

Creeping indigo (*Indigofera endecapylla*), a tropical forage, contains a nitric oxide synthase inhibitor, indospicine, which causes liver damage in sheep, rats, and mice by inhibiting the incorporation of arginine, the amino acid it resembles, into protein (Liener, 1980).

Saponins are bitter-tasting steroidal (C27) or mono-, di-, tri-, and sesquiterpenoid (C30) glycosides from plants, fish, and sponges capable of reducing surface tension, hemolyzing red blood cells, and causing toxic effects in cold-blooded animals. Their occurrence, biological effects, and relevance to food, agriculture, and medicine are reviewed by Walker and Yamazaki (1996a,b). D-*Limonene* and several other chemicals from citrus oils such as ginseng saponins, medicagenic acid, and hederosides in alfalfa and *Hedera helix*, respectively, and oleanolic and ursolic acid in a variety of food, medicinal, and other plants as well as their aglycones (sapogenins) have been studied. Their analgesic, antiatherosclerotic, anticarcinogenic, anticholinergic, antihypercholesterolemic, antihyperglycemic, anti-inflammatory, antitubercular, cardioprotective, diuretic, and hepatoprotective effects are likely to encourage increased dietary, supplemental, and medicinal utilization of saponin-containing plants such as ginseng (Malinow et al., 1982; Rao and Sung, 1995; Xu et al.,

1996). Mechanisms of protection involve Ca^{++}-antagonistic and vasodilatory/venoconstrictive; immune-modulatory, bile acid-binding, antiproliferative, and membrane permeabilizing; and antioxidant and anticytochrome P_{450} effects (Rao and Sung, 1995). Feeding high levels of saponin from a variety of sources, however, resulted in lower growth rate; increased serum lactate dehydrogenase (LDH) and glutamic oxaloacetic transaminase (GOT) associated with hepatocellular necrosis; and increased blood urea nitrogen (BUN), hematuria, and proteinuria associated with renal tubular necrosis (Kobayashi et al., 1993; Nakhla et al., 1991) in animals. Several steroidal and nonsteroidal saponins from pasture weeds such as *Hypericum perforatum* and *Narthecium ossifragum*, vines such as *Tribulus terrestris*, and tropical grasses such as *Brachiaria* and *Panicum* spp. cause primary or hepatogenic photosensitization (Cheeke, 1996). Alphahederin, a saponin that induces metallothionein in maternal tissues, appears to induce visceral and skeletal defects in offspring born to exposed mothers by possibly reducing Zn availability to the fetus (Duffy et al., 1997). Similar to the effects of phenolics, the beneficial effects of saponins can be derived from daily doses present in a balanced diet.

Vaso- and psychoactive substances are present at high levels in cheese, yeast products, fermented foods, beer, wine, pickled herring, snails, chicken liver, coffee, broad beans, chocolate, cream products, and plants such as pineapple, banana, plantain, and avocado. Chemically, they are predominantly amines such as tyramine and its methyl derivatives octopamine, dopamine, epinephrine, norepinephrine, histamine, and serotonin (Lovenberg, 1973). Moderate amounts of cheese and yeast products commonly contain the needed dose of 10 mg of tyramine to cause severe hypertensive crisis in individuals treated with nonselective MAO inhibitors for disorders of mood (Baldessarini, 1985). Inhibition of MAO leads to a combined vasopressor effect of unmetabolized biogenic as well as dietary amines. In addition, tyramine enhances release of catecholamines that are present in supranormal amounts in the adrenal medulla (Baldessarini, 1985). Palpitations, migraine headaches, and in some instances intracranial bleeding and death may ensue. Use of selective (MAO-A or MAO-B) inhibitors for therapy appears not to sensitize individuals to dietary tyramine (Matsumoto et al., 1968). Herbs containing toxic psychoactive agents include California poppy, catnip, cinnamon, hops, hydrangea, juniper, kola nut, nutmeg, periwinkle, thorn apple, and wild lettuce (Beier, 1990).

Miscellaneous Plant Toxicants

A common human intoxication called *milk sickness* was one of the most dreaded diseases from the Colonial times to the early nineteenth century in an area extending from North Carolina to Virginia and to the Midwestern United States (Lewis and Elvin-Lewis, 1977). The disease manifested as weakness, nausea and vomiting, constipation, tremors, prostration, delirium, and even death and resulted from the consumption of dairy products made from milk derived from cows (even healthy ones) grazing on white snakeroot (*Eupatorium rugosum*) or rayless goldenrod (*Haplopappus heterophyllus*). The causative agent appears to be trematol, an unsaturated alcohol, in combination with a resin acid (Lewis and Elvin-Lewis, 1977). Other plant toxins excreted through milk that pose toxic hazards for children and nursing animals include pyrrolizidine, piperidine, and quinolizidine alkaloids; sesquiterpene lactones of bitterweed and rubberweed; and GS (Panter and James, 1990). Animals grazing on high Se forages may excrete high levels of Se in milk and contribute to chronic Se toxicosis in the offspring (Panter and James, 1990). Current processing methods have kept these conditions in check for the most part. In cattle, consumption of 5–10 lb of snakeroot causes weakness and trembling of various groups of muscles, labored respiration, and death.

Marine Toxins

Of the many marine organisms capable of containing toxins (>1200 species), only a few are involved in food poisoning. Modern transportation and recent increase in frequency and intensity of toxic algal blooms have led to an increase in the incidence as well as the spread of seafood poisoning. Toxicants may be produced by the fish itself, by the marine plankton or algae consumed by the fish with or without the aid of certain marine bacteria. A detailed discussion of the toxicology of fish-borne toxins can be found in several studies (Brett, 2003; Leftley and Hannah, 1998; Russell and Dart, 1991).

Shellfish poisoning is one of several (amnesic, digestive, neurotoxic–paralytic) disease entities resulting from the consumption of shellfish (clams, crustaceans, lobsters, mussels, oysters, scallops, etc.) that have ingested toxic marine algae, especially certain dinoflagellates. The shellfish are toxic during seasons of heavy algal bloom (such as red tide) containing 200 organisms/mL or more. Toxicity increases in proportion to the concentration of algae and disappears within 2 weeks after the toxic plankton has disappeared from the waters (Russell, 1986).

Saxitoxin (STX), *neosaxitoxin*, and *gonyautoxins* are the most potent of the more than 20 toxins present in the *paralytic shellfish poison*, produced by the dinoflagellates belonging to *Alexandrium*, *Gymnodinium*, *Gonyaulax*, and *Pyrodinium* spp. STX blocks the action potential in nerves and muscles by preferential blockade of inward flow of sodium ions with no effect on the flow of potassium or chloride ions (Kao, 1967). STX also binds to calcium and potassium channels, neuronal nitric oxide synthase, metabolizing enzymes, and transferrin-like family of proteins (Llewellyn, 2006). Consumption of 1 mg of the toxin (in 1–5 mussels or clams weighing 150 g each) can be mildly toxic, whereas 4 mg can be fatal if not treated vigorously. Toxic signs/symptoms begin as numbness of the lips, tongue, and fingertips within minutes after eating. Numbness then extends to the legs, arms, and neck and is followed by general muscular incoordination, which progresses to respiratory paralysis and death. Decreased heart rate and contractile force, headache, dizziness, increased sweating, and thirst may also be noted. Boiling in bicarbonate-treated

water and discarding the broth is suggested as a means of preventing shellfish poisoning (Halstead, 1978).

Diarrheic shellfish poisoning occurs globally from consumption of shellfish (mussels, cockles, scallops, oysters, cockles, whelks, and green crabs) contaminated by one of several species of Dinophysis and contain a combination of okadaic acid (OA), dinophysis toxins (DPT), pectenotoxins, and yessotoxins (Brett, 2003). Both OA and DPT are powerful inhibitors of protein phosphatases and potent tumor promoters (Korn et al., 1996). Whether protein phosphatase inhibition leads to the observed increase in the permeability of intestinal epithelial cells exposed to OA, its diarrheic effect is unknown.

Neurotoxic shellfish poisoning is characterized by nausea, vomiting, diarrhea, chills, headache, muscle weakness and pain, eye and nasal irritation, and, in severe cases paresthesia, difficulty in breathing, double vision, dysphonea, and dysphagia, tachycardia, and convulsions. It has been reported along the Gulf of Mexico, the eastern coast of Florida, and New Zealand following consumption of shellfish (mussels, oysters, and whelks) or inhalation of airborne blooms containing a heavy load of *Gymnodinium breve* and/or similar organisms. The lipophilic polyether toxin, the brevitoxin, promotes Na^+ influx and thus depolarization by its action on site-5 of the voltage-dependent Na^+ channels (Brett, 2003; Leftley and Hannah, 1998).

Amnetic shellfish poisoning, characterized by short-term and sometimes permanent memory loss associated with GI signs and a hallucinatory state, has been reported mostly from the coastal areas in North America, Canada, France, Portugal, and the United Kingdom. Neuronal degeneration and necrosis in the hippocampus, coma, and death may result in severe cases. A water-soluble acidic nonprotein amino acid, domoic acid (and its isomers), is produced by the diatom *Pseudo-nitzschia* sp. in king scallops with lower levels in blue mussels, queen scallops, crab, razor fish, anchovies, sardines, mackerel, jack smelt, albacore, sand dabs, krill, and humpback whales. Domoic acid is a competitive glutamate antagonist and has been ascribed the etiological role in amnetic shellfish poisoning (Brett, 2003; Leftley and Hannah, 1998).

Azaspiracids, a relatively new class of rapidly acting algal toxins potentially produced in mussels and other shellfish throughout northern Europe by *Protoperidinium* sp., act by unknown mechanism(s) to induce GI symptoms including nausea, vomiting, severe diarrhea, and stomach cramps. Necrosis in the lamina propria of the small intestine, thymus, and spleen; fatty changes in the liver; chronic interstitial pneumonia; and lung tumors were also observed (Brett, 2003). The amount of cyclic imines in shellfish is not regulated and these substances have not been categorically linked to human intoxication (Otero et al., 2011).

Ciguatoxins are present in 300–400 tropical reef and semipelagic species of edible marine animals, including barracudas, groupers, sea basses, snappers, surgeon fishes, parrot fishes, jacks, wrasses, eels, and certain gastropods. These species accumulate, in their liver and other viscera, toxins capable of causing ciguatera poisoning, at an estimated 20,000–50,000 cases/year worldwide (Brett, 2003; Lipp and Rose, 1997). The intoxication, common in the South Pacific and the Caribbean, appears to follow the spatial and temporal pattern of the distribution of a photosynthetic dinoflagellate *Gambierdiscus toxicus*, which is consumed by the smaller herbivorous fish and in turn by the ciguatoxic fish (Russell, 1986). Ciguatoxins, a group of 23 colorless and heat-stable lipophilic polyethers (MW of 1100), appear to play a major role in intoxication with some contribution from the water-soluble maitotoxin (Leftley and Hannah, 1998). Ciguatoxins increase membrane permeability to sodium ions causing depolarization of nerves. In addition, ciguatoxin inhibits subsequent inactivation of open Na^+ channels and possesses anticholinesterase activity in experimental animals (Leftley and Hannah, 1998; Russell, 1986). Maitotoxin, on the other hand, inactivates voltage-dependent and receptor-mediated Ca^{++} channels leading to high intracellular Ca^{++} and cell death (Leftley and Hannah, 1998). Ciguatoxicosis is the most common marine toxicosis in humans manifesting as tingling of the lips, tongue, and throat followed by numbness, nausea, vomiting, abdominal pain, diarrhea, pruritus, bradycardia, dizziness, muscle and joint pain, and ataxia. Severe cases exhibit paresis of the legs and infrequently death due to cardiovascular and/or respiratory failure (Leftley and Hannah, 1998; Russell, 1986). Prevention of ciguatera poisoning is difficult, although extensive evisceration of fish may help.

Puffer fish (fugu fish) poisoning, known to occur as far back as 2000–3000 BC in China and Japan, results from consumption of tetrodotoxin (TTx) present in the liver and ovaries of puffer fish, ocean sunfishes, porcupine fishes, blue-ringed octopus, and certain amphibians of the family Salamandridae (Kao, 1966; Russell, 1986). Toxin accumulation is greatest just prior to spawning in the spring. TTx, with a cyclic hemilactal structure, is highly lethal (LD_{50}, 10 mg/kg) to all vertebrates and is active after boiling for 1 h but is inactivated under alkaline conditions. TTx prevents the increase in the early Na^+ permeability in both motor and sensory neuronal membranes similar to that of STX (Russell, 1986). In humans, numbness of the lips, tongue, fingers, and arms, muscular paralysis and ataxia, hypotension, and respiratory paralysis leading to death progress rapidly beginning 30–60 min after consumption of 1–2 mg of TTx (1–10 g of roe or liver). Training of personnel in proper evisceration techniques and licensing of fugu restaurants are of the essence.

Scombroid poisoning is the most widespread fish-borne intoxication resulting from the consumption of inadequately preserved abalone, amberjack, bluefish, tuna, mackerel, mahi-mahi, and sardines in which histamine and saurine are produced as a result of bacterial scombrotoxic action (Lipp and Rose, 1997). Scombroid fish apparently has a sharp or peppery taste. Signs of intoxication include nausea, vomiting, diarrhea, epigastric distress, flushing of the face, throbbing headache, and burning of the throat followed by numbness and urticaria. Severe cases may lead to cyanosis and respiratory distress and, rarely, to death. These signs appear within

2 h of the meal and disappear in 16 h (Russell, 1986). The disease readily responds to antihistamine treatment.

Other marine toxins such as prorocentrolides, pinnatoxins, and spirolides are thought to activate calcium channels, and some were implicated in over 2500 cases of illness in Japan following consumption of the bivalve *Pinna pectinata*. Certain other compounds from algae including amphidinolides and carbenolides with cytotoxic activity against tumor cells, zooxanthellotoxins with vasoconstrictor activity, and gambierdic acids and goniodomin with antifungal properties may be of therapeutic potential (Cheeke, 1989). Due to their actions on a variety of channels and receptors, small amounts of toxin required to produce effects, their heat-stable nature, and the rapidity with which the effects are produced, marine toxins such as STX, TTx, and OA have become internationally regulated chemical weapons.

Foodborne Bioterrorism

Attacks with biological agents are appealing to organizations with limited resources such as terrorist and radical groups, with intent to scare the masses rather than inflict mass casualties. Biological/toxic agents can be grown inexpensively but are difficult to weaponize for aerosol dispersal making large-scale bioterrorist attack unlikely for most radical groups. Bioterrorism involving food and water supplies is a more practical alternative. CDC (Rotz et al., 2002) groups biological warfare agents into (1) category A (easily disseminated/transmitted from person to person, capable of high mortality rates, with the greatest impact on the public health system and civilian psyche, e.g., variola [smallpox], *Bacillus anthracis* [anthrax], *Yersinia pestis* [plague], *C. botulinum* [botulism], *Francisella tularensis* [tularemia], and filo- and arenaviruses [viral hemorrhagic fever]); (2) category B (moderate dissemination and morbidity and lower mortality rates, e.g., *Coxiella burnetii* [Q fever], *Brucella* [brucellosis], *Burkholderia mallei* [glanders], *Burkholderia pseudomallei* [melioidosis], alphaviruses [encephalitis], *Rickettsia prowazekii* [typhus], toxins [toxicoses], *Chlamydophila psittaci* [psittacosis], food safety threats [*Salmonella*, *E. coli,* etc.], and water safety threats [vibrio, cryptosporidium, etc.]); and (3) category C (emerging pathogens currently limited by availability and difficulty in production, e.g., Nipah virus [encephalitis], Hantavirus [pulmonary syndrome], tickborne hemorrhagic fever virus, yellow fever, multidrug-resistant tuberculosis). Although most biological agents are unstable in the environment; are destroyed by public water treatment methods, boiling of water and cooking of food; cause only short-term vomiting and diarrhea; or would require large amounts to overcome dilution, many steps along the centralized food processing and rapid and wide distribution of foods still present a window of vulnerability for intentional introduction of biological agents (organisms and toxins, most likely including botulinum toxin, *Salmonella*, *Shigella*, *E. coli*, and *V. cholerae*) into food products. Recent reviews of bioterrorism agents include those of Karwa et al. (2005) and Meinhardt (2005).

Compared to conventional chemical weapons, toxins are generally difficult to produce in large quantities, nonvolatile, more toxic by weight, dermally inactive, odorless and tasteless, immunogenic, and slow acting (Karwa et al., 2005). Among the toxins used as aerosols, agents that are highly toxic but hard to produce may be more of a threat in a closed space delivery system, while those that are stable and easily produced and delivered are likely used as open-air weapons. Some toxins are also effective when ingested and others are dermally active. Food bioterrorism, similar to those of a nonterroristic foodborne disease, involves large numbers of people within a geographical area consuming the same contaminated food product(s) exhibiting signs characteristic of the agent involved within a short time frame (hours for toxins and up to 72 h for microorganisms) after consumption. Following the September 11, 2001, terrorist attack on the World Trade Center in New York in preparation for dealing with foodborne terrorism events, under the authority of the Public Health Security and Bioterrorism Preparedness and Response Act passed in June 2002, the FDA developed new regulations that addressed registration of all (domestic and foreign) food facilities: (1) prior notification of importation of food shipments, (2) establishment and maintenance of records of receipts and shipments by all processors, and (3) administrative detention of suspect food. More recently, the FDA added Food Security Preventive Measures Guidance (U.S. FDA, 2003) listing security and testing measures to ensure the physical and chemical safety of milk and food products.

Table 14.5 lists toxicants that could potentially be employed as foodborne/waterborne terrorism agents. Of these, the marine toxins (STX and TTx) are difficult to produce and are considered only remote threats. As discussed in the succeeding text, botulinum toxin, staphylococcal enterotoxin B, ricin, and trichothecene mycotoxins have been stockpiled and/or allegedly used in warfare/terrorism in the past and are most likely to be used in future bioterrorism.

Botulinum toxin, produced by the bacterium *C. botulinum*, in addition to its well-known involvement as a foodborne toxin and its approved therapeutic/cosmetic uses, was once stockpiled, experimented, and/or used by the United States, Russia, Iraq, and the Aum Shinrikyo sect in Japan (Karwa et al., 2005). Its absorption via lung renders is a potential threat by aerosol dispersion (particle size 0.1–0.3 μm). Although aerosolization was thought to be a more efficient means of attack leading to the potential death of 1.5 million people with 1 kg of toxin, recent estimates (Wein and Liu, 2005) suggest that less than 1 g of botulinum toxin introduced into the milk supply at some point along the processing (between milking on the farm and bottling in the processing plant) could result in 100,000 casualties in the absence of testing/detection, suggesting that direct contamination of the food supply may be an even more effective means of releasing the toxin. Other products such as juices and other beverages, subject only to pasteurization temperatures before consumption, are also candidates for such attack. Signs, as described earlier, mainly include vision

TABLE 14.5
Toxins with Potential for Use in Foodborne Terrorism

Toxin Type	Examples	Source	Syndrome
Bacterial	Botulinum toxins	*C. botulinum*	Inhalation: Neurologic (descending paralysis)
	C. perfringens toxins and	*C. perfringens*	Ingestion: gastroenteritis
		S. aureus	Ingestion: gastroenteritis
	Staphylococcal enterotoxin B		Inhalation: toxic shock and pulmonary edema
Fungal	Aflatoxin	*A. flavus*	Inhalation: pulmonary edema and hemorrhage Ingestion: hemorrhagic gastroenteritis
	T-2 toxin	*Fusarium* spp.	Dermal: blistering Inhalation: tracheobronchitis and hemoptysis
Algal	Anatoxin A	Blue-green algae	Ingestion: paralysis
	Microcystin	Blue-green algae	Ingestion: paralysis and liver damage
Marine	STX TTx	Dinoflagellate	Ingestion: neurotoxin
		Puffer fish	Ingestion: neurotoxin
Plant	Ricin	Castor bean	Inhalation: respiratory distress Ingestion: gastroenteritis and shock
	Abrin	Precatory bean	Similar to ricin

Sources: Data extracted from Meinhardt, P.L., *Ann. Rev. Pub. Health*, 26, 213, 2005; Karwa, M. et al., *Crit. Care Med.*, 33(supp), S75, 2005.

disturbances, dysphagia, and dysphonia initially followed by descending paralysis, hypotension, and respiratory failure as early as 24 h after exposure. Treatment involves activated charcoal, respiratory support, and administration of the antitoxin. Early symptomatic detection avoids up to two-thirds of the casualties, whereas rapid ELISA testing to detect the toxin at each point in the sequence of events between milking and bottling would prevent nearly all cases. Thus, employment of security measures and testing at each point of production, collection, processing, and transport of foods such as those proposed by the Food Security Preventive Measures Guidance for milk and other food products (U.S. FDA, 2003) are the best safeguards against terrorism involving food products.

Ricin, a lectin from castor bean (*Ricinus communis*), has a significant bioterror history and potential (Audi et al., 2005). Its recent discovery at a South Carolina post office, a White House mail center, and a U.S. senator's office and the still unknown origin are of concern. Ease of production and high toxic potency by oral, inhalation, or parenteral exposure makes ricin highly attractive for bioterrorists, especially when inhaled. The most likely scenarios of ricin use include aerosol release into the environment or adulteration of food and beverages. Ingestion of ricin leads to nausea, vomiting, diarrhea, and abdominal pain beginning within 12 h and progressing to hypotension, liver failure, renal dysfunction, and death due to multiorgan failure or cardiovascular collapse. Inhalational exposure produces cough, dyspnea, arthralgias, and fever and may progress to respiratory distress and death. Ricin analysis at federal laboratories and supportive measures are the best aids to diagnosis and treatment, respectively.

Staphylococcal enterotoxin B, a common foodborne toxin, is an enterotoxin that can be mass produced from cultures of *S. aureus*. It is stable as an aerosol. Inhalation exposure results in binding of the toxin to the major histocompatibility complex that stimulates T cells leading to massive release of cytokines. This leads to interstitial pulmonary edema including fever, myalgia, cough, chest tightness, dyspnea, headache, and vomiting (Karwa et al., 2005). Signs of toxic shock syndrome (hypotension, shock) also occur. The need for large amounts of the toxin necessary to produce effects makes this toxin less desirable compared to some of the other toxins.

The trichothecene toxins (T-2 toxin) may represent the most promising of the mycotoxins as a bioagent because of its ability (1) to induce effects immediately upon contact, (2) to produce lethality at only a few milligrams, (3) to be easily and inexpensively produced, (4) to remain stable as an aerosol, and (5) to spread readily from person to person (Karwa et al., 2005; Stark, 2005). There is no known antidote or vaccine. Allegations of their use as a bioagent either by the United States against North Korea and China in 1952 or by the Soviet Union to attack Hmong tribesmen in Laos and Kampuchia (as *yellow rain*) in 1981 and later in Afghanistan either remain unsubstantiated or have been disproved. Victims, in the yellow rain incident, appear to have exhibited signs similar to those expected from trichothecene intoxication (blistering of the skin, corneal injury, wheezing, cough, tracheobronchitis, and hemoptysis), and leaf samples from the area contained traces of trichothecenes. Subsequently, these allegations were negated by reports that the material in the so-called yellow rain is likely a mass defecation by swarms of Asian honeybees and the trace levels of trichothecenes likely reflected natural production in this area. The U.S. military still considers these agents as serious bioweapons as evidenced by clearance of a reactive skin decontamination lotion by the FDA in March 2003 for use by the military to remove or neutralize chemical warfare agents and T-2 fungal toxin from the skin. Additional information

on history of bioweapon use, mechanism of action, and signs of intoxication of trichothecenes can be found in Karwa et al. (2005) and Stark (2008).

V. cholerae causes the most severe seasonal diarrheal disease epidemics mostly in Asia, Africa, and Latin America. Its main mode of transmission is via contaminated water and food. The same media could be used by terrorists (Meinhardt, 2005) and apparently was used by the Japanese in World War II in China (Karwa et al., 2005). Serogroup O1 (with two main serotypes, Inaba and Ogawa, and biotypes, classical and El Tor) and the recently identified serogroup 0139 Bengal have caused the most severe disease natural outbreaks. The CT is similar to the heat-labile enterotoxin secreted by *E. coli* and causes diarrhea by the same mechanism: excessive net secretion of electrolytes and water from the upper fifth of the small intestine. Details of the factors that trigger, and the mechanisms involved in, the secretion of CT, its mode of action, and the immunology of the disease have been reviewed by Sanchez and Holmgren (2005). Interestingly, the presence of lytic cholera phages in environmental waters appears to reduce the presence of *V. cholerae*, thus providing a mechanism whereby the emergence and duration of cholera epidemics can be naturally controlled. Recently developed mixed serotype and biotype inactivated *V. cholerae* O1 and attenuated classical *V. cholerae* O1 Inaba vaccines appear to be superior to the earlier vaccines due to improved local (gut) immunity. However, immunity only lasts for 6 months.

For details on disease caused by ETEC and *Shigella* spp., please refer to the discussion on *Foodborne Bacterial Diseases* section in this chapter.

Although contamination of foods with conventional chemical weapons including nerve agents (tabun, sarin, soman, and especially VX), cyanide, incapacitating agents (BZ and agent 15), vesicants (mustards, phosgene, and lewisite), and choking agents (phosgene, chlorine, and bromine), or with radionuclides is possible, the volatile nature of the former (with the exception of VX) and the restricted availability of the latter make threats involving foods less likely. The FDA-approved antidote treatment nerve agent autoinjector (ATNAA) (atropine/pralidoxime) autoinjector to treat nerve gas intoxication and new dosage forms of AtroPen (atropine) autoinjectors for use in children and adolescents to deal with nerve agents; ThyroSafe (potassium iodide) tablets to protect the thyroid from general radiation exposures; Prussian blue to inhibit absorption of radioactive cesium and thallium; and pentetate calcium trisodium (Ca-DTPA) and pentetate zinc sodium (Zn-DTPA) to increase elimination of internal contamination with plutonium, americium, or curium, found in the fallout from nuclear detonation and waste from nuclear power plants, are examples of approaches available to respond to and to treat individuals in such emergencies (Meadows, 2004).

Much progress has occurred in our identification and management of foodborne hazards. According to CDC estimates, annual foodborne disease burden has fallen from 76 million cases, 5,000 deaths, and 325,000 hospitalizations in 1999 to approximately 48 million cases, 3,000 deaths, and 128,000 hospitalizations in 2012. Large gaps in our knowledge, however, exist in the areas of mechanisms of pathogenesis of known human intoxications associated with foods; interactions between multiple toxicants present simultaneously, between toxicants and nutritional components, and between toxicants and antitoxicants (including antimutagens and anticarcinogens) in foods; methods of realistic human health risk extrapolation from animal data; and the development of safer plant varieties and processing, cooking, and storage methodologies that minimize toxic hazards to consumers. Considering the facts that natural dietary toxicants are, at least, as toxic as synthetic additives and that their exposure is generally much greater in quantity and consistency than synthetic toxicants, U.S. and worldwide research resources should be shifted to achieve a more realistic balance, in the study of health hazards, toward the natural components in the food supply. Others have advocated this approach as well (Borchers et al., 2010; Pascal, 2009). This task will be difficult because of the vastly variable composition of individual food ingredients as well as the total human diet.

Increased globalization of the food industry, especially fresh produce, requires implementation of improved food safety programs, with a special emphasis on imported foods. Mandatory rules for food protection and safety need to be developed by the FDA and the food industry to prevent future outbreaks, such as the 1996 outbreak of *Cyclospora cayetanensis* from Guatemalan raspberries and the S. enterica Serotype Newport from Brazilian mangoes. Timely recognition of outbreaks and epidemiologic investigations are also critical to identify new infectious agents, vehicles of transmission, and modes of contamination and to institute corrective measures for prevention. The application of newer molecular methodologies to fingerprint the causative organisms (e.g., CDC's *pulsenet*) to identify patterns of intoxication from bacterial and other biotoxins and intensified activities of the national animal health monitoring system combined with more rigorous application of HACCP or similar control methodologies will lead to significant reduction in currently widespread incidences of microbial diseases from food sources.

Just as important is consumer education. Such consumer education is needed to minimize dietary risks using practicable methods and to shatter the myth that *natural is healthy but man-made is toxic*. An educated populace is less likely to be unduly alarmed and is more likely to accept prudent regulatory actions resulting from realistic scenarios of risk estimation.

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15 Solvents and Industrial Hygiene

David L. Dahlstrom and John E. Snawder

CONTENTS

Industry and the general public use a wide range of organic solvents to develop, synthesize, modify, and improve many of today's most useful chemical products and manufacturing applications. On a global basis, chemical manufacturers produce billions of pounds of organic solvents annually. Common solvent uses include degreasing agents, paint thinners and removers, dry-cleaning agents, chemical intermediates, extractants, and carrier vehicles for paints, varnishes, and industrial coatings. Many of the organic solvents find applications within the chemical industry in the production of waxes, paints, varnishes, lacquers, pharmaceuticals, plastics, pesticides, rubber goods, synthetic textiles, adhesives, shoe polish, floor cleaners, and many other outstanding products in everyday use.

Many organic solvents can prove to be environmental and occupational toxicants when used without proper and adequate administrative and engineering controls. Broad or singular statements to describe adverse health effects associated with solvent exposure are difficult to make. Such adverse effects will differ depending upon the physical and chemical properties associated with each solvent, class, or composition of a mixture of solvents; the physiological route the solvent enters the body; the nature of the environment in which the solvent is being used; how the solvent/mixture is being used (e.g., liquid or vapor, aerosolized or hand-wiped, hot or cold), the variability of the exposure over time, and even the relative health, gender, or age of the individual exposed. The overriding issue for the industrial hygienist and occupational health specialist is not so much identifying that a potential exposure hazard to an individual solvent or mixture exists, but determining at what concentration, frequency, duration, and route(s) of entry (oral, inhalation, skin, and eye contact) a dose of the solvent or solvent mixture will result in a harmful effect (e.g., dermatitis, systemic injury, irritation, narcosis, neuropathy, carcinogenicity).

CHEMICAL CLASSIFICATION FOR SOLVENTS

In general practice, organic solvents are categorized on the basis of their respective structure and composition into one of 11 chemical classes. Figure 15.1 depicts the general chemical structures for each class and examples of common solvents in each group [128,172]. The 2012 American Conference of Government Industrial Hygienists (ACGIHs) 8 h time weighted average (TWA) threshold limit values (TLVs®) and respective U.S. Department of Labor—Occupational Safety and Health Administration's (OSHA) Permissible Exposure Limit (PEL) for each example is provided.

PROPERTIES OF SOLVENTS

To understand the hazards that organic solvents may present, industrial hygienists (IHs) and other health, safety, and environmental (HS&E) professionals need to know about the basic physical, chemical, and toxicological properties, workplace conditions, proximity of use to other chemical use, the nature or the work activity, and the behavior characteristics of the organic solvents and mixtures being used. The following description of key chemical and physical properties of organic solvents should be considered when attempting to evaluate and control the various risks (defined by both the nature of the hazard and toxicity of the material) that can result when working with organic solvents. Additionally, the industrial hygienist must recognize that most technical-grade solvents used in the workplace often contain small amounts of impurities and that these impurities can affect the properties inherent in the pure solvent.

Boiling Point

The temperature at which the vapor pressure of a liquid becomes equal to the pressure of the surrounding atmosphere is the liquid's *boiling point*. In typical use, a pressure of 1 atm (760 mmHg or 14.7 psi) is considered standard atmospheric pressure at sea level. This atmospheric pressure will vary, however, depending on elevation above or below sea level and should be taken into account when estimating boiling temperatures [21,22].

Vapor Pressure

Like many liquids, most organic solvents begin to evaporate at temperatures below their boiling point. As the temperature of the liquid increases, the volume of vapor evolving will likewise increase. The *vapor pressure* of a liquid is the amount of pressure exerted by the saturated vapor above the surface of the liquid within a closed container [120]. Units of vapor pressure are usually expressed in millimeters of mercury, or mmHg at 20°C (68°F). In principle, vapor pressure is inversely related to boiling point in that the lower the boiling point of the solvent, the higher the relative vapor pressure will be, allowing the solvent to more readily volatilize into the surrounding air. In short, the higher the vapor pressure of the liquid, the greater the propensity to evaporate [21,22,64].

The vapor pressure of a liquid will increase as the temperature of the liquid is increased. Therefore, the amount of vapor above the surface of the solvent entering an open

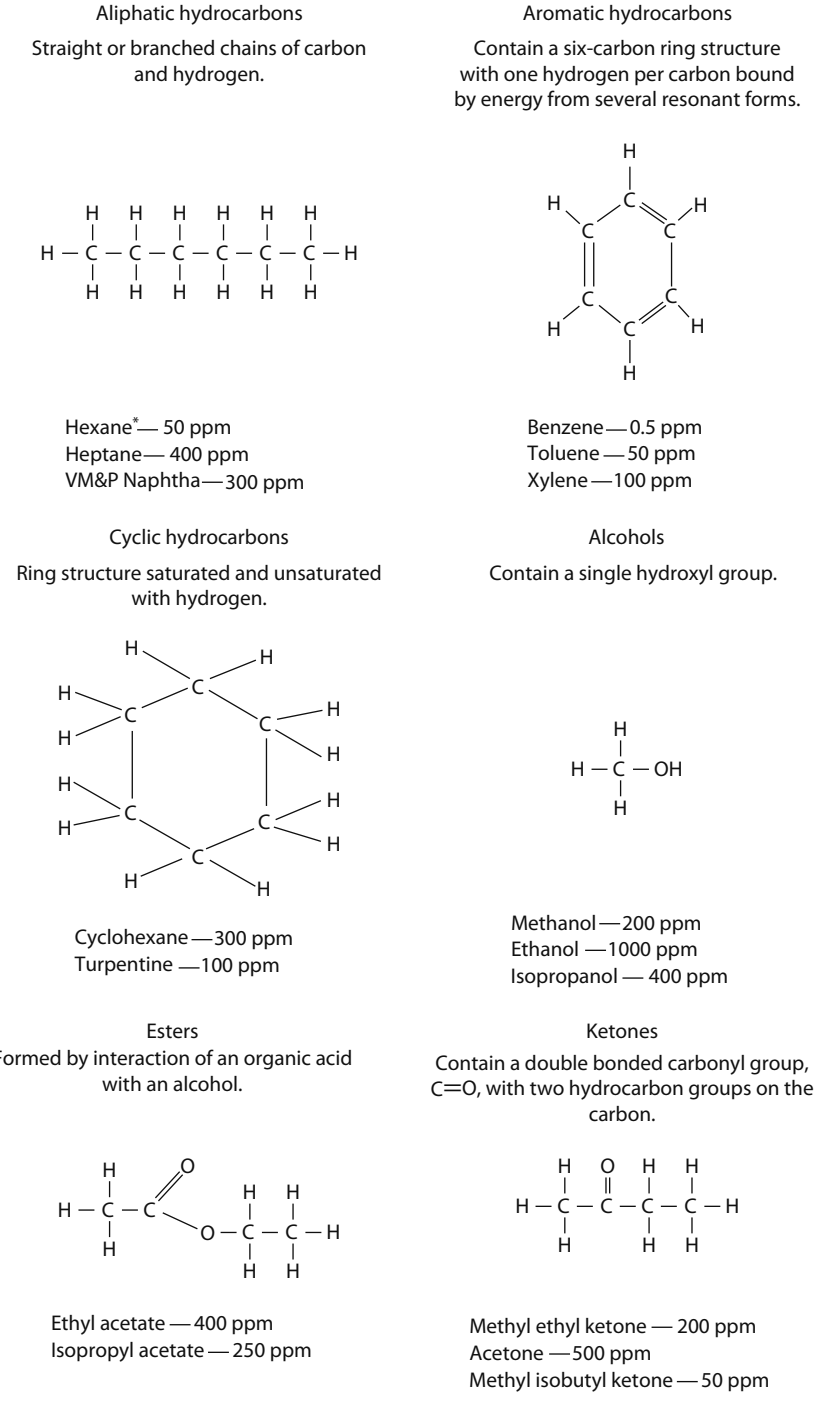
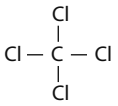


FIGURE 15.1 Classes of organic solvents.

Halogenated hydrocarbon

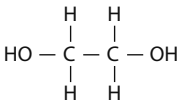
A halogen atom has replaced one or more hydrogen atoms on the hydrocarbon.



Carbon tetrachloride—5 ppm
Methyl chloroform—350 ppm
Chloroform—10 ppm

Glycols

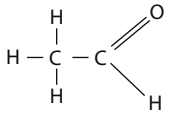
Contain double hydroxyl groups.



Ethylene glycol—100 mg/m^3 (ceiling)
Hexylene glycol— 25 ppm (ceiling)

Aldehydes

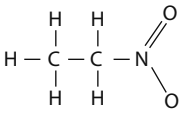
Contain the double-bonded carbonyl group, C=O, with only one hydrocarbon group on the carbon.



Acetaldehyde—25 ppm (ceiling)
Formaldehyde—0.3 ppm (ceiling)

Nitro-hydrocarbons

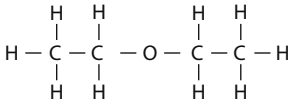
Contain an NO_2 group.



Nitroethane —100 ppm
Nitromethane —20 ppm

Ethers

Contain the C —O —C linkage.



Ethyl ether— 400 ppm
Isopropyl ether—250 ppm

FIGURE 15.1 (continued) Classes of organic solvents.

environment is dependent on the surface area of the liquid, the temperature of the solvent, and the atmospheric pressure.

The IH/HS&E professional is able to use the vapor pressure of a solvent to calculate what the concentration of the solvent vapor in parts per million (ppm) will be at the point of saturation or equilibrium within a given environment. This is performed by dividing the vapor pressure of the solvent by the total atmospheric barometric pressure and then multiplying by 1,000,000:

$$\frac{\text{Vapor pressure of the liquid}}{760\,\text{mmHg}} \times 1{,}000{,}000 = \text{X ppm of vapor}$$

Volatility

Volatility is the ability of a solvent to evaporate. The term *volatile* is commonly used to mean that a material evaporates easily. Volatility is directly related to a substance's vapor pressure. At a given temperature, the substance with higher vapor pressure volatilizes more readily than a substance with a lower vapor pressure. Solvents, or products containing volatile solvents, often present a need for general and/or local exhaust ventilation and other precautions to control vapor concentrations below established exposure and flammability limits [41].

Evaporation Rate

The rate by which liquid evaporates is dependent on a number of intrinsic properties of the liquid and various external factors; the *evaporation rate* is a measure of how quickly the material sublimes to a vapor at normal room temperature. Usually, the evaporation rate is given in comparison to a reference liquid or solvent, such as *n*-butyl acetate or ethyl ether, which evaporates fairly quickly at the same temperature and atmospheric pressure. Because the reference solvent is given an evaporation rate of 1.0, those solvents with an evaporation rate of less than 1.0 evaporate more quickly than the referent and those whose evaporation rate is greater than 1.0 evaporate more slowly [21,22,41]. Often, in many chemical references (i.e., Chemical Safety Data Sheets), the evaporation rate is given only as greater or less than 1, which means the solvent evaporates faster or slower than the reference chemical.

Flash Point

The flash point is the lowest temperature at which a liquid or solid gives off enough vapor to form a flammable air–vapor mixture near its surface. The lower the flash point, the greater the fire hazard. The flash point is an approximate value and should not be taken as a sharp dividing line between safe and hazardous conditions [188]. The flash point of a liquid is often empirically determined by a variety of test methods; each method provides different results. Two types of methods most commonly used are open cup (OC) and closed cup (CC). The flash point is determined based on the temperature at which a solvent within an OC or CC instrument—creates enough vapor sufficient to flash when an ignition source is introduced. Flash point can be used, in part, as a criterion to define under what temperature a solvent may be safely stored in open containers and used. However, solvents introduced into the atmosphere as sprays or mists can be ignited below the flash point of the solvent [21,22,41].

Flammable (Explosive) Range

The range of concentrations of a gas or vapor that, when mixed with air and exposed to an ignition source (spark or flame), will ignite or rapidly combust (explode) is recognized as the *flammability* (explosivity) range for the particular gas or vapor. The flammability (explosivity) range is inclusive of all concentration levels of a gas or vapor from the lowest concentration (lower flammability limit [LFL] or lower explosive limit [LEL]) to the highest concentration (upper flammability limit [UFL] or upper explosive limit [UEL]) a vapor or gas can be made to combust. The range of concentrations between the LFL/LEL and the UFL/UEL is commonly reported in percent concentration (by volume) of the vapor in the air and is typically based on an ambient temperature of 68°F. When considering the LFL/LEL and UFL/UEL of a gas or vapor in conditions other than that of an ambient temperature of 68°F and 1 standard atmosphere, one should recognize that the general effect of increasing the temperature or pressure is to decrease the lower limits and increase the upper limits. Conversely, a lowering of the temperature or pressure will serve to raise the lower limits and lower the upper limit [11,12]. Figure 15.2 provides a graphical depiction of the relationships among flammable/explosive limits, vapor pressure, and flash points [21,22,41].

Specific Gravity

The ratio of the weight of a given volume of a substance (e.g., solvent) at a given temperature (usually given as 75°F) and atmospheric pressure to the weight of an equal volume of water at the same temperature, and atmospheric pressure is known as the *specific gravity* (Sp. Gr.) of the substance. The density of water is about 1 g/cm^3 or g/cc. If the specific gravity of a solvent is less than 1, it will float on top of water; if it is greater than 1, it will tend to sink, depending on its solubility. Therefore, in situations where a solvent is spilled into a water body, it is imperative to determine whether the solvent sinks (Sp. Gr. > 1) or floats (Sp. Gr. < 1) with regard to its ability to catch fire should it reach an ignition source.

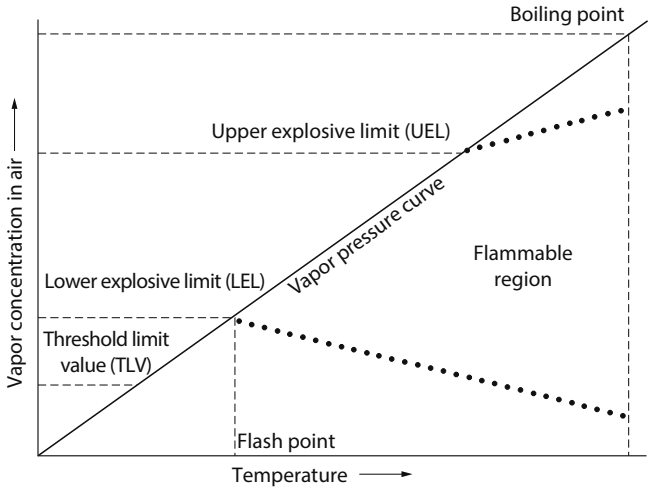


FIGURE 15.2 Diagram of vapor pressure vs. temperature depicts the relationships among upper and lower flammable (explosive) limits, flammable and nonflammable regions, threshold limit value, boiling point, flash point, and vapor pressure curve. This diagram shows what happens to a vapor/air mixture as concentrations and temperature vary. *, TLV®, American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV-TWA), 1998.

Vapor Density

The ratio of the weight of a given volume of vapor or gas to the weight of an equal volume of air at the same temperature and atmospheric pressure defines the *vapor density* of that vapor or gas. Vapors or gases with a vapor density less than 1 (lighter than air) will rise into the air, whereas those with a vapor density greater than 1 (heavier than air) will tend to sink to the lowest point in the immediate surroundings. The vapor density of a solvent can be roughly estimated by dividing the molecular weight of the solvent by the molecular weight of air (MW ~ 29). This value is important when considering the potential for oxygen displacement or fire/explosion should an ignition source be in low-lying areas when a solvent is spilled. Conversely, the likelihood of vapors rising and representing an acute respiratory hazard when the vapor density is less than 1 is equally worthy of consideration [21,22,41].

Partition Coefficient

The *partition coefficient* (P) also called the coefficient of oil/water distribution is the ratio of the solubility of a solvent in an oil to its solubility in water. The P value is typically presented as a logarithm of P (log P). The partition coefficient indicates how easily a chemical can be absorbed into or stored within the fatty tissues of the body [74,157]. The P value is also helpful in predicting the fate of the chemical in the environment through its tendency to partition into the water, soil, and living organisms [41,66,171].

WORKPLACE EXPOSURE LIMITS

Occupational exposure limits (OELs) are developed by various organizations for the purpose of protecting the health of workers who may be exposed to specific hazardous substances during their work. Published OELs typically represent consensus standards at the time and are formulated by qualified experts like toxicologists, occupational physicians, industry experts, and epidemiologists. Such experts utilize the most reliable scientific knowledge available at that time to construct a recommended (i.e., National Institute for Occupational Safety and Health (NIOSH)—Recommended Exposure Limit (REL), ACGIH Threshold Limit Value) or enforceable (i.e., OSHA PEL) maximum airborne concentration (incorporating a safety factor to account for uncertainties) over some specified time period for a specific workplace contaminant. Therefore, these OELs are presented as representative of an airborne concentration of a particular chemical that is deemed an acceptable risk to life and health for the general worker population [193]. It is important to recognize that these OELs do not provide a bright line between what concentration is safe and not safe due to recognized uncertainties, variations of effects due to age, health status, gender, work activity level, or workplace environmental conditions, to name a few considerations.

In the United States, the Occupational Safety and Health Administration (OSHA) is empowered to establish and enforce Permissible Exposure Limits (PEL), likewise the Mine Safety and Health Administration (MSHA) also may develop legally enforced OELs specific to mining. Other federal agencies that publish occupational exposure limits include the National Institute for Occupational Safety and Health (NIOSH) Recommended Exposure Limits (REL), the Environmental Protection Agency's (EPA) New Chemical Exposure Limits (NCELs) and voluntary Acceptable Exposure Limits (AELs), the Department of Energy's (DOE) established personnel radiation dose limits, and finally the Department of Defense's establishment of specific OELs for military personnel. Nongovernmental organizations, such as the American Conference of Government Industrial Hygienists before (ACGIH), develop and present an annual compilation of Threshold Limit Value (TLV®) and Biological Exposure Indices (BEIs®) or American Industrial Hygiene Association's (AIHA) Workplace Environmental Exposure Limits (WEELs). Additionally, many states may develop their own specific workplace- or community-focused exposure regulations. Many manufacturers may establish OELs for their own products or processes. In Canada, OELs are established by each province. Similarly, the Control of Substances Hazardous to Health (COSHH) regulations within the United Kingdom regulate workplace exposure levels by specifying maximum exposure limits (MELs) and occupational exposure standards (OESs). German workforce exposures are regulated by the Deutsche Forschungsgemeinschaft (DFG) maximum concentration values in the workplace (MAKs). Once again, it is important to recognize that many of these established OELs will vary from organization-to-organization that creates them as well as over time as scientific knowledge evolves.

The establishment of new OELs is a complex matter. In general, the process involves the review of reliably established toxicity data from a number of relevant sources to identify critical studies, health effects, and toxic endpoints. After the application of safety and uncertainty factors to the critical data, an OEL value may be derived. Each step of the process requires considerable professional judgment in the selection of the appropriate critical studies, health effects, and derived toxic endpoints, as well as in the application of appropriate safety and uncertainty factors. Because of differences in individual perspective, institutional criteria, and political realities, large differences in final OEL value recommendations can and often do occur.

There are four different types of occupational exposure limits in common use:

1. A TWA exposure limit (i.e., OSHA-PEL or ACGIH-TLV) is the TWA concentration of a chemical in air typically averaged over a normal 8 h work day and 40 h work week to which, it is thought, nearly all workers may be exposed day after day without harmful effects. TWA means that the material's average airborne concentration has been calculated using the duration of exposure to variable concentrations of the chemical over a specific time period. In this way, higher and lower exposures are averaged over the work period, day, or week.
2. A short-term exposure limit (STEL) is generally defined as the average concentration to which workers can be exposed for a short period (usually 15 min) without experiencing irritation, long-term or irreversible tissue damage, or reduced alertness. The number of times the concentration reaches the STEL and the amount of time between these occurrences can also be restricted.
3. A ceiling (C) exposure limit is the concentration of the chemical in air, which should not be exceeded at any time.
4. The Immediately Dangerous to Life and Health (IDLH) designation observed for many chemicals refers to an extremely hazardous, life-threatening concentration of the chemical. It is the estimated maximum concentration of an airborne contaminant from which a worker can escape (e.g., after failure of respiratory protection) without loss of life or suffering permanent health impairment within a specified period of time. A chemical's IDLH is defined by U.S. law as "An atmospheric concentration of any toxic, corrosive, or asphyxiant substance that poses an immediate threat to life or would cause irreversible or delayed adverse health effects or would interfere with an individual's ability to escape from a dangerous atmosphere. [29 CFR* 1910.120]" Workers who enter into an IDLH atmosphere are required to wear the highest form of respiratory protection (positive-pressure, self-contained, or airline breathing apparatus) and usually fully protective clothing ensemble. The purpose of establishing an IDLH value is (1) to

> ensure that the worker can escape from a given contaminated environment in the event of failure of the respiratory protection equipment and (2) is considered a maximum level above which only a highly reliable breathing apparatus providing maximum worker protection is permitted [146].

Regardless of the source or legal standing, OELs should not be taken as sharp dividing lines between safe and unsafe exposures. It is possible for a chemical to cause health effects, in some people, at concentrations lower than the recommended OEL while others may not suffer the health effects expected.

Permissible Exposure Limits

The U.S. Department of Labor's OSHA, publishes a list of regulated PELs as TWAs annually in the Code of Federal Regulations (CFRs), Titles 1910, 1915 and 1926. These regulatory PELs establish the averaged airborne concentrations of over 400 chemicals to which the majority of workers may be exposed over an 8 h day, 40 h a week, over a working lifetime (40–45 years) without suffering adverse impact on health. Likewise, the MSHA regulates miner exposures to specified airborne contaminants during an 8 h day and 40 h work week by a similar list of PELs as applied to various types of mining operations (e.g., coal, metallic, and nonmetallic mining). OSHA PELs are incorporated by law as federal OSHA standards in Section 1910.1000 of Title 29 of the CFRs, Tables Z-1 through Z-3, with particular standards for recognized carcinogens provided in sections 1001 through 1048. These PELs were originally derived from the 1968 ACGIH TLVs® and certain air-quality standards recognized as maximum allowable concentrations (MACs) of the American National Standards Institute. The PELs represent the legal allowable concentrations of airborne contaminants within workplaces regulated by OSHA. Although the PELs remain as created unless changes are made in the law, a number of revisions and additions have been made to the PEL list since 1970. The PELs identify chemical TWAs, ceiling values, and skin notations.

Recommended Exposure Limits

The NIOSH RELs are expressed as TWAs, as ceiling limits, or both. These recommended limits are published as chemical-specific criteria documents and are revised periodically as new research information regarding the particular chemical becomes available. The RELs are applicable to worker exposure assessments for up to a 10 h day and are intended to provide the maximum possible health protection for all workers against acute and chronic effects of exposure. Likewise, the RELs provide skin notations for specific chemicals, where applicable. NIOSH publishes and periodically updates its compendium of chemical exposure information for specific chemicals in its NIOSH Pocket Guide to Chemical Hazards (NPG). The NPG is intended as a source of general industrial hygiene information on several hundred chemicals/classes for workers, employers, and occupational health professionals. The NPG presents key information and data in abbreviated or tabular form for chemicals that are found in the work environment. The information found in the NPG includes: Chemical names, synonyms, trade names, Chemical Abstract Number (CAS), Registry of Toxic Effects of Chemical Substances (RTECS) number, Department of Transportation (DOT) Chemical Identification and Guide numbers, Chemical structure/formula, conversion factors, NIOSH RELs, OSHA PELs, NIOSH IDLH values, Physical description and chemical and physical properties of each chemical, suggested measurement methods, personal protection recommendations, sanitation recommendations, respiratory protection selection recommendations, listed chemical incompatibilities and reactivity agents, exposure routes, potential symptoms, target organs potentially effected, and first aid information [143].

Threshold Limit Values

TLVs® and BEIs® are exposure guidelines developed by the ACGIH. Updates of the TLVs® and BEIs® are published by ACGIH each year. Voluntary committees of experts from a variety of disciplines and industries work collectively, using the best available information using industrial experience, animal and human studies to ensure that the TLVs® and BEIs® represent the opinion of the scientific community, based on the respective review of existing published and peer-reviewed literature in various scientific disciplines (e.g., industrial hygiene, toxicology, occupational medicine, and epidemiology). TLVs® and BEIs® are based solely on health factors, and unlike PELs and RELs, there is no consideration given to economic or technical feasibility. A chemical's TLV®–TWA is the airborne concentration to which the average worker may be exposed during an 8 h workday and 40 h work week without suffering adverse effects over a working lifetime. Most TLVs® are presented as TWA values and allow for periodic short-term exposures above the limit, providing there are compensating equivalent exposures that are below the limit during the workday [4]. BEIs®, as provided in the ACGIH's annual publication of TLVs® and BEIs® are a useful measure to evaluate biological monitoring data from workers. The BEIs® provide levels of the specific chemical or its metabolites as measured from exhaled air, blood, and/or urine of workers exposed to the airborne contaminant. Most BEIs® are directly related to the TLV and reflect internal dose for a worker with inhalation exposure to the same chemical at the TLV®. BEIs® are a measure of the amount of chemical or its metabolites in the body and may be useful when evaluating the possibility of skin absorption, effectiveness of personal protective equipment (PPE), or nonoccupational exposure. BEIs are strictly related to 8 h exposures, 40 h a week, and to the specified timing for the collection of the sample [4].

Workplace Environmental Exposure Limits

The AIHA WEEL Committee develops workplace environment exposure limits (WEELs) for chemical agents in common use in the workplace that have no other current OEL

guidelines established by other organizations [10]. WEELs are expressed as an average concentration measured over a time period, as different time periods of exposure measurement are specified, depending on the properties of the particular chemical. The skin notation for a WEEL is used in the same manner as the ACGIH TLV® [4].

USE OF OCCUPATIONAL EXPOSURE LIMITS IN THE WORKPLACE

Time Weighted Average Calculation

The industrial hygienist and other HS&E professionals regularly rely on reference OEL guidelines when assessing exposure to chemical and physical hazards within the workplace. To illustrate the TWA concept, consider a worker who is degreasing metal parts at two different workstations using trichloroethylene (50 ppm TLV®). The employee spends 240 min at Station 1 with an average exposure of 30 ppm, followed by 45 min with no exposure (lunch), then 195 min at Station 2 with an exposure of 60 ppm. Using the formula

$$\frac{\left(C_1T_1\right)+\left(C_2T_2\right)+\left(C_3T_3\right)+\cdots\left(C_nT_n\right)}{480\,\text{min}\left(8\,\text{h}\right)}=\text{X ppm},$$

where

C denotes the concentration of the chemical
T refers to the respective time period of use of that chemical

For the 8 h scenario above the TWA can be calculated as follows [6]:

$$\frac{(30\,\text{ppm}\times 240\,\text{min})+(0\,\text{ppm}\times 45\,\text{min})+(60\,\text{ppm}\times 195\,\text{min})}{480\,\text{min}\left(8\,\text{h}\right)}$$

$$=39.4\,\text{ppm}$$

In this example, the TLV®–TWA of 50 ppm for trichloroethylene exposure has not been exceeded. However, one may want to evaluate the employee's work practices to consider whether the chemical in use can also be absorbed through the skin or eyes, as well as other aspects of the operation (e.g., provision of local exhaust ventilation) to reduce the exposure as much as possible (refer to the sections on exposure controls and PPE).

Today's workplaces are becoming more and more complex. More and varied chemicals of different compositions, structure, size/dimension, and physical state are being incorporated into manufacturing processes, to create new or modified products for the consumer. Simply consider, for example, the workplace processes associated with the development and use of engineered nanomaterials (ENMs), genetically modified (GM) or chemically treated seeds, or the variety of chemicals used and produced in refineries, chemical manufacturing facilities, or even the average fabrication shop. In all of these environments, the average worker can encounter widely fluctuating levels of exposures to chemical mixtures in the workplace environment over the course of the work day, week, year, and career. How does the industrial hygienist evaluate the potential for adverse health impacts on workers in such workplaces where multiple chemicals and their byproducts of production occur?

Provided the components of a mixture have similar toxicological effects, and the workplace air is analyzed for each component, the TLV® for a mixture of airborne chemicals can be calculated by using the formula [2]

$$\frac{C_1}{T_1}+\frac{C_2}{T_2}+\frac{C_3}{T_3}+\cdots\frac{C_n}{T_n}=X$$

where the letters C and T represent the concentration and TLV® of each chemical, respectively. If the calculated results indicate a value greater than 1, then the TLV® of the mixture measured has been exceeded. As an example, suppose a worker was exposed to a mixture of 25 ppm *n*-hexane (TLV® = 50 ppm) and 200 ppm VM&P naphtha (TLV® = 300 ppm) during the shift. The calculated TLV® would be

$$\frac{25\,\text{ppm}}{50\,\text{ppm}}+\frac{200\,\text{ppm}}{300\,\text{ppm}}=0.5+0.67=1.17.$$

The threshold limit has been exceeded, and action should be taken to reduce this exposure. For other examples of TLV®s for mixtures, refer to the most current annual version of the ACGIH TLV® handbook [4].

Adjusting Occupational Exposure Limits for Extended Work Shifts

The evaluation of potential adverse effects upon workers that may result from chemical exposures during extended work shifts (e.g., 10 h or 12 h days) is likewise a point of focus for the industrial hygienist when characterizing job safety within each workplace. To better evaluate job exposures during extended work periods and to compensate for the potential for higher accumulated doses and shorter daily-recovery times, adjustments of the OELs must be made. A review of the approaches to adjusting occupational exposure limits for unusual work schedules is provided in Patty's Industrial Hygiene [155]. The ACGIH, in the TLV®s and BEIs® documentation, refers to many different methods to adapt existing TLV®s to extended work shifts or workweeks [4]. The Brief and Scala model [37] presents one of the simpler guidelines for industrial hygienist dealing with extended work shifts. The Brief and Scala model does not consider health effects, toxic action, or pharmacokinetics. The model simply adjusts the respective exposure limits according to a reduction factor calculated for a single work day by the formula

$$\text{Adjusted TLV}^{\circledR}=\text{TLV}^{\circledR}\times\left[\frac{8}{\text{h}}\times\frac{\left(24-\text{h}\right)}{16}\right]$$

where h = # of hours worked per day or for a work week

$$\text{Adjusted TLV}^{®} = \text{TLV}^{®} \times \left[\left(\frac{40}{\text{h}} \right) \times \frac{(168 - \text{h})}{128} \right]$$

where h = # of hours worked per week

As an example, suppose a worker assigned to a 12 h shift works on a process using methanol. The TLV® for methanol is 200 ppm. Applying the single day formula, the adjusted TLV® to be utilized to assess worker exposure/overexposure over the extended work period would be

$$200 \text{ ppm} \times \left[\frac{8}{12} \times \frac{(24 - 12)}{16} \right] = 200 \times \left[0.667 \times 0.75 \right] = 100 \text{ ppm}$$

INDUSTRIAL HYGIENE SAMPLING METHODOLOGY

The industrial hygienist can utilize a variety of different sampling techniques, instruments, and analytical methods to identify and measure the concentration of a workplace contaminant (chemical, biological, radiological, or physical hazard) in the workplace. In the case of airborne hazards, the concentration in the workplace is evaluated from the perspective of either the general work environment where the work is performed or by measuring the concentration of the contaminants in the air directly breathed by specific worker by sampling within the personal breathing zone (PBZ), an area approximately one foot in radius around the worker's nose.

There are a number of methods available to estimate the identity and/or concentration of contaminants within the workplace. The primary methods of industrial hygiene sampling include active sample collection (typically utilizing a calibrated sampling pump and specific sampling media), direct reading instrumentation, or passive dosimetry. The respective qualitative and quantitative value of the measurement obtained from each of these sampling methods is dependent on the level of accuracy required, the urgency in obtaining results, the cost of sample collection and analysis, and the level of difficulty for collecting the samples.

As chemical detection capabilities and instrument sensitivities improve, the trend in evaluating workplace environments has moved toward the use of direct-reading instruments (e.g., colorimetric detector tubes and handheld instruments) and passive dosimetry (e.g., organic vapor badges). This is due, in large part, to the desire for immediate feedback and ease-of-use associated with these methods. For additional information on industrial hygiene sampling, refer to the ACGIH text, *Air Sampling Instruments* [1]. NIOSH [144,145], OSHA [150,151], and MSHA [118,119] publish methods and guidelines for sampling specific chemicals in the workplace. The United States EPA also publishes many methods that are useful in monitoring workplace contaminants [60]. The NPG [143] includes information on sampling and analytical methods as described in the NIOSH Manual of Analytical Methods [144] or the OSHA numbered methods [150]. In addition, many manufacturers and commercial laboratories provide chemical sampling guides by chemical name and agency method.

Active Sampling

Active sampling involves the use of a powered sampling pump to draw a measured volume of air from the general work environment (general area sample) or an individual worker's PBZ onto a suitable collection media over a defined time period. The sampling media is then analyzed in the laboratory by prescribed analytical procedure to accurately qualify or quantify the amount of material collected. The sampling pump requires field or laboratory calibration with a primary standard to ensure that it is drawing air through the collection media at the desired flow rate. The collection media is an integral part of the sampling train, being connected to the sampling pump via tubing. The complete sampling train is then positioned in the immediate area of the process (point of generation) or is attached within the PBZ of the worker being monitored with the sampling pump on the hip and collection medium located in the worker's breathing zone (typically the shirt collar). When the sampling process for industrial hygiene purposes is completed, the flow rate calibration of the sampling pump is checked against the initial calibration and the associated collection media sent to an AIHA-accredited analytical laboratory for extraction and analysis. Various types of collection media are available for solvents, depending on such factors as the polarity and complexity of the chemical being evaluated. Examples of the variety of sorbent media include

1. Activated charcoal for sampling solvents such as chlorinated hydrocarbons, gasoline, many alcohols, and ketones
2. Silica gel for amines, methanol, phenols, and aldehydes
3. Chemically treated media, including filters for toluene diisocyanates, naphthylamines, and toluidines

Another active sampling method that is becoming more prevalent is the use of evacuated containers. In this instance, a specific volume of air to be sampled is collected by drawing it through a calibrated orifice at a specific flow rate to provide for either rapid inflow (grab sampling) or over time (1–24 h) into a specially prepared evacuated canister. The air within the canister can then be analyzed in the laboratory, after being first pressurized with nitrogen, aliquots of the air sample withdrawn, cryofocused, and analyzed by gas chromatography/mass spectrometry to determine the concentration of the various chemicals/mixtures present [61,152].

Direct-Reading Instrument Methods

Direct-reading instrument methods allow airborne concentrations of solvents (or other contaminants) to be measured with nearly instantaneous results. Direct-reading instrument methods are particularly useful in a number of applications,

including identifying potential process leak locations, determining peak exposure areas and occurrences, evaluating the effectiveness of engineering controls, or for continuous monitoring applications [7]. A wide variety of direct-reading devices and instruments is available for measuring solvent concentrations. The measurement methods generally incorporate the use of colorimetric detector (or indicator) tubes and badges or direct-reading instruments [11–13,62].

Colorimetric detector tubes and badges contain reagent-containing media that react with airborne solvent vapor (categorically or specifically) to produce a color change. The intensity of color change or the length of stain is compared with a calibration scale to determine the concentration (generally within ±25%) of the solvent vapor. The use of these tubes generally incorporate the use of a calibrated bellows-type or battery-powered pump. Such pumps are easy to use, relatively inexpensive, and may be used for short (minutes) or long (hours) sampling intervals. Disadvantages include possible interfering compounds, lower accuracy, and some subjectivity in the readings. Many of these methods are referred to as *grab samples* providing data regarding the presence and general concentration of the air contaminant only at that location and point in time. The results are considered semiquantitative or semiqualitative, due to larger accepted standard error and method limitations [14].

Direct-reading instruments are preferred over detector tubes when multiple readings are desired. Types of direct-reading instruments suitable for measuring solvents include analyzers with flame ionization (FID) and infrared (IR) detectors, combustible gas/vapor meters, photoionization detectors (PIDs), and portable gas chromatographs [5]. Direct-reading instruments can be either handheld (for portability) or fixed (for continuous area monitoring) [137]. The FID and PID are portable, rapid responding, and sensitive instruments but are nonspecific. They are particularly suitable for monitoring many solvents into the part per billion range. Infrared instruments are often capable of both qualitative and quantitative analysis of workplace contaminants but often lack portability and may generally provide slower response times. Combustible gas/vapor monitors are useful from a safety standpoint (detecting flammable/explosive atmospheres); however, for many solvents, the toxicity hazard may be at a lower concentration than the lower flammability/explosive limit [6]. Portable gas chromatographs are often as accurate as laboratory instruments in both qualitative and quantitative analysis of workplace contaminants. Shortcomings include power requirements, the need for specialized compressed carrier gas and sample collection; however, technological advances are providing solutions to many of these issues [144,147].

Passive Dosimetry

General area or PBZ sampling with passive dosimeters (e.g., organic vapor monitors) relies on the principle of molecular diffusion into the collection media rather than actively pulling the air through a sampling train. This detection and measurement technology is particularly well suited for personal sampling because these devices [29]

1. Are lightweight
2. Are unobtrusive
3. Require no external power source
4. Require no calibration
5. Can be used to obtain short-term or full-shift exposures

Organic vapor monitors are accurate (within a specified standard error and limitations) and can be used to sample for many industrial solvents. Analysis generally involves chemical desorption and gas chromatographic methods.

EXPOSURE CONTROLS

Worker overexposure to solvents may be avoided through the selection and implementation of proper administrative policies and practices, management programs (e.g., employee training, education, housekeeping, waste disposal, job safety analysis, medical surveillance), workplace planning (e.g., process design and location, equipment design, materials storage, chemicals used), and the use of engineering controls (e.g., general supply and local exhaust ventilation), where necessary. The selection of appropriate exposure control methods will depend on the nature of the hazard, how the potential hazard enters the work space, and the anticipated exposure pathway (inhalation, dermal, ingestion). Specific exposure-control methods may be mandated by federal health and safety regulations (as in the case of benzene and vinyl chloride) or when exposure levels exceed established OELs (e.g., PELs and TLV®s).

The preferred approaches to controlling workplace exposures to solvents are through the use of administrative or engineering controls. Types of controls (in order of preference): chemical or engineering process changes to eliminate/reduce solvent usage, substitution with a less hazardous solvent (Green Chemistry), isolation of the process to minimize worker exposures, local exhaust ventilation to reduce the concentration at the source in the work environment. The choice of using appropriate PPE to defray worker exposures should be viewed as a control method of last resort when no other practical means are available to control worker exposure. Recognize that the control of solvent exposures within the workplace is generally not resolved by a single control measure but is most often achieved by the application of a combination of these methods [9].

Administrative and Management Control

The first line of defense in the management of worker health and safety is the implementation and management commitment to effective workplace policies and procedures. These policies and procedures set the expectations for personnel practices, workplace behavior, product quality, and supervisor commitment. It is the measure of management

commitment and adherence to established policies and procedures that defines the level of success in workplace safety. Typical management programs that serve to enhance workplace safety and employee well-being include

1. Employee education (hazard communications, hazard awareness, emergency procedures, frequent safety meetings, proper use of PPE)
2. Employee training (e.g., process procedures, materials use, equipment operation and maintenance, standard operating procedures, safe work practices)
3. Community outreach programs (e.g., community right-to-know practices) [15]

Engineering Controls

Engineering controls play a key role in the design and use of production equipment, the general workplace environment, and employee well-being. To maintain safe and healthful work environments, significant consideration must be given to the design and integration of applicable engineering controls in workplace layout, selection and use of production equipment, chemical and material usage, process design, operating procedures, ventilation system configuration, fire suppression, utilities, etc.

Engineering controls should figure prominently in the design of process operations, including the use of solvents and other chemical hazards. In this regard, useful engineering controls can include practices to the following:

1. Eliminate use of the solvent in the process.
2. Substitute a less toxic solvent in the process.
3. Isolate the process from surrounding operations.
4. Enclose the process to minimize worker exposure.
5. Revise processes to minimize or eliminate process hazards by changing from manual to mechanical systems, wet methods from dry methods, water-based cleaners from organic solvent-based ones, etc.
6. Use effective local exhaust or dilution ventilation to eliminate or minimize hazardous levels of solvent vapors from the worker's breathing zone.

Elimination

Recent and ongoing developments in the field of Green Chemistry demonstrate that organic solvent-based processes can often be redesigned to eliminate the use of particular solvents/chemicals in the operation. Eliminating the solvent/chemical is considered the best approach to controlling worker exposure. From a cost perspective, available options should be evaluated during the initial design of the process, but can be instituted whenever safer process methods arise. The practice of process solvent/chemical elimination has been utilized in various organic solvent-based processes such as metals degreasing, cleaning, printing, painting, and treatment. These initiatives are the result of a combination of health and safety awareness, materials cost control, good business practice, and government regulation. Industry has recognized that the elimination of organic solvents may have additional benefits as well, including the reduction of hazardous air pollutant emissions into the ambient environment, the cost savings associated with decreases in waste treatment and disposal costs, and significant reductions in PPE purchases. Examples of organic solvent elimination include replacing chlorinated solvent degreasers with water-based detergent or subcritical carbon dioxide systems (discussed later); replacing solvent-based paints with water-based paints; improving flux application systems in circuit-board manufacturing to eliminate the need for cleaning with chlorinated compounds; and using water-based or vegetable oil-based inks to eliminate solvent-based inks.

Substitution

When the elimination of solvents from a process is not practical, it is often possible to replace the more toxic solvent with another of lower toxicity or less hazardous physical properties (e.g., higher flash point, lower vapor pressure). Often, substitutions may be made within a chemical series by retaining the active group; for example, substitution of butyl cellosolve for methyl cellosolve. In many cases, it may be possible to retain the general chemical group, such as in the substitution of aromatic naphtha for toluene or toluene for benzene. Substituting a solvent with similar polar characteristics but different toxicity, such as ethanol for methanol, may also be possible. Other examples include replacing perchloroethylene with citrus-based products in metal degreasing, substituting isocyanate-containing coatings with toluene-based materials, and replacing formaldehyde used in preserving laboratory specimens with glycol-based compounds.

Isolation and Enclosure

A process can sometimes be enclosed or automated to isolate the worker from the hazards of operation. When total enclosure of a solvent-based process is not possible, the operation can be separated from adjacent areas to minimize the number of workers potentially exposed to the vapor. The isolation by enclosure of a solvent-based process usually requires the introduction of local exhaust or dilution ventilation (see following text) to prevent or minimize the accumulation of toxic concentrations of vapors within the workspace or process enclosure (fire/explosion hazard). Examples of isolation are found in most manufacturing environments; for example, manual painting in automotive assembly plants and manual metal-plating operations have been replaced with robotic systems. These automated processes often can be operated and monitored from remote locations.

Process Revision

The revision of production processes is also a viable engineering control. Often, an engineering cost–benefit analysis that includes consideration of potential decreases in

employee health costs, medical surveillance testing, the use of PPE, and the return on investment for new equipment will determine the practicality of the option whenever newer techniques and production equipment become available. Two examples of process revision related to the potential for exposure to solvents in industrial practice are seen in the spray-painting practice, such as replacing spray-painting with paint dipping and replacing compressed air spray painting with electrostatic methods, resulting in a decrease in the volume of paint overspray into the workplace.

Workplace Ventilation

When the engineering control methods discussed earlier are not feasible or available, the incorporation of mechanical ventilation methods for the control of worker exposure to airborne contaminants such as solvent vapors is appropriate. Within the occupational workplace setting, this involves the balanced delivery of a sufficient supply of uncontaminated air into the work area (dilution ventilation) or direct removal of contaminated air by both general and local exhaust ventilation methods.

Dilution Ventilation

Mechanical ventilation in the workplace to manage worker exposures to airborne contaminants can, in some situations, be accomplished by the introduction of sufficient fresh air in specific work locations to dilute vapors to acceptable levels. Typical applications for dilution ventilation are controlling heat exposures (as in foundries) or regulating humidity and odor. If dilution ventilation is to be used to reduce the concentration of solvent vapors in the ambient air, at least four conditions must be met [3]:

1. The concentrations of solvent vapor generated must be relatively low or the air volume necessary for dilution will become so costly and inefficient as to be impractical.
2. The worker must be positioned a sufficient distance from the immediate source of solvent vapor generation to ensure that the established PEL, TLV®–TWA, STEL, and ceiling limit are not exceeded.
3. The solvent must have a relatively low toxicity rating.
4. The solvent vapor must be released into the work environment at a uniform rate.

Local Exhaust Ventilation

In contrast to dilution ventilation, *local exhaust ventilation* (LEV) functions to remove the solvent vapors (air contaminant) at their point of generation. In most instances, the use of local exhaust ventilation proves to be more effective in protecting the worker from exposure and less expensive to operate because lower air volumes and smaller fans are required. LEV systems can consist of a canopy hood or slotted capture collection system (depending on the vapor density of the solvent), duct work, a suction fan, and an optional filtration system for contaminant removal prior to discharge to the outside environment. Figure 15.3 shows a typical local exhaust ventilation system [9]. The decision of whether or not to install LEV for solvent exposure control is based on a number of factors, including

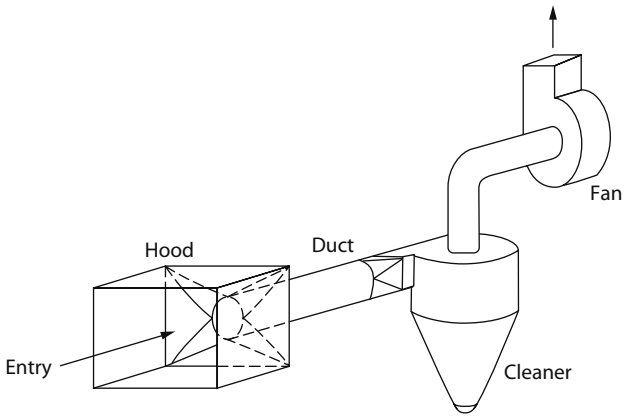


FIGURE 15.3 Typical local exhaust system components. (From DiNardi, S.R., ed., *The Occupational Environment: Its Evaluation and Control*, AIHA Press, Fairfax, VA, 2003. With permission of the American Industrial Hygiene Association.)

1. Lack of more cost-effective controls
2. Volume and toxicity rating of the solvent vapor generated
3. Regulatory requirements
4. Good management practice

In LEV systems, the contaminated air is exhausted to the outside ambient environment either directly or by passing the air stream through some variety of contaminant collection or filtration system. The three types of LEV hood designs for solvent vapor control are *enclosing*, *exterior* (or capture), and *receiving*. Figure 15.4 provides an illustration of each [9].

Enclosing hoods partially or completely enclose the process so the point of contaminant generation is located inside the capture area of the hood. Enclosing the process as much as possible increases the effectiveness and efficiency of LEV systems. Examples of enclosing systems include laboratory chemical fume hoods and spray-paint booths. Exterior hoods, also called *capture* or *slotted hoods*, are located near the point of contaminant generation but do not enclose it. Examples of exterior hoods are slot-type hoods used on vapor degreasing processes and flexible hoods used to exhaust solvent-based

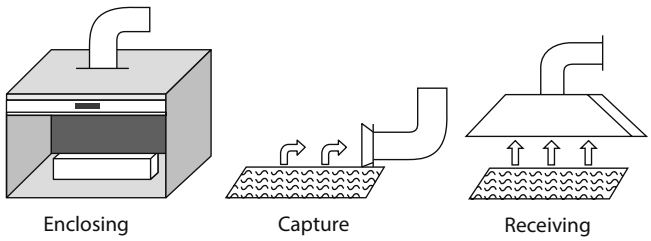


FIGURE 15.4 Three types of local exhaust ventilation hoods.

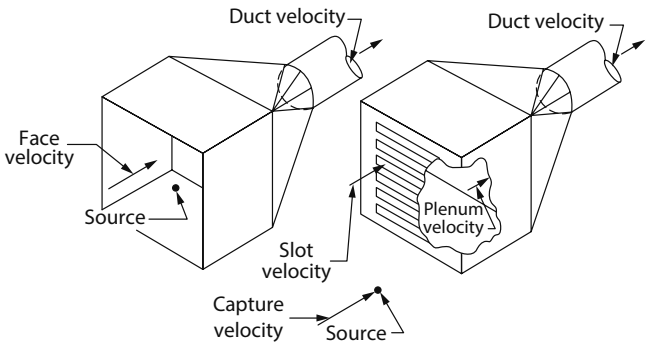


FIGURE 15.5 Principles of exhaust hoods. (From ACGIH, *Industrial Ventilation: A Manual of Recommended Practice*, 23rd edn., American Conference of Governmental Industrial Hygienists, Cincinnati, OH, 2007. With permission.)

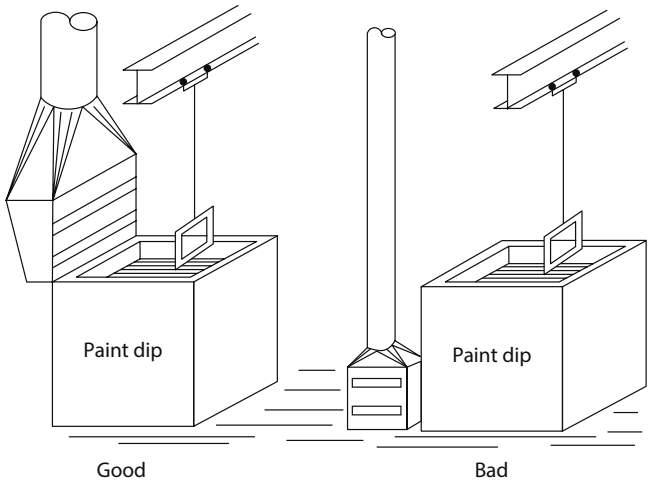


FIGURE 15.6 Principles of exhaust hoods. (From ACGIH, *Industrial Ventilation: A Manual of Recommended Practice*, 23rd edn., American Conference of Governmental Industrial Hygienists, Cincinnati, OH, 2007. With permission.)

mixing processes. Receiving hoods are typically canopy-type hoods used for exhausting hot processes (e.g., ovens and detergent baths). They are generally less suitable for solvent operations such as metal cleaning and degreasing.

Careful evaluation of the specific industrial process should be performed by qualified professionals prior to selection and installation of a LEV system. Input should be obtained from various disciplines, including engineering, planning, industrial hygiene, and ergonomics, and from employees who will be involved with the process operation. In addition to calculating the correct air flow rates and capture velocities, ventilation designers must ensure that the arrangement of the hood and ductwork does not interfere with the work or other aspects of the facility's operation. In general, designers of LEV systems should take into account the flammability limits (e.g., use of approved wiring and motors), vapor density and toxicity of the solvent, the anticipated concentration and volume of vapor generated, possible interfering air currents in the room, whether access to the work area is needed, and, the amount of airflow or capture velocity required to adequately exhaust the contaminant [9].

Capture velocity is the air velocity at any point in front of the hood or at the hood opening that is necessary to overcome surrounding air currents to capture the contaminated air at the point of generation by causing it to flow into the hood. Recommended capture velocities for solvents vary between 50 and 500 ft/min depending on the nature of the operation, toxicity of the vapor, and conditions of solvent dispersion into the air [3].

Figures 15.5 through 15.7 provide design detail principles of local exhaust ventilation, including hood nomenclature and design considerations. For further reading on local exhaust ventilation systems, refer to the fundamental text *Industrial Ventilation: A Manual of Recommended Practice*, published by the ACGIH [3].

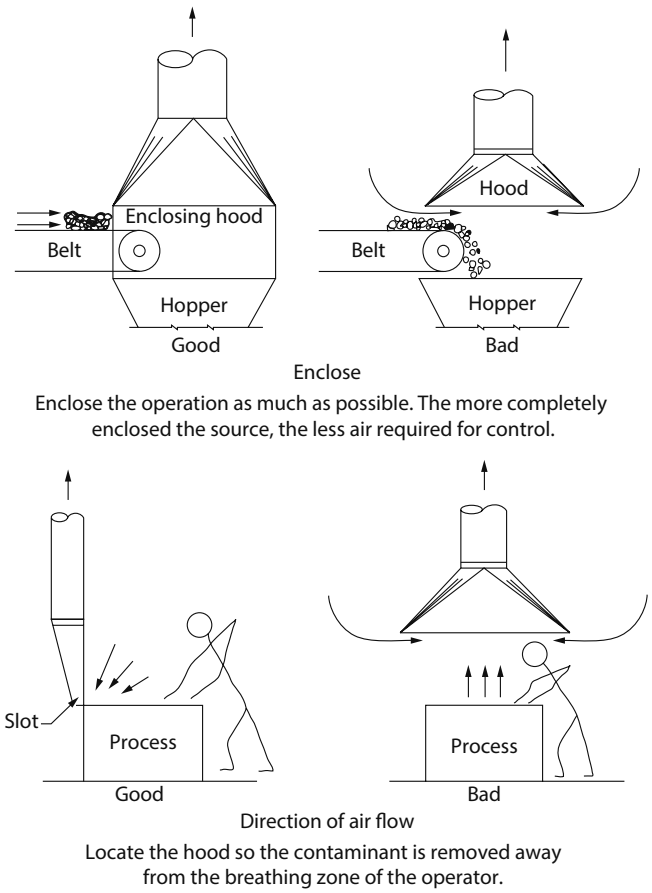


FIGURE 15.7 Principles of exhaust hoods. (From ACGIH, *Industrial Ventilation: A Manual of Recommended Practice*, 23rd edn., American Conference of Governmental Industrial Hygienists, Cincinnati, OH, 2007. With permission.)

Personal Protective Equipment

When engineering or administrative controls are not feasible or do not provide adequate protection, PPE must be used to minimize exposures. PPE should always be considered a last

resort and managed carefully by qualified individuals. This is due to a number of limiting factors associated with PPE, including the following:

a. PPE does not eliminate the hazard. When PPE (e.g., air purifying respirator and elastomeric gloves) is used to control solvent (workplace) exposures, the PPE functions as a barrier that separates the worker from the airborne and liquid hazards present. Should this PPE be compromised, either by tearing or degradation, the worker will be directly exposed to the hazard.
b. The selection and use of the correct PPE are relative to its effectiveness. Because no single respirator type, filtration cartridge or canister, or glove/barrier clothing elastomer is effective in all conditions of solvent use, it is mandatory that the worker and the industrial hygienist/OHS specialist work together to ensure that the PPE selected remains effective against the hazards it is intended to protect against. It is therefore recommended that the employer select only that PPE that is approved by NIOSH whenever possible.
c. The safe use and care of the PPE selected for protection of the worker require proper worker training and are mandated by federal OSHA regulations, as specified in Title 29 CFR 1910 (General Industry), 1915 (Shipyards), 1917 (Marine Terminals), 1918 (Longshoring), and 1926 (Construction). It is therefore incumbent upon management to ensure that proper PPE use programs and procedures are written and available to the respective employees and that the employees are sufficiently trained to know when and how to properly use the PPE available to them. Likewise, it is incumbent upon each employee to participate in such training programs and to use the PPE made available to them in the proper and intended way.
d. The use of PPE may provide a false sense of security to the user. Depending on the frequency of use and the level of training the worker receives, the PPE user may believe that the PPE selected will provide complete protection under all circumstances, leading them to enter into circumstances for which the PPE is not intended or effective. This type of mistake is most commonly evident in chemical spill or emergency incident situations when the volume and concentration of solvent (chemical) vapors and liquid are sufficient to quickly compromise the effectiveness of the standard workplace PPE.

The type and variety of PPE used within the workplace are numerous, making the proper selection and use of this equipment a necessary and sometimes difficult task for management and the worker alike. The PPE selected for use in a particular job setting should match the following:

- The level and type of hazard to be confronted, including the chemical and physical properties of the solvents in use, the workers' level of direct contact with the hazard [solvent], and the nature of the work to be performed)
- The worker's activity, vision, and dexterity needs while wearing the PPE

Often, the worker must confront a combination of hazards/chemical mixtures during job performance, making PPE selection and use decisions complex. Consequently, it is not only advisable for management to consult with respective PPE manufacturers regarding the intended use of PPE but also for the worker to be ever vigilant while using PPE to ensure that the integrity of the PPE remains protective against the hazards encountered. The primary categories of PPE in common use within the workplace include respirators, clothing (suits, gloves, foot coverings, sleeves, and aprons), eye, and face protection. The combined use of PPE representing these categories is dependent on the nature of the hazard, the frequency and duration of exposure, and the nature of the work to be performed. The NPG provides information on exposure hazards and appropriate PPE for over 400 chemicals [143].

Respirators

Respiratory protection is used to provide the wearer with clean breathing air while working in the presence of potentially harmful concentrations of airborne contaminants. Often, the use of respirators in the workplace are employed to protect the worker from intermittent exposures that can occur during process operations or during emergency repair and maintenance. However, respirators may also be the only feasible method of protection for exposures that may occur during normal work operations.

When workplace engineering (e.g., local exhaust or dilution ventilation) or other control methods are not feasible, the employer should implement an effective respiratory protection program, including worker training and medical surveillance (as applicable), and provide workers with respiratory protection appropriate to the hazard. The goal is to ensure that the appropriate type of respiratory protection is selected and used correctly. OSHA's Respiratory Protection Standard for General Industry, as established in 29 CFR 1910.134, specifies the employer and employee responsibilities when respiratory equipment is to be used within the workplace. This standard mandates that the employer develop and implement a written respiratory protection program that describes when respiratory equipment is to be used, what respiratory equipment is to be used, which employees are qualified to use the respiratory protection specified, and how the respiratory equipment is to be used and maintained. OSHA provides guidance documents with basic information to workers and employers who may find themselves using respiratory protection for the first time. The guidance provides information on what respirators are, how they work, and what is needed for a respirator to provide protection [153]. Elements of the program include respirator selection, user training and fit testing, medical approval, and specific instructions for cleaning and maintenance (refer to the 2005 updated standard). Only NIOSH-approved respirators should be selected for use.

NIOSH approval program requirements are specified in 42 CFR Part 84. OSHA and NIOSH also provide information and guidance for the proper selection of respirators [146,154].

The two major categories of respirators are air-purifying and atmosphere-supplying/supplied air. Air-purifying respirators for chemical and particulate (e.g., solvents) exposure provide protection to the user by removing the hazardous contaminant from the stream of the air prior to its inhalation by the user. This air-cleaning process is accomplished by drawing the contaminated air through specially prepared cartridges or canisters containing various filtering or sorbent materials (e.g., activated charcoal). Air-purifying respirators are available in various face-piece configurations, including quarter-, half-, and full-face mounts, with or without eye protection. They are designed to be disposable or reusable and are either operated by negative pressure (user inhales) or battery-powered. It is important to recognize that air-purifying respirators do not provide an independent source of breathing air and should not be used in environments that are oxygen deficient or when the airborne contaminants present do not have good warning properties (e.g., noticeable taste, odor, minor irritating effects below the established occupational exposure limit) to indicate cartridge or canister overloading, improper face-piece fit, or damage to the respirator. Air-purifying respirators should never be worn for protection against airborne chemicals with poor warning properties [6]. Because people vary greatly in their ability to detect odors, other methods such as cartridge replacement schedules or visible end-of-service-life indicators are being developed by various groups to ensure greater safety when air-purifying equipment is used. It is also important to recognize that, because people have different facial configurations, no one make or model of respirator will provide a sufficient face-to-face-piece seal for all users, therefore fit-testing is an integral part of ensuring worker protection. Respirator users need to be trained to check their respirator for proper fit each time they are donned to ensure they provide adequate protection.

Atmosphere-supplying/supplied air respirators provide the user with an independent source of clean breathing air separate from the local environment. Examples of atmosphere-supplying respirators include air-line devices and self-contained breathing apparatus (SCBA). Whereas air-line devices are designed to provide the user with an independent source of air for extended periods of time, the SCBA is designed to provide the user with an independent source of breathing air ranging from 5 min (emergency escape only) to 60 min, depending on user activity and equipment configuration.

Other factors that must be considered when selecting respiratory protection are the nature of the hazard, including oxygen deficiency, concentration of the airborne contaminant, and adequacy of warning properties. The expertise of the industrial hygienist is necessary for proper identification and assessment of the airborne contaminant levels, flammable limit status, and ambient oxygen concentration; evaluating the configuration and location of the work area in relation to an available area of clean air; implementing appropriate and periodic workplace and user monitoring procedures during respirator use; and determining the proper respiratory equipment for use.

Protective Clothing

Protective clothing is used to protect the user from dermal contact and exposure to chemicals by forming a barrier between the skin and the hazard (e.g., solvent). The proper use of protective equipment, including clothing, eye, face, hand, and foot protection, is mandated by OSHA standards found in 29 CFR 1910 Subpart I and should be referred to by employer and employee alike.

Protective clothing includes gloves, laboratory coats, rubber aprons, chemical resistant suits, and boots. Various configurations of chemical-resistant clothing can be selected, depending on the nature and concentration of the solvent (hazard) of concern, the kind of work activity and time period to be performed, and the level of protection required. Chemical-resistant elastomers used as barrier coatings for protective clothing include neoprene, nitrile, natural, or butyl rubber; polyvinyl chloride or polyvinyl alcohol; and Viton™. Some operations may require only partial protection (such as a protective apron, sleeves, or leggings), while others may require the use of full-body enclosures (such as those used by emergency response workers). It must be recognized that the type of protective clothing configuration, barrier material, and level of protection chosen can significantly affect the mobility, vision, and manual dexterity of the worker. Depending on the characteristics of the work environment, the use of protective clothing, especially encapsulating suits, can present potential heat stress hazards and may therefore require the close monitoring of workers and workplace conditions.

The degree of protection afforded by a given type of chemical-protective clothing is related to three primary performance factors: permeation, degradation, and penetration. Permeation is the ability of a chemical to pass through the molecular configuration of a protective barrier (e.g., the clothing or glove) and is defined on the basis of the permeation rate for the particular material being challenged. Degradation of a material results from a reduction in one or more of the physical properties of protective clothing or gloves due to direct contact with a chemical and is defined by the degradation rate for the material with respect to the specific chemical challenge. Penetration is measured as the rate of flow of a chemical through physical aspects of the clothing or glove, such as zippers, seams, pores, or imperfections in the material. Manufacturers of protective clothing determine product-specific performance data via laboratory tests conducted in accordance with methods established by the American Society for Testing and Materials (ASTM). All three factors should be considered when choosing protective clothing, because data for some but not all of these factors may not correlate with a given type of clothing and target chemical; for example, a glove may have acceptable degradation ratings for use with a specific chemical, but the chemical

may readily permeate the material. No single glove or type of protective clothing provides adequate protection against every hazard. Furthermore, a glove type from one manufacturer often has different performance data from the same glove type produced by another manufacturer. Another general source of information is Chemical Protective Clothing, published by the AIHA [8]. This two-volume set provides the data required to select and use chemical protective clothing. Included in the document is a discussion of permeation theory, testing methods, and available vendors.

Eye and Face Protection

Eye and face protection is used to prevent injuries that may occur while handling or transporting solvents and other chemical liquids, vapors, fumes, or particulates. Two types of protective eyewear in common use to prevent exposure are chemical splash goggles and face shields. Chemical splash goggles are designed to completely enclose the eyes (as opposed to safety glasses, which are designed to prevent physical injuries that may result from an object striking the eye). Some goggles may also prevent vapor exposure to the eye in addition to contact with the liquid. Face shields are often worn in conjunction with goggles to protect the face and neck. Face shields and goggles that meet recognized safety standards bear the engraving of ANSI Z-87, which indicates that the device has passed safety performance tests conducted by the American National Standards Institute. OSHA regulates the safe use of eye and face protection within the workplace as described in 29 CFR Part 133.

ABSORPTION OF SOLVENTS AND INHALATION EXPOSURE

Inhalation is the easiest and fastest means of exposure to solvents because solvent vapors in the atmosphere are readily accessible to the respiratory tract when one breathes contaminated air. The respiratory tract has a large surface area and the lining of the respiratory tract is NOT effective in preventing absorption of toxic substances into the body. The respiratory tract consists of the nasal passages, trachea (windpipe), larynx (voice box), and the lungs. The following factors affect inhalation of solvents:

1. Concentration of toxic substance in the air
2. Solubility of substance in the blood and tissue
3. Respiration rate
4. Length of exposure
5. Condition of respiratory tract

A key factor in determining how a solvent enters the body is the solubility of that chemical into blood and tissues. When considering solvent exposure via the inhalation route, the rate of solvent uptake and the subsequent equilibrium concentration in tissues are also dependent on individual activity, breathing rate, and the minute volume of blood flow through the lung and other organs.

Solvents that are highly soluble in water-based systems, such as blood and tissues, are absorbed very readily into the system by the inhalation route, causing blood concentrations to rise rapidly. The driving force is the difference in concentration of the solvent between inspired air and blood. The amount of solvent diffusing through the alveolar capillaries is dependent on the air–blood partition coefficient. Tissue equilibrium concentrations with solvents such as xylene, styrene, and acetone, which are highly soluble in blood and tissues, are not limited by pulmonary ventilation because the tissues act as a sink for the inhaled solvent. As pulmonary ventilation is increased, the blood and tissue concentrations continue to rise. The limiting factor in attaining the tissue equilibrium concentration is the blood flow through the tissues and the blood–tissue partition coefficient [66].

Solvents such as methyl chloroform, methylene chloride, trichloroethylene, and toluene, which have lower solubility in blood and tissues, reach equilibrium rapidly because of low solubility or low blood–air partition coefficients [171]. Tissue concentrations also will reach equilibrium rapidly because of low tissue–blood partition coefficients. In this case, the limiting factor in tissue concentration is the solubility of the solvent in the tissue and the individual's pulmonary ventilation rate [157]. To achieve a higher concentration in tissues and blood, pulmonary ventilation must increase, allowing more solvent to enter the blood and a new blood–tissue equilibrium to be reached [23,24].

DERMAL UPTAKE OF SOLVENTS

The opportunity for solvents to enter the body via contact with the skin is enhanced, in part, due to the large surface area of the skin (18 ft^2). Fortunately, the barrier properties of the skin associated with filamentous proteins and lipids of the stratum corneum naturally inhibit penetration by harmful non-lipid-soluble substances. Disruption of this barrier, however, by injury, illness, or removal of lipids, can facilitate passage of these materials; for example, treatment of the skin with polar organic solvents, detergents, and some surfactants can remove the lipids, thereby increasing the skin's permeability.

Penetration of the skin by a solvent depends on a number of other factors such as the thickness of the skin layers, the integrity of the skin, the concentration gradient of solvent on either side of the epithelium, and a number of physical constants. In addition, the degree to which the skin hydrates can increase absorption by affecting its permeability. Movement of water-soluble compounds may be impeded, however, when the stratum corneum is highly hydrated. Although hair follicles and sweat glands comprise only a small proportion of the skin's surface area, they, too, provide pathways for solvent penetration.

Solvents can denature the lipids in the skin, resulting in drying and irritating effects, cellular hyperplasia, and swelling; for example, the careless use of solvents without the use of barrier creams or proper hand and arm protection frequently leads to cases of dermatitis in the workplace. In studies on the effect of solvents on the lipid barrier in the skin,

the ability of a solvent to penetrate the skin is dependent on the polarity of the solvent and the surface charge of the skin. Results comparing penetration or removal of skin lipids by several solvents indicate that ethanol, the solvent with the greatest polarity, extracts the most lipids, followed by acetone and ether [27]. Treatment of the skin with solvents can also enhance the penetration rate of other compounds. In a study using excised human skin, the effect of several solvents, including dimethyl sulfoxide (DMSO), dimethylacetamide, formamide, and diethyl formamide, on the penetration rate of sarin was examined [121]. The results of this study demonstrate that solvent pretreatment of the skin increases the rate of sarin transport across the skin barrier by a factor of 10–100 over that of sarin alone on control skin.

In studies using toluene, xylene, and styrene vapors to assess the rate of skin penetration of these aromatic solvents in human volunteers, human volunteers were exposed to 300 ppm or 600 ppm concentrations of the solvent vapors for 3.5 h in a dynamic exposure situation. The subjects wore full-face respirators to prevent pulmonary absorption of the solvents via the inhalation route. Each subject exercised for a 10 min period, sufficient to make the subjects perspire and to raise the skin temperature about 0.5°C. Perspiration and warm skin temperature enhance the hydration of the skin and subsequent percutaneous absorption. After termination of exposure, these solvents displayed biphasic elimination from the blood into exhaled air with a short half-life of about 1 h and a much longer half-life of approximately 10 h. Xylene and styrene showed a slight delay in excretion in exhaled air after percutaneous exposure when compared with similar exposure via the inhalation route. Delayed excretion after dermal exposure may be accounted for by a slow release from the skin after termination of exposure.

Overall percutaneous absorption of the xylene, toluene, and styrene concentrations corresponded to only about 0.1% of the amount estimated to be absorbed by the pulmonary route. This observation indicates a very small absorption potential for these solvents by the percutaneous route. When the percutaneous absorption of xylene vapor is compared to earlier work with xylene liquid, the vapor displays an approximate 10-fold greater efficiency in penetrating the skin than does the liquid. According to Riihimäki and Pfäffli [168], it is not uncommon to observe greater penetration with vapor exposure because liquid solvents remove the lipids from the stratum corneum and thus interfere with absorption. Additionally, exercise promoted the absorption of solvents because of the warm hydrated skin. In general, percutaneous absorption of solvent vapors would not contribute significantly to the total blood concentrations of these solvents.

Chemical Interactions Effecting Toxicity

It is not unusual for a toxic chemical to enter the body and interact with another toxic substance or with a medical drug. The result of this interaction of chemical agents, drugs, etc., can be mediated in several ways through effects on absorption, protein binding, biotransformation, or excretion of the drugs or chemicals. The possible results of chemical interactions within the body are listed as follows:

1. *Additive effect* (e.g., 2 + 2 = 4): The effect observed when the combination of two chemicals having independent toxicities results in a combined toxic effect equal to the addition of the two. Example: Two organophosphates simultaneously used as pesticides will depress cholinesterase levels equal to the additive concentrations of each agent.
2. *Synergistic effect* (e.g., 2 + 1 = 20): The toxicological effect that occurs when chemicals having independent toxicity produce a toxic response significantly greater in effect than the additive sum of the two substances observed individually. Example: The hepatotoxicity of carbon tetrachloride is significantly increased in the presence of ethyl alcohol.
3. *Potentiation* (e.g., 2 + 0 = 10): The effect that is observed when one substance acts as a catalyst in the presence of another substance to enhance the toxicity of the second substance. Example: A steady diet of corn oil potentiates the effect of incomplete carcinogens.
4. *Antagonism* (e.g., 2 + 2 = 1): A substance recognized as an antagonist is one whose effect tends to decrease the adverse effect of a second substance. There are various types of antagonist actions. *Functional antagonism* occurs when two chemicals produce opposing physiological effects and result in an overall no net-effect exposure. *Chemical antagonism* occurs when the interaction of the two chemicals results in an interference with the normal chemical transformation of the chemicals so less toxic agent is available. *Dispositional antagonism* occurs when absorption, distribution, or excretion of the chemical is altered; therefore, less of the chemical mixture reaches the target tissue. *Receptor antagonism* occurs when competition for the same receptor results in less of either chemical reaching the receptor. Example: Many of the principles of antagonism are used in the design of antidotes in clinical toxicology or for the poisoning of humans.

TOXICOLOGY OF SELECTED SOLVENTS

This section provides insight into various solvents of occupational concern due to their propensity to produce neurotoxic, reproductive, or carcinogenic effects in humans. Examples are provided for these, as well as less toxic, alternative solvents. Generally speaking, acute exposure to high levels of solvents can result in temporary or long-term alterations of central nervous system (CNS) function.

Effects of Acute Solvent Exposure on the Central Nervous System

Although varying widely in chemical structure and physical properties, organic solvents produce a rather stereotypical

set of toxicological manifestations upon acute exposure [20], the significance of which is dependent on dose concentration, duration, and frequency. Most commonly, acute exposure is evidenced by a varied level of CNS dysfunction and, if exposure is sufficiently severe, narcosis. The systemic toxicity of solvents is observable either throughout the body or in an organ with selective vulnerability distant from the point of entry of the chemical, as with solvents and peripheral neuropathies. Exposure to certain solvents can be associated with some temporary alteration of cognitive and psychomotor function following short-term exposures at or near the TLV® Exposure to greater concentrations may provoke such symptoms as headache, dizziness, ataxia, euphoria, drowsiness, lightheadedness, disorientation, confusion, tremors, and nausea. Exposure to potentially lethal (IDLH) levels of solvents can result in stupor, loss of consciousness, coma, respiratory depression, and abnormal cardiac function.

Toxic Effects of Solvent Exposure on the Peripheral Nervous System

Exposure to solvents at concentrations too low to induce many of the acute symptoms cited earlier is of special concern with regard to neurotoxicity. This concern that results from the capacity of nerve tissue for post-toxicity regeneration is limited and repeated insults may lead to cumulative damage. The more subtle symptoms of chronic solvent exposure include relatively mild alterations of mood and behavior not accompanied by quantifiable evidence of dysfunction on neurobehavioral tests [28,84]. Although dose–response and causal relationships have been difficult to study in the absence of animal models, symptoms of chronic solvent exposure can include

- Increased irritability
- Decreased span of attention
- Loss of interest in daily activities

More severe damage to the nervous system, both central and peripheral, occurs upon repeated exposure to certain solvents such as carbon disulfide and *n*-hexane, as discussed later in this chapter.

Numerous neurobehavioral and functional tests have been used to detect such changes in both clinical and experimental settings [97,163]. Whether the acute effects of solvents play a role in determining the pathogenesis of toxic lesions observed after chronic exposure to the same solvents is uncertain. However, current thought is that the acute effects on the nervous system are mediated through nonspecific interactions of solvents with the cell membrane by increasing membrane fluidity or functional alteration of cell surface receptors, while the effects of chronic exposure are mediated by specific biochemical actions of solvents.

It is well known that neurotoxic chemicals can have a negative impact on sensory function. Often, the symptoms reported following chemical exposure to such chemicals are related to effects observed among the five senses [70]. Toluene, xylene, styrene, trichloroethylene, and carbon disulfide are examples of solvents associated with adverse effects on the auditory system, for example [135]. In the industrial environment, workers are often exposed to solvents as well as high levels of noise, which is known to damage the inner ear and result in hearing loss. Evidence from workplace studies and animal experimentation indicates that the combined effects of noise and ototoxic solvents may increase individual susceptibility to hearing loss [96,135]. In one animal study, rats were exposed to toluene, to noise, or to toluene followed by noise followed by direct testing of their auditory functions. The study results demonstrated that rats exposed to toluene followed by noise exhibited a decrease in auditory sensitivity greater than the sum of the effects of toluene and noise alone [135]. The risk for hearing loss may be increased by factors other than noise, such as drugs or other chemicals, and can also be influenced by heredity and aging [135]. It is important to take all of these factors into account when evaluating hearing loss in the workplace.

Toxic Effects of Solvents on Organ Systems

Organs that receive a high percentage of the cardiac output are exposed to greater doses of absorbed toxicants than poorly perfused tissues. A major determinant of target organ selectivity for the toxicity of solvents is xenobiotic metabolism. While pharmacokinetics define the quantity of solvent reaching a particular organ or tissue after absorption, metabolism may yield products with increased toxic potential relative to the parent chemical. Thus, well-perfused organs with high capacities for specific types of biotransformation reactions, mainly those catalyzed by the cytochrome P450, are common targets for solvent-induced toxicity. In particular, the liver is vulnerable to the toxicity of many solvents, owing to its high capacity for xenobiotic metabolism. Many common hepatotoxic solvents yield toxic intermediates or end-products upon biotransformation. Such hepatotoxic solvents include carbon tetrachloride [164], chloroform [160], and trichloroethylene [26]. However, some solvents, such as ethanol, may exert their hepatotoxic effects indirectly by altering cellular reduction–oxidation balance during metabolism and thereby deranging normal liver function and structure [169].

The kidney, as a filtering and concentrating organ of excretion, receives not only untransformed solvents but also the products of hepatic metabolism of solvents. These biotransformation products—for example, conjugates of trichloroethylene—may be more toxic than the parent chemical and produce renal-specific toxicity [114]. The ion transport and solute concentrating functions of renal tubules also contribute to the vulnerability of the kidney to certain chemical toxicants [115]. In addition, biochemical peculiarities of certain species and genders may play a major role in bringing about solvent-induced renal toxicity. A notable example is the susceptibility of the male rat to renal toxicity caused by 1,4-dichlorobenzene, Stoddard solvent, VM & P naphtha, and other hydrocarbon solvents. This has been attributed to the male rat-specific abundance of the low molecular weight

protein 2μ-globulin, which acts as a carrier for lipophilic molecules [180]. 2μ-Globulin is normally degraded in renal tubule lysosomes and binding to a solvent ligand slows degradation of the protein so that the 2μ-globulin-hydrocarbon complex is sequestered by lysosomes [109]. The sequestered protein apparently disrupts lysosomal function and cytotoxicity results when large amounts of 2μ-globulin accumulate [173]. There is apparently no counterpart to this type of nephrotoxicity in species other than the rat [16].

Organs that catalyze relatively few types of chemical biotransformation reactions or have low rates of xenobiotic metabolism, such as lung, nasal mucosa, and testes, may also be target organs for the toxicity of some solvents. For example, ethylene glycol monomethyl ether and related glycol ethers are testicular toxicants [85,132] as are hexane and the hexane metabolite 2,5-hexanedione [32]. Special attention to the potential susceptibility of the tissues lining the upper airways and nasal mucosa to solvent-induced toxicity is evidenced in recent studies. These tissues have high levels of certain xenobiotic-metabolizing enzymes and, in addition, are exposed to high solvent concentrations relative to the lung and other organs. In particular, esters such as propylene glycol monomethyl ether acetate, dimethylphthalate, and dimethyl succinate are enzymatically transformed by carboxylesterase in the nasal area to yield acidic products that may accumulate to toxic levels in the nasal mucosa [132,178,180,185]. Certain solvents need no metabolism to adversely affect the tissues of the upper respiratory tract; vapors or aerosols of aldehydes cause local tissue damage to the nasal epithelium [134], presumably due to the activity of these solvents in forming protein–protein and protein–DNA crosslinks [88].

Solvent Mixtures

Humans are often exposed to multiple chemicals at work or home. An example reported by Worksafe Australia (the Australian National Occupational Health and Safety Commission), involves solvent exposure and health effects observed in spray-paint apprentices [197]. This study identified 32 different solvents contained in 20 thinner products used by the painters. Within this group, six different categories of solvents were represented: alcohols, aromatic hydrocarbons, esters, glycol ethers, ketones, and mixtures. Of significance was the fact that the workers commonly perceived these 20 thinners to be equivalent products and consequently equally safe to use. This common, but mistaken perspective underscores the need for chemical hazard communication programs to adequately inform workers of the inherent and potential hazards of working with solvent mixtures and chemicals.

As noted previously, exposure to multiple solvents and other chemicals, either simultaneously or sequentially, may alter the toxicological impact of the individual chemicals upon the body. Thus, a combination of certain chemicals may affect (positively, negatively, or not at all) the absorption, distribution, metabolism, and excretion of the chemical mixture within the body of the worker [103]. The study of chemical interactions has evolved most extensively in the area of therapeutic drugs.

Although some information exists on interactions of industrial chemicals, most research on chemical toxicity to date has dealt with single, pure chemicals. These single chemical studies are important because they allow researchers to gather fundamental knowledge about the mechanisms of toxicity under conditions that are well controlled. However, further research is required in the evaluation of potential health effects associated with exposures to multiple chemical compounds [202]. Occupational Exposure Limits (OELs) for defined chemical mixtures may be calculated for many organic solvent mixtures by a procedure called the Reciprocal Calculation Procedure (RCP). The RCP, first published in Appendix H of the ACGIH, 2009 *Threshold Limit Values and Biological Exposure Indices* [2] provides for the calculation of an OEL for a mixture of refined hydrocarbon solvents containing saturated aliphatic, cycloaliphatic, and/or aromatic hydrocarbons consisting of 5–15 carbon atoms and having boiling points in the range of approximately 35°C–330°C.

Glycol Ethers

Glycol ethers represent an important category of solvents that are widely used in mixtures for industrial and consumer applications. They are grouped as ethylene glycol, propylene glycol, or butylene glycol with the ether component of the molecule containing methyl, ethyl, propyl, butyl, or higher molecular weight moieties [76]. Additional members of this class of compounds are the corresponding acetate esters. The miscibility of glycol ethers with water and many organic compounds make them ideally suited as solvents in oil–water compositions. Production capacity of the ethylene-based ethers in 2010 exceeded 3 billion pounds, with the coatings (paint) industry being the major consumer [76,160]. In addition to coatings, glycol ethers are found in many household goods such as brake fluids, waxes, cleaners, dyes, detergents, degreasers, and inks. In particular, 2-butoxyethanol has been formulated into hundreds of consumer products [38].

The current ACGIH TLV®–TWAs and German MAKs for three widely used glycol ethers—2-methoxyethanol (ME), 2-ethoxyethanol (EE), and 2-butoxyethanol (BE)—are 5, 5, and 20 ppm, respectively. The NIOSH RELs for ME, EE, and BE are significantly lower at 0.1, 0.5, and 5.0 ppm, respectively. All have skin notations. ACGIH bases their limit for ME on possible blood, reproductive, and CNS effects. For EE and BE, effects on reproduction and the blood are considered, respectively. The TLV® and REL for propylene glycol monomethyl ether (PGME) are both 100 ppm and are based on potential irritation and CNS effects [2,4,143].

The commonly encountered glycol ethers are colorless liquids with mild odors. The primary routes of exposure in the industrial environment are inhalation and skin absorption [76]. Outside of the workplace, some cases of accidental or intentional ingestion of products containing glycol ethers by children and adults are reported [38]. In general, the ethylene glycol ethers exhibit low acute oral toxicity [76]. Experiments

in rats have shown that the methyl, ethyl, and butyl ethers are readily absorbed through the skin [170]. As the molecular weights of the glycol ethers increase, the potential for inhalation exposure and skin absorption decreases. Because the methyl and ethyl ethers of ethylene glycol and their acetates have demonstrated adverse reproductive, embryotoxic, teratogenic, and developmental effects in animal studies [85–87,141,142], their use in consumer products has declined [76].

Metabolically, the monoalkyl ethers of ethylene glycol are converted to their respective alkoxyacetic acids via the actions of alcohol dehydrogenase [38]. Many of the observed adverse effects caused by ethylene glycol ethers in animals, such as hematotoxicity (e.g., 2-butoxyethanol) and testicular toxicity, are attributed to these toxic metabolites. Whereas rat erythrocytes have demonstrated vulnerability to the hemolytic effects of 2-butoxyacetic acid (from BE), human erythrocytes have been shown to be much less susceptible to these effects [38]. PGME and its acetate (PGMEA) are relatively innocuous compounds when compared to the ethylene glycol ethers discussed earlier. Overexposure to PGME has been associated only with increased liver weight and CNS depression. Studies have shown that EE and PGME are metabolized by different routes and the types of metabolites produced are responsible for the marked differences in toxicity; for example, methoxyacetic acid is the primary metabolite of EE, and propylene glycol is the main biotransformation product of PGME and PGMEA [131,133].

Investigators have studied the potential interaction of ethanol and EE due to the recognition of similar metabolic pathways and the likelihood of concomitant exposure to ethanol due to personal behaviors in some individuals [142]. When dose levels of EE are presented to rats alone or in combination with ethanol, researchers have noted an apparent increase in the duration of pregnancy. Exposure to dose levels of EE during gestational days 7–13 resulted in observation of a decrease in certain behavioral tests such as rotorod performance; however, when test animals were exposed to dose levels of EE and also consumed ethanol, the behavioral deficits observed were diminished. When dose levels of EE were administered alone during late gestation, the motor activity levels of pups were depressed and performance during avoidance conditioning trials was retarded. Observers note that the combined administration of EE and ethanol appears to generate a synergistic effect on the behavioral deficits induced by EE and to depress both activity and learning. Also, it has been observed that ethanol during late gestation altered the neurochemical effects of EE.

In summary, concomitant exposure to ethanol and EE can have differential effects depending on the stage of gestation. Ethanol administration during the early period of gestation tended to improve both the behavioral and neurochemical effects of EE to approximately 50% of the response produced by EE alone. In the late stage of gestation, however, the combination of ethanol with EE exaggerated the effects of EE alone. These scientific observations indicate that the possibility exists for ethanol-induced exaggeration of the potential toxic effects of EE exposure in pregnant workers.

Retrospective epidemiological studies of workers exposed to ME and EE report evidence of adverse effects on the male reproductive system, with increased frequency of reduced sperm counts [199]. Evaluations of sperm production in humans and several other animal species indicate that the output of human sperm is about one fourth that of other mammals when compared on a per-gram tissue basis. This finding suggests that humans may be more susceptible to occupational toxicants than predicted by laboratory animals [182]. As is the case with many widely used chemicals with potentially harmful effects, substitutes are being considered. PGMEA and ethyl-3-propionate have been identified as useful and less-toxic alternatives to ethylene glycol ether solvents [33].

Benzene

Benzene is utilized extensively as a raw material in the manufacture of polymers, detergents, pesticides, dyes, plastics, and resins and as a solvent for waxes, oils, natural rubber, and other compounds [93,123]. In addition, benzene is a component of gasoline, cigarette smoke, and some foods and is generally present at low levels throughout the ambient environment [91]. Exposure to benzene in the workplace is primarily through inhalation, although skin absorption may also contribute to the overall body burden. OSHA regulates benzene specifically by standards established in 29 CFR 1910.1028 and recognizes benzene as an occupational carcinogen [148]. The OSHA PEL for benzene is 1 ppm and the STEL is 5 ppm [148]. The 2005 ACGIH TLV®–TWA and STEL for benzene are 0.5 and 2.5 ppm (skin notation), respectively. The ACGIH designates benzene as a confirmed human carcinogen [2,4]. The NIOSH REL and STEL for benzene are 0.1 and 1 ppm, respectively. NIOSH identifies benzene as an occupational carcinogen [2,4,143]. The ACGIH BEI for benzene is 25 pg (pictograms) of the metabolite *S*-phenylmercapturic acid per gram of creatinine in urine, as measured at the end of the work shift [4].

Due to its high lipid solubility, acute exposure to benzene can depress the CNS to the point of narcosis. Headache, dizziness, nausea, and vomiting are all features of benzene overexposure. Exposure to benzene at high concentrations can lead to blurring of vision, unconsciousness, convulsions, ventricular irregularities, and respiratory failure. Death as a result of exposure to extremely high concentrations of benzene may occur because of respiratory failure or cardiac arrhythmias [175,198]. Concomitant exposure to benzene and high concentrations of catecholamines can sensitize the heart and lead to ventricular fibrillation.

Benzene is hemato toxic [174] and carcinogenic following repeated exposure to high concentrations [126]. A cross-sectional study that compared peripheral blood cell counts of 250 Chinese workers exposed to benzene to 140 age and sex-matched controls found that that total white blood cells, granulocytes, lymphocytes, B cells, and platelets significantly declined with increasing benzene exposure. The researchers found that benzene exposure decreased colony formation

from myeloid progenitor cells, and that these progenitors were more sensitive to benzene toxicity than were mature WBCs. Genetic variation in myeloperoxidase (MPO) and NAD(P)H:quinone oxidoreductase (NQO1) conferred susceptibility to benzene-induced lowering of WBC counts [105].

Numerous rodent studies demonstrate that benzene can also cause cytogenetic damage in vivo [91]. In addition, examination of the chromosomes of humans exposed to high levels of benzene reveals an elevated rate of chromosomal aberrations that persist after cessation of exposure [69]. Chronic exposure to benzene leads to a progressive depression of bone marrow function [116]. Epidemiological studies demonstrate that blood dyscrasias such as pancytopenia, aplastic anemia, and acute myelogenous leukemia can develop in humans as a result of this exposure [126,175]. Furthermore, some clinical investigations indicate that it may take several years after the termination of exposure for benzene-induced leukemia to appear [192].

Enzymes linked to the metabolic activation of benzene and its metabolites are the cytochrome P450 monooxygenases and myeloperoxidase [126]. The major metabolic pathway for benzene appears to be oxidation to a phenol, which is then converted to a sulfate conjugate and excreted in urine. Other hydroxylated metabolites include hydroquinone and catechol. Benzene metabolism can be affected by interactions of benzene with its metabolites or other compounds. As an example, experiments in mice suggest that benzene can inhibit the oxidation of phenol. Furthermore, animal and human studies have demonstrated that co-exposure to toluene may significantly alter the formation of benzene metabolites. Finally, treatment with ethanol induces benzene and phenol metabolism in the liver, resulting in higher levels of active metabolites [126].

The actual mechanism of benzene-induced leukemia is not known. Potential mechanisms for benzene-induced bone marrow disease include metabolism of the parent compound to phenols and other metabolites, in particular, quinone-type metabolites such as catechol, quinol, and pyrogallol, which could react with chromosomes and interfere with mitosis. Another possibility is the depletion of sulfur available for glutathione detoxification, thereby leading to interaction of toxic intermediates with critical elements of the bone marrow. Another suggested mechanism involves transfer of benzene metabolites from the liver to the bone marrow [175]. Researchers have investigated the metabolism and binding of radioisotope-labeled benzene in the isolated hind limb of rats in which benzene was administered directly into the bone marrow space. Metabolites of benzene were found covalently bound to macromolecules in the bone marrow, indicating that the bone marrow has the potential of metabolizing benzene to reactive intermediates [95]. The fact that benzene or benzene metabolites have been shown to inhibit the multiplication of erythrocyte precursor cells in the bone marrow may imply an additional mode of action [106]. The potential for benzene to induce leukemia in experimental animals has been difficult to demonstrate. In a 2-year carcinogenicity study, rats and mice fed benzene in corn oil developed dose-related leukopenia and tumors in multiple organs, but the study failed to show benzene-associated leukemia [91].

Toluene

Toluene is a flammable, aromatic solvent used extensively in the chemical, rubber, paint, and drug industries. It is also useful as a solvent for paints, inks, lacquers, dyes, and other compounds and as an additive for gasoline. Sources of toluene in the ambient environment include manufacturing plants, automobile emissions, gasoline evaporation, and cigarette smoke [44,129]. Various exposure limits and biological indicators of exposure apply to toluene. The ACGIH TLV®–TWA and German MAK for toluene are 50 ppm (skin notation) and 50 ppm, respectively. The NIOSH REL is 100 ppm as a time-weighted average and the STEL is 150 ppm. The established IDLH for toluene is 500 ppm. The current OSHA PEL is 200 ppm, with a 300 ppm ceiling limit as a 10 min peak per 8 h work shift [2,4]. The ACGIH BEIs are 0.05 mg of toluene per liter of venous blood, collected before the last shift of the work week; 1.6 g of hippuric acid per gram of creatinine in the urine, collected at the end of the shift; and, 0.5 mg of o-cresol per liter of urine, collected at the end of the shift [2,4,143]. Toluene in expired air has also been evaluated to determine its usefulness as an indicator of exposure [25]. Analysis of expired air in toluene-exposed workers revealed that the toluene concentration was correlated to the exposure environment, representing approximately 15%–20% of the environmental concentration [39].

The principal toxic effect of toluene is injury to the nervous system. Toluene is most rapidly absorbed by inhalation, followed by ingestion and skin contact. A substantial amount of inhaled toluene is retained in the body. The toxicity of toluene is similar to that of benzene except that it does not exhibit the hematopoietic effects characteristic of benzene. Toluene is an eye and skin irritant, and animal studies indicate that its acute oral toxicity is less than that of other alkyl benzenes [44]. In humans, acute effects of toluene exposure can resemble alcoholic intoxication by first stimulating and later depressing the CNS.

Exposure to high concentrations of toluene, as seen in cases of solvent abuse (e.g., glue sniffing), may cause death by sensitizing the myocardium [167,198]. In chronic abusers of toluene, irreversible neurological toxicity and reversible renal damage have also been reported [181,196]. Symptoms associated with the intentional inhalation of high concentrations of toluene include euphoria, mild tremors, unsteady gait, and changes in behavior. Encephalographic examination of these individuals has shown abnormalities indicative of cerebellar atrophy [100]. Toluene is a lipid-soluble compound that readily crosses the placenta and, as such, may pose a teratogenic risk in cases of high exposure, as with intentional abuse. A pattern of teratogenicity, like that of fetal alcohol syndrome (described in the section on ethanol), is prevalent in human studies relating to excessive in utero exposure to toluene. Simultaneous abuse of alcohol and toluene may heighten the risks [196].

Toluene is metabolized to benzoic acid, which is subsequently conjugated with glycine or glucuronic acid to form hippuric acid or benzoyl glucuronates, respectively. These conjugates, as well as another metabolite, o-cresol, are excreted in the urine [104]. In human studies, ethanol has been shown to inhibit the metabolism of toluene at blood ethanol concentrations of 21 mmol/L [58]. Test results indicate that the concentration of toluene in the alveolar air of the toluene/ethanol-exposed group can be significantly higher than that of the toluene control group. In these studies, hippuric acid and o-cresol excretion is significantly reduced as compared to controls. Additionally, during the 24 h following the last exposure, excretion of both hippuric acid and o-cresol was about 40% to 50% of that excreted by subjects who received only toluene. These results suggest that ethanol may alter the metabolism of inhaled toluene and prolong its elimination from the body; therefore, the possibility of ethanol consumption should be considered during biological monitoring, as ethanol intake could lead to an underestimation of the actual toluene exposure [58].

In contrast to the earlier observations, pretreatment of rats with phenobarbital (PB) indicates that the metabolism of toluene can be enhanced to form benzoic acid. The pretreatment did not, however, appear to effect the rate of conjugation of benzoic acid with glycine to form hippuric acid. The hippuric acid concentration in the urine of PB pretreated rats was about three times that of rats receiving toluene only. In addition, the toluene concentration in the blood of the PB pretreated group was only about half that in the toluene-exposed rats. Not only did the phenobarbital pretreatment enhance metabolism of toluene to benzoic acid (with subsequent conversion to hippuric acid), but it also reduced the blood concentration of toluene, thus shortening the sleeping time induced by the narcotic effect of toluene [94].

At present, the mechanisms of the neurotoxic effect of toluene are not well understood. Some experimental work with rats indicate that exposure to 30,000 ppm of toluene for a few minutes reduced the concentration of tryptophan and tyrosine in plasma by about 50%–20%, respectively, compared to controls. Tryptophan and tyrosine are known to be precursors of the neurotransmitters noradrenaline, dopamine, and 5-hydroxytryptamine. The reason for the decrease in the precursors is unknown, but it is speculated to be an alteration in the hepatic uptake or utilization of these amino acids [195]. A potential factor in toluene-induced neurotoxicity is the production of reactive oxygen species that can result in cell damage. Experiments using rats suggest that benzaldehyde, a metabolite of toluene, accelerates the production of these reactive oxygen species within the nervous system and may also contribute to the overall neurotoxicity [123].

N-Hexane

N-Hexane is a flammable liquid and one of the most toxic of the alkanes. It is an excellent organic solvent that has been used in industrial applications such as printing, low-temperature thermometers, adhesives, extractions, and cleaning processes [43,89]. The primary routes of exposure in the industrial setting are by inhalation and skin contact. The ACGIH TLV®–TWA, NIOSH REL, and German MAK are all 50 ppm. ACGIH and the German MAK recognize *n*-hexane with a skin notation. NIOSH recognizes the IDLH concentration for *n*-hexane as 1100 ppm (10% of the LEL). ACGIH set the TLV® based on possible neuropathy, CNS effects, and irritation [2,4,143]. Acute toxic responses after accidental ingestion include nausea, gastrointestinal irritation, and CNS effects. Inhalation overexposure leads to dizziness, a sense of euphoria, and numbness of the extremities. Exposure to high concentrations causes vertigo and a marked anesthetic effect. Hexane is also an irritant to the skin upon dermal exposure [43].

Many cases of polyneuropathy in workers exposed to *n*-hexane have been noted, with the earliest occurring in Japan [201]. The severity of symptoms in the Japanese workers varied directly with degree and duration of exposure, and in some cases, there was incomplete recovery [89]. Polyneuropathy has also been reported in cases of solvent abuse [43]. The neurotoxic effect of *n*-hexane has characteristically been a progressive motor or sensorimotor neuropathy with symptoms usually reported after several months of exposure [89]. In cases from occupational exposure, symptoms have often been sensory, with numbness and paresthesia in the distal extremities, most notably the feet or hands. Improvement of symptoms is noted after cessation of exposure, and mild cases can recover completely.

Hexane is readily absorbed in laboratory animals and has an affinity for tissues high in lipid content [34]. It is rapidly metabolized to hydroxylated compounds prior to being converted to a keto-form [101,117]. 2,5-Hexanedione and methyl *n*-butyl ketone are the metabolites suspected of being responsible for the production of neurotoxicity.

The mechanism of 2,5-hexanedione-induced neuropathy is not known but several hypotheses have been presented [53,54]. These include a reduction in energy production in the axon resulting in disruption of axonal transport, alteration of protein structure, and inadequate proteolysis of neurofilaments in the nerve terminal. 2,5-Hexanedione has been shown to interact with glyceraldehyde-3,5-dehydrogenase and phosphofructokinase, inhibiting their glycolytic properties and resulting in decreased energy production and possible disruption of axonal flow. Reaction of 2,5-hexanedione with lysine amine moieties to form pyrrole adducts and modification of neurofilament or axonal skeletal proteins is also an attractive hypothesis [40]. Modification of the proteins may lead to cross-linking of the neurofilaments, which could cause difficulty in neurofilament passage through narrow regions of the axon, such as the node of Ranvier, and therefore an accumulation of proteins at the site of constriction. Possible biophysical membrane changes as a result of 2,5-hexanedione may influence the degeneration of the axon. 2,5-Hexanedione binding and inactivation of calcium-dependent proteases that are important for degradation of neurofilament proteins are the last mechanisms mentioned that might lead to accumulation of neurofilaments. Although

none of the mechanisms mentioned fully answers all of the questions concerning *n*-hexane-induced neurotoxicity, these hypotheses offer some contributions to the understanding of the toxic response. It may be that several mechanisms act in parallel to produce the neurotoxic effects.

Repeated exposure of rats to *n*-hexane not only produces the characteristic pattern of neurotoxicity but also results in testicular lesions [200]. The testicular effects are linked to disruption of the cytoskeleton of Sertoli cells. Secondary effects, caused by a loss in functional spermatogonial cells, are seen in affected tubules. Acute exposure led to reversible effects but inhalation or oral exposures of 2–5 weeks led to irreversible effects. Although the neurotoxic effect of *n*-hexane is observed in humans, the testicular effect seen in rats has not been well documented in humans.

Methyl *N*-Butyl Ketone

Industrial uses of methyl *n*-butyl ketone (2-hexanone, MBK) as a solvent or co-solvent (e.g., with methyl ethyl ketone) include the manufacture of adhesives, lacquers, vinyl coatings, printing inks, oils, varnish removers, and other materials [35,102]. Occupationally, the principal routes of exposure to MBK are via inhalation and skin contact with the liquid. The OSHA PEL for MBK is 100 ppm. Since 1998, the ACGIH has identified the TLV®–TWA for MBK at 5 ppm (skin notation) to protect against possible neuropathy. The German MAK is also 5 ppm. The NIOSH REL and IDLH for MBK are 1 ppm and 1600 ppm, respectively [2,4,143].

Methyl *n*-butyl ketone has low acute oral toxicity. The inhalation of high vapor concentrations of MBK can result in eye and respiratory tract irritation followed by CNS depression and narcosis [183]. MBK easily penetrates the skin, and inhalation exposure yields approximately 80%–85% pulmonary retention. In addition, MBK is widely distributed in the tissues, the highest concentrations being found in the blood and the liver [35]. Chronic exposure to low doses may produce degenerative axonal changes, primarily in the peripheral nerves and long spinal cord tracts [176,177,183]. Depending on the route of administration, a number of metabolites in varying amounts can be detected in the blood. The primary neurotoxic metabolite, as with *n*-hexane, is 2,5-hexanedione. Other metabolites identified following oral, intraperitoneal, or respiratory exposures include 2-hexanol and 5-hydroxy-2-hexanediol [35].

Since the 1970s, MBK has been considered a neurotoxic agent after instances of neurotoxicity were reported in the printing and painting industries [18,127]. Inhalation appears to be the primary route of exposure, with the severity of the toxicity being proportional to the extent of exposure. The characteristic disorder associated with methyl *n*-butyl ketone exposure begins several months after chronic exposure commences. Symptoms include weight loss and distal sensory neuropathy marked by a tingling sensation in the hands or feet. The muscular weakness that develops usually involves the hands and feet, but in severe cases may extend to the legs and thighs. The sensory loss is symmetrical and a moderate reduction of nerve conduction velocity is found in peripheral nerves [17,18].

When volunteers were given MBK by inhalation, orally, or by dermal application, 2,5-hexanedione was detected in the serum. Radioactivity associated with the radiolabeled MBK was found to be excreted slowly, indicating that repeated exposures to high concentrations of methyl *n*-butyl ketone may lead to prolonged exposure to its neurotoxic metabolites [57]. The relative neurotoxicity of methyl *n*-butyl ketone, *n*-hexane, and their metabolites was investigated in rats. Potency was estimated by the time required to produce evidence of severe hind limb weakness or paralysis. Results showed 2,5-hexanedione to be most toxic followed by 5-hydroxy-2-hexanone, 2,5-hexanediol, methyl *n*-butyl ketone, 2-hexanol, and *n*-hexane. An examination of the data showed that the neurotoxic potency was related to the amount of 2,5-hexanedione metabolically produced [102].

Carbon Disulfide

Carbon disulfide (CS_2) is a toxic and highly flammable solvent in extensive use in the manufacture of rayon, soil disinfectants, carbon tetrachloride, and electronic vacuum tubes. It is commonly used as a solvent in industrial hygiene analytical procedures. Other applications include its use as a fumigant for grain and a corrosion inhibitor [31,129]. Inhalation and skin contact are the main routes of occupational exposure. Because the sense of smell is quickly fatigued and sensitized to carbon disulfide's characteristic rotten-egg odor, this warning property is not useful in judging exposure. The ACGIH TLV®–TWA exposure limit is 10 ppm with skin notation. The NIOSH REL is 1 ppm with a STEL/ceiling limit of 10 ppm and skin notation. The OSHA PEL–TWA is 20 ppm with a ceiling limit of 30 ppm as a 30 min peak over an 8 h work shift [2,4,143]. The ACGIH TLV®–TWA was set to protect against cardiovascular, CNS, and neuropathic effects. NIOSH has established an IDLH value of 500 ppm [143]. In addition to these levels, proposals in the literature have suggested lowering the occupational exposure limit to 4 ppm to prevent neurological sequelae [90]. The BEI recommended by the ACGIH is 0.5 mg of the metabolite 2-thiothiazolidine-4-carboxylic acid (TEA) per gram of creatinine in urine, measured at the end of the work shift [4].

Acute exposure to high concentrations of carbon disulfide can result in restlessness, euphoria, nausea, vomiting, headache, mucous membrane irritation, unconsciousness, and fatal convulsions. Chronic exposure can lead to abnormalities such as irritability, hallucinations, auditory and visual disturbances, and weight loss [31,79,110,129,191]. Distal sensorimotor neuropathy is the most common chronic effect associated with CS_2 exposure. This has been confirmed in experimental animals as a neurofilamentous axonopathy that affects long axons in the CNS and peripheral nervous system [50,78]. Peripheral neuropathy takes place only after frequent and prolonged exposures to CS_2 and is characterized by a loss of distal sensory and motor function. The condition can progress more proximally with continued exposure.

Chronic exposure to CS_2, as well as hexane, 2-hexanone, and their metabolite 2,5-hexanedione, results in large swellings of the distal axons, which are filled with neuron filaments. Continued exposure causes axonal degeneration distal to the axonal swellings [48,78]. In addition to these effects, encephalopathy, detected by neurological examination and neuropsychological testing, has been reported. Evidence suggests that exposure to CS_2 accelerates the rate of atherosclerosis [78]. In addition, an investigation to determine a possible association between CS_2 exposure and ischemic heart disease mortality found that the relationship is meaningful only for workers exposed to high levels for many years. Price has suggested a safe level of between 15 and 20 ppm [162]. Approximately 70%–90% of absorbed CS_2 is metabolized and excreted in the urine. The remaining 10%–30% is exhaled in the breath unchanged. In addition to TTCA, mentioned earlier, other metabolites found in workers' urine include 2-mercapto-2-thiazolin-5-one and thiocarbamide [90,158,159,189,190].

In a study of rayon production workers with long-term exposure to CS_2 at concentrations well above the TLV®, evidence of neuropathy was observed in a significant number of workers and consisted of distal sensory loss, altered tendon reflexes, reduced muscle power, and reduction in nerve conduction velocity. These abnormalities persisted for up to 10 years after removal from exposure and were considered to be permanent impairments in nervous system physiology [49].

Methanol

Synthetic methanol (or methyl alcohol, wood alcohol) production exceeded 1 billion pounds in 2005. The largest use of methanol was in the production of methyl *t*-butyl ether (MTBE), an additive in gasoline. However, beginning in 2005 because of concerns with ground water contamination from MTBE, its use in reformulated gasoline has declined significantly. It is also utilized as a denaturant for ethanol, a raw material in the production of numerous other chemicals such as formaldehyde and acetic acid, and as a solvent or antifreeze in paints and strippers, cleaners, and windshield washer compounds [55,59].

The major routes of exposure to methanol in the industrial environment are through inhalation and dermal contact. The ACGIH TLV®–TWA of 200 ppm (250 ppm STEL) is based on potential ocular toxicity and CNS effects. The OSHA PEL, German MAK, and NIOSH REL are all set at 200 ppm. NIOSH has further established an IDLH value of 6000 ppm for methanol, and the ACGIH and NIOSH have added skin notations as indications that skin absorption can be a contributor to the overall body burden. The ACGIH BEI is 15 mg methanol per liter of urine, collected at the end of the work shift [2,4,143].

Most information regarding methanol toxicity in humans is gathered from acute exposures, primarily from ingestion, but adverse health effects from inhalation and dermal exposures have been reported [112]. In one NIOSH study, teachers' aides reported headaches, blurred vision, and other symptoms following inhalation exposure to methanol used in duplicating machines. Concentrations at the site were about 2–15 times the current REL. Adverse effects have also been reported following skin applications of methanol for various purposes, although inhalation may have also contributed to these exposures [112].

Methanol is readily absorbed following oral, inhalation, or dermal exposure and is distributed throughout the body according to the water content of the tissues [112]. Ingestion of as little as 2 teaspoonfuls may cause toxicity, whereas the fatal dose in humans is between 2 and 8 oz [77]. In the absence of medical treatment, a dose of between 4 and 10 mL of methanol taken internally can lead to blindness [165], and, depending on the amount of methanol ingested, mild to severe CNS depression can occur. A latent period, commonly 12–24 h, usually ensues followed by severe abdominal pain, difficult breathing, blurred vision, and pain in the eyes, among other symptoms. Visual impairment or total blindness can occur within days depending on individual susceptibility and the time when treatment began [112]. Metabolic acidosis due to formic acid production is thought to be the cause of the delayed symptoms and ocular toxicity [165].

Metabolism of methanol in the liver accounts for a high percentage of absorbed methanol in both nonhuman primates and rats. Lesser amounts are excreted unchanged in the urine and breath. Metabolism is important not because of its primary role in clearance but because of the connection between its metabolites and the acute toxic effects mentioned earlier. Methanol is oxidized by the catalase–peroxidative system in rats, rabbits, and guinea pigs and an alcohol dehydrogenase system in humans and primates. The metabolic sequence proceeds from methanol to formaldehyde to formic acid (formate) and finally to carbon dioxide and water. Formic acid is metabolized in both rats and primates via a folate-dependent pathway. Rats are able to utilize this pathway more efficiently than primates, allowing for a more rapid conversion to carbon dioxide. Because the process is slower in humans and primates, high doses of methanol cause a buildup of formate in tissues, including the eye, resulting in the observed toxicity [165]. Administration of ethanol has been used in treating methanol poisoning because ethanol inhibits the oxidation of methanol by competing for the same metabolic pathway. Prompt hemodialysis to remove both methanol and formate, coupled with concurrent administration of ethanol and bicarbonate, is a successful treatment in many poisoning cases [77]. Fomepizole, a potent inhibitor of alcohol dehydrogenase that does not cause inebriation and is simpler to deliver intravenously than ethanol, is approved by the U.S. Food and Drug Administration for treatment of methanol intoxication.

Ethanol

Ethanol (ethyl alcohol, grain alcohol) is produced in large quantities and is utilized extensively as a solvent in industry, in numerous consumer preparations, and as an additive to up to 95% of all gasoline sold in the United States. Ethanol–gasoline blends are sold as blends ranging from 10% (E10) to

85% (E85) ethanol to gasoline [56]. It is used industrially as a raw material in the production of pharmaceuticals, plastics, perfumes, cosmetics, and other compounds. Other applications include products such as hairsprays, mouthwashes, cleaning products, and drug formulations [77,112]. Denaturants (e.g., methanol) are added to the alcohol in a number of these products to discourage ingestion. The majority of industrial ethanol is synthesized by the acid-catalyzed hydration of ethylene and represents the largest source of ethanol; however, significant amounts are made from the fermentation of natural materials, particularly starchy grains [56,112].

Human exposure to ethanol is primarily through ingestion of alcoholic beverages and inhalation of ethanol vapors from industrial processes and consumer products. Percutaneous absorption appears to be much less important [112]. OSHA, ACGIH, and NIOSH have established the exposure limit of 1000 ppm for ethanol [2,4]. The German MAK is 500 ppm. The NIOSH IDLH of 3300 ppm is set because of safety concerns (10% of the LEL) rather than toxicological considerations [143].

Although there is no clear evidence that ethanol is carcinogenic in animals, it has been shown to be a tumor promoter. Additionally, the International Agency for Research on Cancer (IARC) has classified alcoholic beverages as a Group 1 carcinogen based on the occurrence of a variety of tumors in humans that have been causally related to ingestion of these beverages [112]. An unfortunate occurrence associated with chronic maternal consumption of large amounts of alcohol is a pattern of congenital abnormalities commonly called fetal alcohol syndrome. Effects may include growth retardation, microcephaly, mental deficiency, facial abnormalities, and poor coordination. Children who have been affected may display a few or many of the features characteristic of the syndrome [47,112,161].

Ethanol is a CNS depressant that is capable of inducing all stages of anesthesia. It is readily absorbed by the gastrointestinal tract and the lungs and is distributed throughout the body water [77]. Absorption can be delayed, however, by food in the stomach. Subjects exposed to 5000–10,000 ppm of ethanol vapor experienced eye irritation and coughing [165]. Individuals with tolerance to alcohol experienced headache, drowsiness, and stupor when exposed to concentrations of 9400–13,200 mg/m^3 (5000–7000 ppm) for a period of 110 min [165]. Ingestion of approximately 1 L of an alcoholic beverage (45%–55% ethanol) within several minutes can result in death [77]. Individuals with blood alcohol levels of approximately 0.05%–0.15% (50–150 mg/dL) may exhibit decreased inhibitions, poor coordination, blurred vision, and slowed reaction time. Increasing blood levels to 0.15%–0.30% can result in slurred speech, visual impairment, hypoglycemia, and staggering. At 0.3%–0.5% blood alcohol content (severe intoxication), symptoms can include poor muscular coordination, hypothermia, vomiting and nausea, and convulsions. In adults, coma and death are typically associated with levels exceeding 0.5% [112,165]. The wide ranges reported earlier reflect the differences in tolerance and susceptibility of individuals to the effects of alcohol.

Like methanol, ethanol is metabolized primarily (about 90%) by the liver. Elimination from the body by urinary excretion and pulmonary exhalation is minimal [112]. Oxidation of ethanol to acetaldehyde occurs via alcohol dehydrogenase within the cytosol. Acetaldehyde is then converted to acetic acid by the action of aldehyde dehydrogenase. Both enzymes utilize oxidized nicotinamide adenine dinucleotide (NAD) as a cofactor [77]. Following release to the blood, acetic acid is metabolized to carbon dioxide and water in the peripheral tissues [112]. Alternative, but less active, metabolic pathways have been demonstrated in humans and other species. These include catalase and microsomal ethanol-oxidizing systems [36,111]. Adults metabolize ethanol at a rate of about 7–10 g/h. This rate remains essentially constant for each individual within a wide range of exposure. Metabolic rates are higher for chronic alcoholics and children [112,165].

The interaction of ethanol with other hepatotoxicants is well known. Ethanol pretreatment has been shown to increase the toxicity of carbon tetrachloride, chloroform, trichloroethylene, dimethyl-nitrosamine, chlorpromazine, and other compounds [179]. The induction of cytochrome P450 isozymes may be responsible for their metabolic effects [112].

Methylene Chloride

Methylene chloride (dichloromethane) is widely used in a number of diverse applications, including the manufacture of polyurethane foams, pharmaceuticals production, boat building, paint stripping, vapor degreasing, extraction of caffeine from coffee and tea, and in various consumer products. Its high volatility; good solvent properties for fats, oils, and other compounds; and relatively good water solubility compared to other chlorinated compounds have made it quite valuable [149,184].

Due to the high vapor pressure of methylene chloride, the primary route of human exposure is through inhalation; however, dermal contact can be significant, depending on the method of use. The ACGIH TLV®–TWA of 50 ppm was set to protect against CNS effects and anoxia. In addition, the ACGIH has designated methylene chloride as a confirmed animal carcinogen but also states that available epidemiological studies do not confirm an increased risk of cancer in exposed humans [4]. NIOSH recommends that methylene chloride be regarded as a potential occupational carcinogen [143]. OSHA regulates methylene chloride in the workplace under 29 CFR 1910.1052 [149]. OSHA considers methylene chloride a potential human carcinogen and has reduced the PEL for methylene chloride from 500 to 25 ppm, with a STEL of 125 ppm (15 min) and an action level of 12.5 ppm that triggers certain requirements [149]. The current German MAK is 100 ppm [2].

The primary acute hazards associated with exposure to methylene chloride are due to its narcotic effect and can result in CNS depression and eye, skin, and respiratory tract irritation. In addition, one of the products of methylene chloride metabolism is carbon monoxide, which can impair health in a manner similar to direct exposure to carbon monoxide.

The resulting carboxyhemoglobin levels reduce the supply of oxygen to the heart and may aggravate preexisting heart disease [149].

Metabolism of methylene chloride can proceed via two pathways, one by a route involving cytochrome P450 monoxygenases (CYP) and the other by a route utilizing glutathione *S*-transferase (GST). Carbon dioxide is an end-product in both systems, but carbon monoxide is only produced via the MFO route. At low concentrations, the CYP enzymes appear to dominate, but at higher concentrations (above 300–500 ppm), the glutathione pathway increases in a disproportionate manner [184].

Methylene chloride was shown in a 1986 National Toxicology Program inhalation study to produce lung and liver tumors in male and female mice and benign mammary tumors in male and female rats [80]. Recent research has suggested that mice may be uniquely sensitive at high exposures to methylene-chloride-induced lung and liver cancer [80]. The tumors appear to be caused by a genotoxic mechanism involving metabolites of the GST pathway. The particular metabolites responsible are not found in high concentrations in lung or liver tissue in humans or rats.

In a study to determine the effects of alcohols and toluene upon methylene chloride-induced carboxyhemoglobin in the rat and monkey, it was shown that ethanol, methanol, isopropanol, and toluene inhibited the formation of carboxyhemoglobin. In addition, neither the rat nor the monkey demonstrated the methanol potentiation of carboxyhemoglobin that has been reported to occur in humans [46].

A study of the pharmacokinetics of [14C]-methylene chloride in rats at 50, 500, and 1500 ppm for 6 h showed that metabolic processes were saturated above the 50 ppm exposure concentration. At 48 h after exposure, approximately 95% of the body burden attributable to the 50 ppm exposure was metabolized, in contrast to 69% and 45% at 500 and 1500 ppm, respectively [125]. In addition, the production of carboxyhemoglobin reached a steady-state range of 10%–13% regardless of the exposure concentration, suggesting that the CO metabolic pathway was saturated.

Tetrachloroethylene (perchloroethylene) is another solvent in which patterns of elimination are altered when metabolic pathways become saturated [156]. In a study comparing oral and inhalation exposure of rats to [14C]-tetrachloroethylene, it was found that, with increasing dose, metabolism was saturated, resulting in more of the parent compound being eliminated unchanged at 72 h after exposure [156]. These results with methylene chloride and tetrachloroethylene indicate that simply increasing the exposure concentration does not always increase the body burden in a linear manner. Such information may be useful for safety evaluations to avoid the overestimation of body burden.

1-Bromopropane

1-Bromopropane (*n*-propyl bromide, 1-BP) is a colorless to pale yellow liquid with a strong, characteristic odor. The boiling point is 71°C and the vapor pressure is 110.8 mmHg at 20°C [130,186]. 1-Bromopropane is less flammable than many other halogenated alkanes at room temperature [81,186]. In 2006, worldwide annual production capacity of 1-bromopropane was estimated at greater than 20,000 metric tons (44 million pounds/year), of which 5000 metric tons were thought to be used as a pharmaceutical intermediate or process agent. The United States production was estimated at approximately 5000 metric tons (11 million pounds/year) and growing at a rate of 15%–20% per year [187]. Because 1-bromopropane has a relatively short atmospheric half-life (16 days), it is considered to have a relatively low ozone depletion potential and was marketed as a replacement for ozone-depleting refrigerants (e.g., chlorofluorohydrocarbons and hydrochlorofluorocarbons) [65].

Prior to the late 1990s, 1-BP was primarily used in enclosed processes as an intermediate in the production of pesticides, quaternary ammonium compounds, flavors and fragrances, pharmaceuticals, and other chemicals [82]. Beginning in the later part of the decade, 1-BP was introduced as a presumed less toxic replacement for methylene chloride degreasing operations and critical cleaning of electronics and metals. Because it is relatively nonflammable, 1-BP can be used safely in metal cleaning processes where heating is required. 1-Bromopropane was also introduced as a nonflammable, nontoxic, fast-drying, and inexpensive solvent for adhesive resins. Aerosol-applied adhesives containing 1-BP were used extensively by foam fabricating companies [82]. As usage of 1-BP became more prevalent, case reports of adverse events and toxic effects of 1-BP began to increase [93].

1-Bromopropane is absorbed in animals by all exposure routes and occupational exposures to humans can occur by both inhalation [139] and dermal routes [71]. Metabolism studies in rats and mice, have shown 1-BP can directly conjugate with glutathione forming *N*-acetyl-*S*-propylcysteine, or may be oxidized by P450 enzymes, primarily CYP2E1, to reactive intermediates that can also be further oxidized by other P450 enzymes and/or conjugated with glutathione. Potential biological markers of 1-BP exposure to workers that have been investigated include measurements of bromide ion $Br^{(-)}$, *N*-acetyl-*S*-(*n*-propyl)-L-cysteine (AcPrCys), and 1-BP in urine, and serum bromide level [45,82,83,122].

Although no study was identified that defined the absorption, metabolism, and disposition of 1-BP in animals and humans, it is possible to make some general assumptions based on the previously described reports. Exposure to 1-BP can occur by inhalation, oral, and dermal routes with 1-BP being rapidly distributed through the body tissues. Depending on species and activity levels, 30%–70% of the absorbed dose is eliminated unchanged in exhaled breath. Of the retained 1-BP, it may be eliminated by conjugation with glutathione GSH directly or by GST enzymes, or undergo oxidative biotransformation by the CYP450 monooxygenases. Animal studies strongly suggest that toxicity of 1-BP is dependent on the metabolic pathway of the compound. GSH-dependent metabolic pathways are integral to toxic actions, but it is not likely that the GSH-1-BP-conjugates are the source of toxicity. Instead, a stronger case can be made that toxicity of 1-BP

is dependent on the generation of reactive oxidative metabolites of 1-BP by CYP450 monooxygenases that are conjugated with GSH for elimination. Toxicity of 1-BP likely results when GSH levels are depleted from neutralizing reactive metabolites; as free GSH is utilized, GSH-1-BP-conjugates increase until GSH is consumed. At this point critical cellular components can be damaged, and toxicity results. The strongest support for a mechanism such as this is derived from experiments using sensitive species or strains or more elegantly, genetically engineered animal models that are missing the key step in the toxic pathway [75,113].

1-Bromopropane has been found to be neurotoxic, a developmental and reproductive toxicant, immune-toxic, and hepatotoxic in rodents and has produced neurological and possible reproductive effects in humans [107,108,138,139]. In a 2-year inhalation study by the National Toxicology Program (NTP), nonneoplastic lesions increased in 1-bromopropane-exposed rats (nose, larynx, and trachea) and mice (nose, larynx, trachea, and lung). In addition, predominantly in the nose and skin of exposed rats, there was an exposure-related increase in inflammatory lesions containing Splendore-Hoeppli material, indicative of immunosuppression [136,139]. 1-Bromopropane was shown to be immunosuppressive in rats and mice after whole-body inhalation exposure [19].

Currently, neither NIOSH nor OSHA have established a REL or PEL for workplace 1-BP. ACGIH in the 2005 TLV®s and BEIs recommended an 8 h TWA of 10 ppm as protection against the potential for neurotoxicity, hepatotoxicity, and reproductive and developmental toxicity in 1-BP exposed workers. A Notice of Intended Change (NIC) has been proposed to lower the TLV® to 0.1 ppm (8 h TWA). Other professional organizations and manufacturers have RELs ranging from 20 to 100 ppm. The U.S. EPA stated in May 2007 that exposures within or below the range of 17–30 ppm are anticipated to be protective against reproductive effects in men and women. The only, legally enforceable, occupational standard for regulating 1-bromopropane in the United States is a California OSHA PEL of 5 ppm [40]. Other professional organizations and manufacturers have RELs ranging from 20 to 100 ppm. In 2007, EPA, in response to adverse health findings, proposed to limit uses of 1-BP to metal cleaning and degreasing with appropriate controls; uses as aerosol solvents and adhesives were found to be unacceptable.

NONTRADITIONAL SOLVENTS

Given the negative health and environmental impacts created by some of the more widely used solvents, a great deal of effort has gone into finding suitable replacements. The following compounds are examples of nontraditional materials that show promise as replacement solvents.

D-Limonene

D-Limonene is a naturally occurring monocyclic terpene found in citrus peel oils, spices, evergreens, and human milk [194]. It is considered to have low acute toxicity and is listed as generally recognized as safe (GRAS) as a food additive by the U.S. Food and Drug Administration (21 CFR 182.60). It has found wide application as a solvent in numerous cleaning and degreasing applications, replacing more toxic and environmentally undesirable chlorinated solvents, glycol ethers, xylene, and chlorofluorocarbons (CFCs) [68]. Skin contact with D-limonene may cause irritation and sensitization (attributed to the oxidation product D-limonene oxide) [194]. D-Limonene has been shown to produce hyaline droplet nephropathy and renal tubular tumors in male rats; however, these effects are attributed to the unique presence of α2μ-globulin in the male rat and are not deemed relevant to other species, including humans [67]. Among the attributes of D-limonene are its antimicrobial, antiviral, antifungal, and antilarval properties [42]. D-Limonene and related monoterpenes have also demonstrated chemopreventive and chemotherapeutic efficacy in experimental cancer-therapy models [51]. Based on similar metabolic pathways in rats and humans and the therapeutic successes in rodents, it has been suggested that D-limonene may be an efficacious chemotherapeutic agent for human malignancies [51].

Vegetable Oil–Based Solvents

Vegetable oils and their derivatives (oleo-chemicals) have many industrial uses. They are used as solvents for printing inks, in the production of paints and coatings, as lubricants and fuels, and as feed stocks for many products that are historically manufactured from petroleum hydrocarbons [63]. Fatty acid methyl esters (FAMEs) derived from vegetable oils have been used for fuels (biodiesel), cleaning and degreasing agents, and concrete and asphalt release and cleaning agents [99]. These products emit little or no VOCs, and are less toxic than many organic solvents. However, they can be irritating to the skin with prolonged contact, and mists and vapors are irritating to the eyes and respiratory system. No OELs have been set by NIOSH or OSHA for exposure to workplace levels of FAME compounds. FAME compounds are not on the ACGIH list of TLV®s and BEIs. The OSHA PEL of 5 mg/m^3 for oil mists may apply to some operations (spraying), with products containing FAMEs. Because of the growing use of oleo-chemicals as fuels and solvent substitutes, methyl soyate, a FAME made from soybeans and used extensively as a biodiesel fuel, was nominated for the National Toxicology Program for further testing. Initial tests looking at micronuclei formation in male B6C3F1 mice were negative as were Salmonella tests for mutagenicity [140].

Carbon Dioxide

Carbon dioxide (CO_2) is a gas under standard temperature and pressure conditions. It can be converted, however, to the liquid and supercritical phases by increasing pressure and temperature. The critical point of carbon dioxide is 31°C and 73 atm. Below this point, CO_2 can be maintained in a liquid state (e.g., 65 atm and 25°C), whereas above 31°C no amount of pressure can be applied to liquefy it

(supercritical phase) [92]. In either of these dense phases, CO_2 exhibits good solvent properties.

Beneficial characteristics include liquid-like density, gas-like diffusivity, and low surface tension. In particular, liquid CO_2 acts like a hydrocarbon solvent, it has good homogenizing properties (immiscible liquids form a single phase when mixed with CO_2), and it is a good solvent for many aliphatic hydrocarbons and most small aromatic hydrocarbons. Other chemical groups such as halocarbons, esters, ketones, and low-molecular-weight alcohols also exhibit good solvency in CO_2 [92]. Since the mid-1970s, supercritical CO_2 technology has been employed in the food, beverage, pharmaceutical, and perfume industries. Applications include the production of spice extracts, natural dyes, decaffeinated coffee and tea, plant extracts, active substances from drugs, and volatile oils [30,52,124]. It has also been used in wastewater treatment, chemical analysis, and at times as an aerosol propellant. More recently, liquid CO_2 has found favor as an alternative for metal parts degreasing and as a solvent for dry-cleaning clothes [52,98].

One such CO_2 degreasing system is being used in a pen manufacturing operation to replace perchloroethylene. It consists primarily of two separate systems: a hot oil pretreatment process and an automated system that employs liquid carbon dioxide in a pressure vessel. The application is to degrease and remove chips from ball points after machining. The hot oil unit is used to displace fatty esters contained in machining oil and to remove chips in the point cavity. The automated unit then removes oil from the points using liquid carbon dioxide. The carbon dioxide and oil are separated in a recycling system, and the carbon dioxide is used again during the next cleaning cycle.

Advantages of CO_2 usage over conventional solvents are numerous. Carbon dioxide is nonflammable, noncorrosive, nonreactive, nontoxic, inexpensive, and plentiful. Products obtained are solvent free. Selective separations are possible. Finally, environmental problems are eliminated, because the gas is recovered for future use. One of the disadvantages of CO_2 systems involves the relatively high start-up costs for equipment; however, these may be recouped through improved productivity and reduced costs for waste disposal, for example.

Ionic Liquids

Ionic systems, which are made up of salts that are liquid at room temperature, are finding applications in a number of chemical processes. Ionic liquids have good solvent properties for many inorganic, organic, and, polymeric materials and, in some cases, these compounds can serve as both catalyst and solvent [72]. Research has indicated that partitioning of organic solvents between an ionic liquid and water corresponds closely with that found for molecular organic solvents and water; thus, ionic liquids have the potential to replace the toxic, flammable, and volatile organic compounds currently used in liquid–liquid separations [73]. The room-temperature ionic compounds, such as 1-butyl-3-methylimidazolium hexafluorophosphate and 1-butylpyridinium nitrate, consist of nitrogen-containing organic cations and inorganic anions. Their physical and chemical properties can be altered according to the choice of ions. Advantages compared to conventional organic solvents include low volatility and relative ease of recycling [73]. Other potential uses include removal of organic contaminants from wastewater, soil cleanup, replacement of corrosive mineral acids in refinery processes, and spent nuclear fuel treatment [72]. The safety and toxicological profiles of these compounds have yet to be thoroughly developed; therefore, caution must be exercised before they are put into general use.

OPPORTUNITIES IN THE TOXICOLOGICAL EVALUATION OF SOLVENTS

Human exposure to solvents is quite common in today's society. These exposures frequently involve multiple chemicals that are found in numerous products such as cleaning agents, paint thinners, and fuels. Although most toxicological research to date has dealt with single chemicals, questions remain about the long-term health effects associated with low-level exposures to multiple chemicals and the sensitivity of the toxicological endpoints that are currently being relied upon. Development of innovative experimental protocols and new quantitative mechanistic approaches to the study of chemical interactions may be beneficial in this regard [103,202]. Economic concerns and the desire for less toxic and more environmentally friendly chemicals have resulted in the introduction of numerous alternative compounds into the marketplace. In some cases, little may be known about the health and environmental impacts of these materials; examples include the ionic liquids discussed earlier. It is therefore essential that sufficient toxicological and environmental data be gathered before replacements are introduced on a wide scale. Research has shown that many neurotoxic chemicals are capable of adversely affecting the sensory function. Minor changes in vision or hearing, for example, can dramatically alter job performance and the overall quality of life. While most reports to date have dealt with changes in the visual system, additional investigations into the effects of solvents on hearing, taste, and smell would provide important new information on this subject [70].

QUESTIONS

15.1 Which of the following Occupational Exposure Limits are legally enforceable by statute?
   a. Threshold Limit Values
   b. Recommended Exposure Limits
   c. Workplace Environmental Exposure Limits
   d. Permissible Exposure limits
   e. New Chemical Exposure Limits

15.2 You are a toxicologist with industrial hygiene responsibilities in a large manufacturing company. Your boss has just told you that the solvent the factory is using to degrease metal parts will be banned by the EPA within the next

6 months. Your job is to lead a team of employees, who have a vested interest in the current solvent, in coming up with a suitable alternative material. What are your considerations in recommending a replacement? Explain.

15.3 Assume that the solvent chosen earlier will be used in six locations in the factory. You surmise that some sort of ventilation will be required to protect the employees. What factors must you take into account in recommending the proper system?

15.4 One of your employees has begun using a solvent mixture containing xylene and toluene. To ensure the safety of the worker, you have conducted personal air monitoring throughout the day and have come up with the following sampling times and monitoring results: 0800–1000, 60 ppm xylene and 25 ppm toluene; 1000–1200, 92 ppm xylene and 45 ppm toluene; 1200–1300, no exposure because employee left for lunch; 1300–1600, 110 ppm xylene and 47 ppm toluene. Calculate the TWA exposure for each chemical. Assume that there is no dermal exposure and that the toxic effects contributed by each solvent are additive. Has the TLV®–TWA been exceeded? Toluene TLV® = 20 ppm, Xylene TLV® = 100 ppm.

15.5 Which of the following represents the level of a biomarker of exposure that is most likely to be observed in specimens collected from healthy workers who have been exposed to a specific chemical to the same extent as workers with inhalation exposure at the Threshold Limit Value (TLV®):

a. Biological Exposure Index (BEI®)
b. Biological Limit Value (BLV®)
c. Biological Exposure Limit (BEL®)
d. Biological Benchmark Dose (BMD®)
e. Biological Recommended Limit (BRL®)

15.6 Match each solvent or metabolite with the appropriate fact listed as follows:

Solvent/Metabolite	Fact
1. D-Limonene	a. Antidotal in methanol poisonings
2. Carbon disulfide	b. Associated with bone marrow disease in humans
3. 2,5-Hexanedione	c. Potentially useful in cancer therapy
4. Toluene	d. Teratogen and embryotoxic
5. Methanol	e. Metabolism produces carboxyhemoglobin
6. 2-Butoxyacetic acid	f. Frequently—sniffed to obtain euphoric effect
7. Ethylene glycol monomethyl ether	g. Used in rayon production
8. Benzene	h. A few milliliters can lead to blindness
9. Ethanol	i. Primary causative agent in polyneuropathy
10. Methylene chloride	j. Produces hemolytic effects in rats

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16 Crop Protection Chemicals
Mechanism of Action and Hazard Profiles

James T. Stevens, Trent D. Stevens, and Charles B. Breckenridge

CONTENTS

INTRODUCTION

The use of chemicals to control pests dates back more than 3000 years to the Chinese, who discovered that sulfur was effective as a fumigant, and then in the sixteenth century, they discovered that arsenic could be used as an insecticide.[1] Tobacco leaf (nicotine) and the seed of *Strychnos nux vomica* (strychnine) were used as rodenticides in the eighteenth century,[2] and the insecticidal active botanicals, including rotenone derived from the root of *Derris elliptica* and pyrethrum from the flowers of chrysanthemums, were used as insecticides in the mid-1800s. Bordeaux mixture (copper sulfate, lime, calcium hydroxide, and water) was introduced in France for mildew control in grapes in 1880.[1] Paris green (copper arsenite) and calcium arsenite were used extensively by the turn of the twentieth century to control the Colorado potato beetle.[2]

The era of modern agricultural production, which began after World War II, depended on (1) the introduction of highly mechanized farming practices; (2) the use of fertilizers, as production was diverted from a large munitions manufacturing capacity that had developed during the war; (3) the use of pesticides aimed at controlling pests; and (4) optimizing yield, especially in monoculture staple crops, such as corn, soybeans, rice, and wheat. The discovery of more efficacious (e.g., low use rate) and selective pesticides (e.g., tolerance to beneficial plants, insects, and animals) has largely been based on the use of screening methods, and more recently, combinatorial chemistry coupled to high-throughput screening techniques to discover new classes of biologically active ingredients. The drive to discover new pesticides comes from the following business imperatives: (1) cost-effectiveness, which confers competitive advantage; (2) societal pressure for improved safety; and (3)

the development of pest resistance (e.g., pests evolve over generations by the selection of polymorphic forms that have developed a tolerance to the pesticide). The incorporation of genes that confer pesticide tolerance to relatively inexpensive, and comparatively safe, nonselective herbicides in key crops is a recent development. More recent still is the insertion of genes into plants to produce an insecticidal protein, delta-endotoxin derived from *Bacillus thuringiensis*, which is thought to create a lytic pore 1–2 nm in diameter in the midgut of the insect.[3] On the immediate horizon is the use of genetic engineering, perhaps combined with chemical-induced changes in plant metabolism, to create functional food and fiber, which exhibit traits that have enhanced nutritional value, facilitate processing, or have other desirable attributes.

In this chapter, the hazard of pesticides is presented for agents that have either significant economic value or are representative of a group of active ingredients with a specific biochemical mode of action (MOA) in targeted species. The MOA, where known, and the hazard profile of organic pesticides, such as those described in the first paragraph or others that have subsequently been discovered, have been included for the sake of comparison with those of synthetic pesticides, because of increased interest in organically grown food and in natural pesticide usage.[4]

Pesticides are grouped according to their MOA in targeted species. This method of classifying fungicides,[5] herbicides,[6] and insecticides[7] was developed by agronomists to assist growers in preventing the development of resistance in targeted species (see Table 16.70). Grouping pesticides according to their MOA is more indicative of potential biological outcomes, and in some instances, this provides data that are directly relevant to toxicologists, because the MOA in the pest species may be more or less conserved in mammals. This is also relevant to pesticide registration, because in 1996, the U.S. Environmental Protection Agency (EPA) was directed by Congress to conduct cumulative risk assessments on chemicals that share a common *mechanism of action* in mammals. In the guidance developed by EPA,[8] they considered an initial grouping of chemicals based upon one of the following four criteria: structural similarity, the mechanism underlying the pesticide effect on target species, the general mechanism of mammalian toxicity, or a specific toxic effect in mammals.

This chapter organizes chemicals by their mechanism of action in the targeted species of fungi, insects, or plants. Chemical structures are provided, but structure–activity relationships are not discussed extensively. Hazard profiles for members of a pest-based *common mechanism class* have the potential to reveal whether there should be an animal-based *common mechanism group,* although such judgments are usually based upon MOA studies in animals. Even if it is established that a group of chemicals belong to a *common mechanism class*, this still leaves unaddressed the important question as to whether the MOA elaborated in the target species or in animal models are relevant to man, and if so, how doses should be scaled between species.

HAZARD CHARACTERIZATION OF PESTICIDES

Federal Insecticide, Fungicide, and Rodenticide Act

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) was passed by the U.S. Congress in 1947.[9] The legislation was administered by the U.S. Department of Agriculture (USDA) and remained primarily a labeling requirement for many years. FIFRA has been amended several times and its registration provisions strengthened.[10] Pesticide use in the United States is also regulated under the Federal Food, Drug, and Cosmetic Act (FFDCA). FFDCA was amended in 1954 (Section 408; Miller Amendment) to require the establishment of pesticide tolerances on food.[11] An additional amendment in 1958 (Section 409) created the requirement to establish tolerances for food additives present in processed foods.[11] Section 409 of FFDCA contains the Delaney Clause, which prohibited the use of carcinogens as food additives. The EPA applied this Section 409 of FFDCA to those circumstances when pesticide residues found in processed food were greater than those found in the raw agricultural commodity. Under such circumstances, food additive tolerances are also required, and such additives must not be carcinogenic.

Food Quality Protection Act of 1996

In 1996, the Food Quality Protection Act (FQPA) reauthorized FIFRA provisions, and this act requires tolerances to be reassessed as part of reregistrations.[12] FQPA amendments to the FFDCA[11] and FIFRA[9] directed the EPA to consider a number of factors in assessing risk as part of the tolerance-setting procedure.[12] FQPA provides for a single health-based standard and eliminated the problem posed by having different standards for pesticide concentrations in raw and processed foods. FQPA required that in the process of setting tolerances for pesticide residues in food, the EPA must evaluate the aggregate risk arising from exposure to pesticides from all routes of exposure, including oral, dermal, or inhalation exposure. Occupational exposure assessment, however, remained outside the jurisdiction of FQPA.

FQPA also directed the EPA to consider pesticides having a common mechanism of toxicity and to evaluate the cumulative effect of exposure to pesticides sharing a common mechanism. Finally, the agency was charged with developing techniques for evaluating the potential for pesticides to affect the endocrine system. Most of these provisions reflect concerns that children may be more susceptible to chemicals than adults, thereby taking into account key recommendations of a National Academy of Sciences report, *Pesticides in the Diets of Infants and Children*.[13] Under FQPA, the EPA assumes an extra 10-fold uncertainty factor to account for increased susceptibility of children, including effects of in utero exposure, unless there are data to suggest otherwise.

STUDY REQUIREMENTS

Toxicology testing guidelines have been promulgated by the EPA[14]; the Japanese Ministry of Agriculture, Forestry, and Fisheries[15]; the European Community[16,17]; and the Organization for Economic and Cooperative Development.[18] These guidelines have been harmonized among the various regulatory authorities[15–20] and are revised or enhanced[19] as new testing procedures are developed (Table 16.1).

Acute toxicity studies are conducted by administering a chemical by oral, dermal, or inhalation routes to estimate the dose that is expected to cause mortality in 50% (LD_{50}) of the test animals. Studies are also conducted to evaluate the irritation potential of chemicals when applied to skin and eyes. The potential of chemicals to cause allergic reactions when applied to skin (e.g., skin sensitization) is also determined. Acute oral and inhalation studies are usually conducted in rats, dermal and eye irritation studies are typically conducted in rabbits, and the sensitization study is carried out in guinea pigs. The results from these studies are used to establish the precautionary language (Table 16.2) used on product labels for crop protection chemicals.[21]

Oral toxicity studies are conducted in rats, mice, or dogs fed diets containing the pesticide for various durations of time (28 days, 90 days, or 1 year) or for the lifetime of the animal (24 months for rats and 18 months for mice). Animals are randomly assigned to either a control group or one of several treatment groups, which are comprised of 10–50 rats or mice, or alternately 4–6 dogs per group. Typically, there are at least four groups in each study, with one control group and three groups of animals that receive either a low, medium, or high concentration of the pesticide in their diets. The high-dose group is typically administered a maximally tolerated dose,[22] or if the pesticide is nontoxic, a maximum limited dose of 1000 mg/kg/day. Lower doses are established to have minimal-to-moderate effects, and one dose ideally should have no effect. Typically, clinical signs, effects on survival, body weight, feed consumption, blood chemistry, and hematological and urinary parameters are evaluated on multiple occasions during the in-life phase of the study. At study termination, individual organs are weighed, and gross and microscopic examinations are conducted on approximately 50 tissues/animal. The effects of the tested pesticide are described (hazard identification), and the lowest observed effect level and the no observable effect (NOEL), or alternately the no observable adverse effect, are determined.

Dermal toxicity is evaluated by applying the chemical to the skin for 6 h a day for 21 days in rats, or for 28 days in rabbits. Developmental toxicity studies are conducted to evaluate the potential of the pesticide to affect the development of offspring, including an evaluation of birth defects in both rats and rabbits. In addition to developmental toxicity studies, a reproduction study is conducted in rats. This study involves feeding diets containing the chemical to young adult male and female rats for approximately 3 months prior to mating. The females are allowed to produce a litter of offspring that are then reared to adulthood. The animals are fed diets containing the test chemical during this entire period of time. After reaching sexual maturity, a second generation of animals is allowed to mate and produce a second litter of offspring (the F_2 generation) that are in turn administered the pesticide until they reach adulthood, at approximately 90 days of age. A variety of toxicological and reproductive parameters are assessed to determine the effect of the pesticide on neonatal development and reproductive function in young male and female animals.

The mutagenic potential of a pesticide is typically assessed by evaluating its possible interaction with (1) genes (gene mutation tests), (2) chromosomes (clastogenic tests), and (3) directly with the DNA (classified as other tests).

The carcinogenic potential of a pesticide is evaluated in mice and rats that are typically fed the chemical in their diets for 18 or 24 months, respectively. The dietary concentration of the chemical administered in chronic studies is generally selected based on the results from a 90-day feeding study.[22] Approximately 50 tissues from each animal are examined for the presence of tumors or for other evidence of tissue damage. Pesticides are considered to be potential human carcinogens if (1) they significantly increase the incidence of any tumor above the incidence observed in concurrent or historical control animals; (2) they increase the incidence of rare or malignant tumors; and/or (3) they shorten the latency to tumor development.

To determine whether a chemical is likely to be carcinogenic in humans, regulatory agencies around the world[20,23,24] conduct a weight-of-the-evidence assessment using methods similar to those described in the EPA cancer classification scheme.[20,24] In a weight-of-the-evidence assessment, the carcinogenic potential of a chemical is evaluated by considering the results from animal studies including (but not limited to) the details of the tumor responses seen in animal bioassays (dose–response functions, including evaluations of the evidence for nonlinearity, structure–activity considerations, and details on MOA, including descriptions of key events, temporal/dose congruity, biological plausibility, and alternative MOA, which include but are not limited to assessments of genotoxic potential). Results from epidemiological investigations are weighted heavily, if such data are available. The resulting assessment includes the classification of the pesticide into one of several categories shown in Table 16.3 for the EPA and International Agency for Research on Cancer (IARC).

In addition to assessments of mutagenicity, developmental toxicity, reproductive toxicity, and oncogenicity, it is possible to determine immunotoxicity, neurotoxicity, and other effects from associated EPA Series 870 Guideline studies.

However, the existing data related to these other studies is not consistently available or relevant, so these results will not be covered here.

FUNGICIDES

The Fungicide Resistance Action Committee (FRAC) has classified fungicides according to their mechanisms of action in fungi.[5] In Table 16.4, pesticides are grouped according to their assigned FRAC codes. Not all FRAC codes have been included in this chapter, and some commercially important members of

TABLE 16.1
Series 870 Health Effects Test Guidelines

OCSPP No.	Guideline Names	Other Existing Reference Numbers		
		OPPT	OPP	OECD
Group A—Acute toxicity test guidelines				
870.1000	Acute toxicity testing–background	None	None	None
870.1100	Acute oral toxicity	798.1175	81-1	401, 420, 423, 425
870.1200	Acute dermal toxicity	798.1100	81-2	402
870.1300	Acute inhalation toxicity	798.1150	81-3	403
870.2400	Acute eye irritation	798.4500	81-4	405
870.2500	Acute dermal irritation	798.4470	81-5	404
870.2600	Skin sensitization	798.4100	81-6	406
Group B—Subchronic toxicity test guidelines				
870.3050	Repeated-dose 28-day oral toxicity study in rodents	None	None	407
870.3100	90-day oral toxicity in rodents	798.2650	82-1	408
870.3150	90-day oral toxicity in nonrodents	None	82-1	409
870.3200	21/28-day dermal toxicity	None	82-2	410
870.3250	90-day dermal toxicity	798.2250	82-3	411
870.3465	90-day inhalation toxicity	798.2450	82-4	413
870.3550	Reproduction/development toxicity screening test	None	None	421
870.3650	Combined repeated-dose toxicity with the reproduction/development toxicity screening test	None	None	422
870.3700	Prenatal developmental toxicity study	798.4900	83-3	414
870.3800	Reproduction and fertility effects	798.4700	83-4	416
Group C—Chronic toxicity test guidelines				
870.4100	Chronic toxicity	798.3260	83-1	452
870.4200	Carcinogenicity	798.3300	83-2	451
870.4300	Combined chronic toxicity/carcinogenicity	798.3320	83-5	453
Group D—Genetic toxicity test guidelines				
870.5100	Bacterial reverse mutation test	798.5100, 798.5265	84-2	471
870.5140	Gene mutation in *Aspergillus nidulans*	798.5140	84-2	None
870.5195	Mouse biochemical-specific locus test	798.5195	84-2	None
870.5200	Mouse visible-specific locus test	798.5200	84-2	None
870.5250	Gene mutation in *Neurospora crassa*	798.5250	84-2	None
870.5275	Sex-linked recessive lethal test in *Drosophila melanogaster*	798.5275	84-2	477
870.5300	In vitro mammalian cell gene mutation test	798.5300	84-2	476
870.5375	In vitro mammalian chromosomal aberration test	798.5375	84-2	473
870.5380	Mammalian spermatogonial chromosomal aberration test	798.5380	84-2	483
870.5385	Mammalian bone marrow chromosomal aberration test	798.5385	84-2	475
870.5395	Mammalian erythrocyte micronucleus test	798.5395	84-2	474
870.5450	Rodent dominant lethal assay	798.5450	84-2	478
870.5460	Rodent heritable translocation assays	798.5460	84-2	485
870.5500	Bacterial DNA damage or repair tests	798.5500	84-2	None
870.5550	Unscheduled DNA synthesis in mammalian cells in culture	798.5550	84-2	482
870.5575	Mitotic gene conversion in *Saccharomyces cerevisiae*	798.5575	84-2	481
870.5900	In vitro sister chromatid exchange assay	798.5900	84-2	479
870.5915	In vitro sister chromatid exchange assay	798.5195	84-2	None
Group E—Neurotoxicity test guidelines				
870.6100	Acute and 28-day delayed neurotoxicity of organophosphorus substances	798.6450, 798.6540, 798.6560	81-7, 82-5, 82-6	418, 419
870.6200	Neurotoxicity screening battery	798.6050, 798.6200, 798.6400	81-8, 82-7, 83-1	424
870.6300	Developmental neurotoxicity study	None	83-6	None
870.6500	Schedule-controlled operant behavior	798.6500	85-5	None
870.6850	Peripheral nerve function	798.6850	85-6	None
870.6855	Neurophysiology: sensory-evoked potentials	798.6855	None	None

(*continued*)

TABLE 16.1 (continued)
Series 870 Health Effects Test Guidelines

OCSPP No.	Guideline Names	Other Existing Reference Numbers		
		OPPT	OPP	OECD
Group F—Special studies test guidelines				
870.7200	Companion animal safety	None	None	None
870.7485	Metabolism and pharmacokinetics	798.7485	85-1	417
870.7600	Dermal penetration	None	85-3	None
870.7800	Immunotoxicity	None	85-7	None
Group G—Health effects chemical-specific test guidelines				
870.8355	Combined chronic toxicity/carcinogenicity testing of respirable fibrous particles	798.3320	None	None

Source: U.S. EPA, Pesticide assessment guidelines: OPPTS harmonized 870 health effects test guidelines/series, U.S. Environmental Protection Agency, Washington, DC, 2012.

TABLE 16.2
USEPA Acute Toxicology Classification Scheme

Toxicology Category	Signal Word	Oral LD_{50} (mg/kg)	Dermal LD_{50} (mg/kg)	Inhalation LC_{50} (mg/L)	Eye Irritation	Skin Irritation
I	Danger[a]	Up to 50	Up to 200	Up to 0.2	Corrosive; corneal opacity not reversed in 7 days	Corrosive
II	Warning	From 50 to 500	From 200 to 2000	From 0.2 to 2.0	Corneal opacity reversed in 7 days; irritation persisting 7 days	Severe irritation at 72 h
III	Caution	From 500 to 5000	From 2000 to 5000	From 2.0 to 20	No corneal opacity; irritation reversed within 7 days	Moderate irritation at 72 h
IV	Caution	Greater than 5000	Greater than 5000	Greater than 20	No irritation	Mild or slight irritation at 72 h

Source: Stevens, J.T. et al. Agricultural chemicals: The impact of regulations under FIFRA on science and economics, in Primer on Regulatory Toxicology, Chenzelis C, Holson J, Gad S, eds., Raven Press, New York, 1995, pp. 133–163.

[a] The word *Poison* is used on the label if the *Danger* category is based on oral, dermal, or inhalation toxicity.

the selected FRAC codes may have been omitted. Table 16.4 provides a summary of the mechanisms of action of pesticides in fungi for the selected FRAC code. It also provides conclusions as to (1) whether the identified mechanism of action in fungi is likely conserved in humans and (2) whether there are any other bases for establishing a *common mechanism of toxicity group*, as set forth by FQPA and elaborated by EPA.[8]

Acylalanines and Oxazolidinones (FRAC Code A1)

The acylanine fungicides are represented here by metalaxyl and by mefenoxam, which is the active R-enantiomer of metalaxyl. These chemicals inhibit RNA synthesis in fungi by interfering with RNA polymerase I.[5] The spectrum of activity of mefenoxam is restricted to the control of downy mildew and late blight in a broad spectrum of crops.

There are data suggesting that structural features of ribosome gene promoters are conserved from plants to humans, but specific base sequences are not.[25] Therefore, it is unlikely that a pesticide selected to interfere with RNA polymerase I in fungi would have the same effect in mammalian cells. Furthermore, since RNA polymerase I is central to cell replication and growth,[26] it is unlikely that an effect on RNA polymerase I in animal bioassays would go unnoticed unless the chemicals were poorly absorbed, or rapidly metabolized and eliminated. The hazard profile for metalaxyl is unremarkable, and there is no indication of any effect on the cell cycle, although specific studies have not been conducted (Table 16.5).

Benzimidazoles and Thiophanates (FRAC Code B1)

The benzimidazole (thiabendazole) and thiophanate (thiophanate-methyl) fungicides have broad-spectrum activity, as indicated by a MOA involving the inhibition of mitosis by preventing polymerization of β-tubulin, one of the constituent building blocks of microtubules.[5] Microtubules play a critical role in both the plant and animal kingdoms during mitosis[27,28] and provide the basis for a cytoarchitecture that permits intracellular transport of molecules using the motor proteins dynein and kinesin.[27–29] Recently, it has

TABLE 16.3
Schemes for the Classification of Carcinogens

U.S. Environmental Protection Agency Original Classification Scheme[24]				International Agency for Research on Cancer 2012 Guidelines[264]	
		Risk Character			
Carcinogen Category	Criteria for Classification	RfD	Q_1*	Descriptive Characterization	
A—Human	Sufficient evidence in humans		X	Carcinogenic to humans	Group 1: The agent is carcinogenic to humans
B—Probable human					
B1	Limited evidence in humans Sufficient evidence in animals (two species with tumors)		X	Likely to be carcinogenic to humans	Group 2A: The agent is probably carcinogenic to humans
B2	Inadequate human evidence Sufficient animal evidence		X		
C—Possible human	No evidence in humans Limited evidence in animals	X	X	Suggestive evidence of carcinogenic potential	Group 2B: The agent is possibly carcinogenic to humans
D—Not classifiable	Inadequate animal or human evidence	X		Inadequate information to assess carcinogenic potential	Group 3: The agent is not classifiable as to its carcinogenicity to humans
E—Not a human carcinogen	Sufficient animal testing with no evidence of carcinogenicity and human experience	X		Not likely to be carcinogenic to humans	Group 4: The agent is probably not carcinogenic to humans

been suggested that microtubules may provide the network for laying down cellulose structural support in plants.[30] While the α- and β-tubulin subunits that make up microtubules are conserved between kingdoms, microtubules in plants are fundamentally different from those found in fungi and animals, because plant microtubules do not arise from discrete organizing centers, such as the centrosome or the spindle pole body, as they do in both fungi and animals.[31] During mitosis in fungal and animal cells, γ-tubulin serves as a microtubule nucleating factor, playing a role in assembling the α- and β-tubulin dimers that are embedded in, and grow out of, a pair of centrioles and their associated proteins. Recent data suggest that γ-tubulin may also play a role in microtubule nucleation in plant cells.[32]

Against this background of new and developing information, and considering that a number of isoforms of β-tubulin have been described in mammalian cells,[33] it is not known, but it is certainly possible, that the benzimidazole and thiophanate fungicides could interfere with microtubule formation in animal cells.

The structures, uses, and hazard profiles of the benzimidazole and thiophanate fungicides are presented in Table 16.6. The hazard characteristics of thiabendazole and thiophanate-methyl have been reviewed by the EPA and by members of the Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR).[34–36]

Pyridinylmethyl-Benzamides (FRAC Code B5)

The pyridinylmethyl-benzamides, represented here by fluopicolide, act by delocalizing spectrin-like proteins that are believed to support membrane stability in *Ascomycetes* and *Oomycetes* fungus species.[5,37,38] Fluopicolide is applied to various crops in combination with other fungicides as a mesosystemic treatment, where it affects zoospore release, cyst germination, sporulation, and plant tissue mycelial growth.[37,39] 2,6-Dichlorobenzamide (BAM) is a common metabolite of both fluopicolide and dichlobenil that the EPA has classified as a possible human carcinogen (Group C), but the expected BAM residues are lower than those found after dichlobenil application.[40]

The structure, uses, and hazard profiles of fluopicolide are presented in Table 16.7. The hazard characteristics have been reviewed by both the EPA and the JMPR, and both claim to have sufficient data for safety assessment.[38–40]

Phenyl-Benzamides, Pyridinyl-Ethyl-Benzamides, and Pyrazole-Carboxamides (Carboxamides; FRAC Code C2)

There are three groups of carboxamide fungicides represented here: phenyl-benzamides, pyridinyl-ethyl-benzamides, and pyrazole-carboxamides. Carboxamides inhibit mitochondrial respiration by blocking electron transport at the succinate dehydrogenase stage (complex II) in the Krebs cycle (see Figures 16.1 and 16.2). A recent characterization of the crystalline structure of succinate dehydrogenase in *Escherichia coli*[41,42] has permitted structural modeling of the active site(s) of the enzyme.[43,44] Future work may permit a determination of where carboxamides bind to succinate dehydrogenase in fungi, and it may address the question of whether or not homologous binding sites are present in human mitochondria. If it is assumed that carboxamides bind to human mitochondrial targets, one would predict that they would be highly toxic. Flutolanil, for example, is remarkably nontoxic, which belies any effect as an inhibitor of mitochondrial respiration in mammals.

TABLE 16.4
Fungicides Listed according to FRAC Classification System

Chemical Group	Common Name	FRAC Code	FRAC MOA	MOA Conserved in Mammals?	Other Common MOA in Mammals
Acylalanines	Mefenoxam (Table 16.5)	A1	RNA polymerase I	Unlikely	None known
Benzimidazoles Thiophanates	Thiabendazole Thiophanate-methyl (Table 16.6)	B1	ß-Tubulin assembly in mitosis	Likely; in cancer chemotherapy, inhibition of mitosis at metaphase through interaction with tubulin	Induction of apoptosis in cancer cells
Pyridinylmethyl-benzamides	Fluopicolide (Table 16.7)	B5	Delocalization of spectrin-like proteins	Likely; spectrin-based skeleton, identified in erythrocytes and in many cellular processes	None known
Phenyl-benzamides Pyridinyl-ethyl-benzamides Pyrazole-carboxamides	Flutolanil Fluopyram Penflufen Penthiopyrad Sedaxane (Table 16.8)	C2	Complex II: succinate dehydrogenase	Likely: SDG has vital role in ATP production in the mitochondrial respiratory chain[265]	Glutathione activity in liver mitochondria
Methoxy-acrylates Oximinoacetates Dihydro-dioxazines Imidazolinones	Azoxystrobin Trifloxystrobin Fluoxastrobin Fenamidone (Table 16.9)	C3	Complex III: cytochrome bc1 (ubiquinol oxidase) at Q_o site (*cyt b gene*)	Potentially: superoxide generation site of complex III in cardiac myocytes may increase in TCA cycle activity[266]	None known
Cyano-imidazoles Sulfamoyl-triazoles	Cyazofamid Amisulbrom (Table 16.10)	C4	Complex III: cytochrome bc1 (ubiquinone reductase) at Q_i site	Likely[267]	None known
Organotins	Triphenyltin acetate/hydroxide (Table 16.11)	C6	Inhibitors of oxidative phosphorylation, ATP synthase	Likely but the active ingredient does not appear to reach target site	None known
Triazolopyrimidines	Ametoctradin (Table 16.12)	C8	Complex III: cytochrome bc1 (ubiquinone reductase) at Q x (unknown) site	Potentially[268]	None known
Anilinopyrimidines	Cyprodinil Pyrimethanil (Table 16.13)	D1	Methionine biosynthesis (proposed) (*cgs gene*)	Potentially: methionine sulfoxide reductase A and methionine metabolism are associated with oxidative stress[269]	None known
Quinolines	Quinoxyfen (Table 16.14)	E1	Signal transduction (mechanism unknown)	Possible[270]	None known
Phenylpyrroles	Fludioxonil (Table 16.15)	E2	MAP/histidine kinase in osmotic signal transduction (*os-2, HOG1*)	Possible as mammalian analogs of HOG1 exist	None known
Dicarboximides	Iprodione Vinclozolin (Table 16.16)	E3	MAP/histidine kinase in osmotic signal transduction (*os-1, Daf1*)	Likely as NAZDH cytochrome c reductase in lipid peroxidation (formerly F1)	Share a common metabolite 3,5-dichloro-aniline[85]; potential antiandrogen
Triazoles	Cyproconazole Difenoconazole Fenbuconazole Flutriafol Metconazole Myclobutanil Propiconazole	G1	C14-demethylase in sterol biosynthesis (*erg11/cyp51*)	Likely[271]	Aromatase (CYP19) inhibition[272]

TABLE 16.4 (continued)
Fungicides Listed according to FRAC Classification System

Chemical Group	Common Name	FRAC Code	FRAC MOA	MOA Conserved in Mammals?	Other Common MOA in Mammals
	Prothioconazole Tebuconazole Triadimefon Tetraconazole Triadimenol Imazalil				
Imidazoles	Prochloraz (Tables 16.17 and 16.18)				
Hydroxyanilides	Fenhexamid (Table 16.19)	G3	3-keto-reductase, C4-demethylation (*erg27*)	Unlikely, plants only[273]	None known
Peptidyl pyrimidine nucleoside	Polyoxin (Table 16.20)	H4	Chitin synthase	Unlikely, absent in plants and animals[274]	None known
Mandelic acid amides	Mandipropamid (Table 16.21)	H5	Cellulose synthase	Unlikely, plants only	None known
Phenylacetamides	Cyflufenamid (Table 16.22)	U6	Unknown	Unknown	Unknown
Benzophenones	Metrafenone (Table 16.23)	U8	Actin disruption (proposed)	Potentially[275]	None known
Guanidines	Dodine (Table 16.24)	U12	Cell membrane disruption (proposed)		
Dithiocarbamates	Ferbam, thiram, and ziram (Table 16.25)	M3	Interferes with oxygen uptake and inhibits sulfur-containing enzymes	Possible[276]	(Cholinesterase inhibition (ziram and metam sodium): neurotoxic (distal peripheral neuropathy); potential to chelate cations; mancozeb, maneb, and metiram share a common metabolite, ETU[98]
Ethylenebisdithiocarbamates	Mancozeb, maneb, and zineb (Table 16.26)		Breaks down cyanide that then reacts with sulfhydryl groups in cells		
Phthalimides	Captan (Table 16.27)	M4	Preferentially reacts with protein sulfhydryl groups; enzyme inhibitor	Likely	None known
Chloronitriles	Chlorothalonil (Table 16.28)	M5	Conjugates with cellular thiols	Likely	None known

Source: FRAC, FRAC code list 2012: Fungicides sorted by mode of action, 2012. Available from: http://www.frac.info/frac/index.htm.

Flutolanil represents the phenyl-benzamides. It is a systemic fungicide with protective and curative action. Flutolanil is used to control sheath blight, white mold, and snow blight in rice, cereals, sugar beet, and other crops.[37] The structure, uses, and hazard profile of flutolanil are given in Table 16.8.

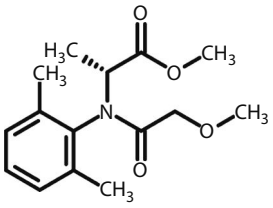
The pyridinyl-ethyl-benzamides include fluopyram, which is used to combat gray mold, powdery mildew, and other fungal diseases in grapes, various fruits, and vegetables.[37] The structure, uses, and hazard profile of fluopyram are also provided in Table 16.8.

Penflufen, penthiopyrad, and sedaxane represent the pyrazole-carboxamide fungicide category. These are effective on many different types of fungal pathogens, including *Rhizoctonia*, rusts, gray mold, powdery mildew, and apple scab.[37] Structures, uses, and hazard profiles for these three fungicides appear in Table 16.8.

Methoxy-Acrylates, Dihydro-Dioxazines, and Imidazolinones (Strobilurins; FRAC Code C3)

The first member of the strobilurin family (Strobilurin A) was isolated independently from *Oudemansiella mucida* found growing on beech trees[45,46] and from the pine cone fungus, *Strobilurus tenacellus*.[47] Strobilurins are also known as quinone outside inhibitors, or QoI-fungicides.[5]

TABLE 16.5
Structure, Uses, and Hazard Profile for the Acylalanine (Metalaxyl, Mefenoxam) Fungicides (FRAC Code A1)

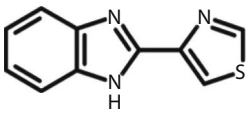
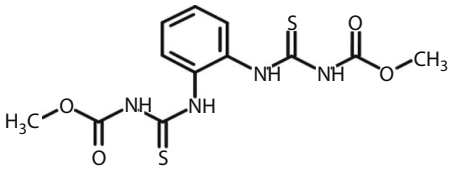
Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Used on alfalfa, apples, asparagus, avocadoes, berries, citrus, cole crops, cotton, cucurbits, hops, peanuts, stone fruit, soybeans, sugar beets, tobacco, and vegetables; also used as a seed treatment	100–1000 8.25–300 g/100 kg (seed)

Irritation		LD_{50} (mg/kg)				
Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Severe irritant	Slight irritant	490	>2000	>2.3	Negative	Warning

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	13	Mutagenicity	Not mutagenic
Dog/52 weeks	8	Developmental	Not teratogenic
Mouse/18 months	38	Reproductive	No evidence
RfD (based on the 6-month dog study)	0.08	Oncogenicity	No evidence

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, 2011; U.S. EPA, Metalaxyl: Reregistration Eligibility Decision (RED), EPA-738-R-94-017, U.S. Environmental Protection Agency, Washington, DC, 1994; U.S. EPA, *Fed. Regist.*, 60(244): 65579; U.S. EPA, 62(149), 42019, *Fed. Regist*. 1997; Royal Society of Chemistry. Chemical structure. Cambridge, U.K.: ChemSpider, 2012 [September 12, 2012]. Available from: http://www.chemspider.com

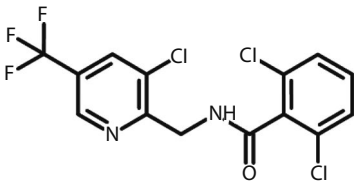
TABLE 16.6
Structures, Uses, and Hazard Profiles for the Benzimidazole (Thiabenazole) and Thiophanate Fungicides (FRAC Code B1)

Fungicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Thiabenazole (Mertech®)[37,280]		Used for the control of *Aspergillus, Botrytis*, and others in vegetables, bananas, cereals, cabbage, stone fruit, citrus fruit, and hops	0.2–2.2 g/L
Thiophanate-methyl (Topsin-M®)[37,280]		Used for eyespot on cereals; scab and rot on apples and pears; and powdery mildew on pome fruit, stone fruit, vegetables, strawberries, and vines	30–50

	Irritation		LD_{50} (mg/kg)				
Fungicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Thiabendazole	Nonirritant	Nonirritant	3100	>2000	0.07	Not sensitizer	Warning
Thiophanate-methyl	Mild irritant	Mild irritant	>5000	>10,000	1.7	Not sensitizer	Caution

Fungicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Thiabendazole[2,281]	Rat/2-year oral	40	Mutagenicity	Not mutagenic
	Dog/52-week oral	10.0	Developmental	Not teratogenic
	Mouse/18-month oral	8.3	Reproductive	Not a reproductive toxin
	ADI (human study)	0.035	Oncogenicity	Not oncogenic
Thiophanate-methyl[282,283]	Rat/2-year oral	8.0	Mutagenicity	Not mutagenic
	Dog/52-week oral	50	Developmental	Not teratogenic
	Mouse/18-month oral	23	Reproductive	Not a reproductive toxin
	RfD (based on the 2-year rat study)	0.08	Oncogenicity	Not oncogenic

TABLE 16.7
Structure, Uses, and Hazard Profile for the Pyridinylmethyl-Benzamide Fungicide Fluopicolide (Volare®) (FRAC Code B5)

Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Used to control downy mildew, late blight, and *Pythium* species in conjunction with other fungicides	—

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Nonirritant (rabbits)	Nonirritant (rabbits)	>5000	>5000	>5.16	—	Caution

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	20	Mutagenicity	Not mutagenic
Dog/52 weeks	4.5	Developmental	Only at high doses
Mouse/18 months	7.9	Reproductive	Not a reproductive toxin
cRfD	0.2	Oncogenicity	Not oncogenic, unclassified

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; U.S. EPA, Fluopicolide: Pesticide fact sheet, U.S. Environmental Protection Agency, Washington, DC, 2007; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.

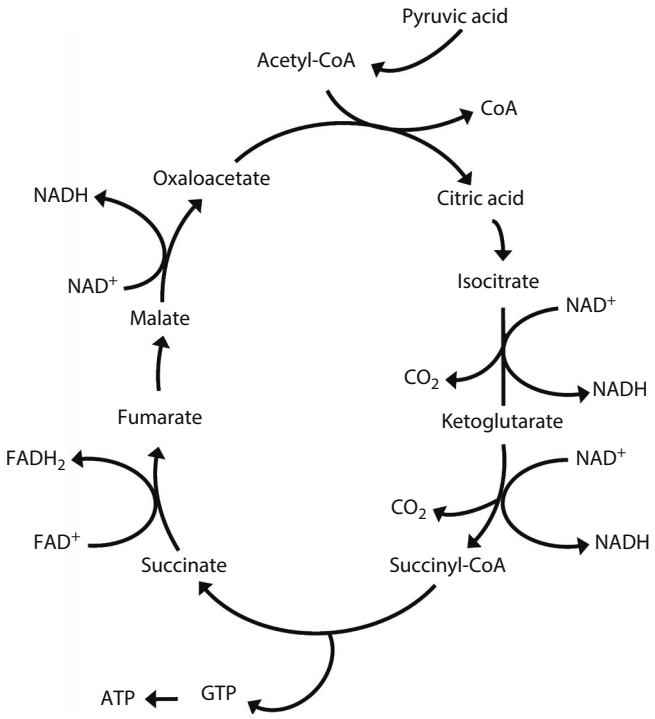


FIGURE 16.1 Krebs cycle.

The strobilurins are selective, yet they have broad fungicidal activity that includes protective, eradicant, and antisporant effects. The strobilurins inhibit mitochondrial respiration by binding to the Qo site on cytochrome b (Figure 16.2), thereby blocking electron transfer between cytochrome b and cytochrome c1 in the Krebs cycle (Figure 16.1), resulting in a disruption of ATP synthesis.[48] The crystalline structure of cytochrome bc1 complex has been elucidated alone[49] and bound to its substrate cytochrome c,[50] and details on the binding to the dimeric structure have been proposed.[51]

The Qo binding site for stigmatellin, an analogue of the strobilurins, has been identified by crystallization of cytochrome bc1 in the presence of an excess of the inhibitor.[49] The amino acid sequence of the Qo binding site appears to be highly conserved across species, including mammals, and tests carried out on 14 strobilurins on mitochondrial enzyme preparations from fungi, housefly, rats, and corn showed no selectivity.[48] The structures, uses, and hazard profiles for two economically important members of the methoxy-acrylates, as well as for the dihydro-dioxazine fluoxastrobin and the imidazolinone fenamidone, are presented in Table 16.9. The fact that the strobilurins are relatively nontoxic to animals suggests that they do not reach these mitochondrial targets.

Cyano-Imidazoles and Sulfamoyl-Triazoles (FRAC Code C4)

The quinone inside inhibitor fungicides (QiI-fungicides) include the cyano-imidazole fungicide cyazofamid and the sulfamoyl-triazole fungicide amisulbrom.[5] Like the strobilurins, cyazofamid and amisulbrom inhibit at complex III, or ubiquinol-cytochrome c reductase, in the mitochondrial respiratory chain.[37] But apparently because of differences in target enzyme susceptibility, both cyazofamid

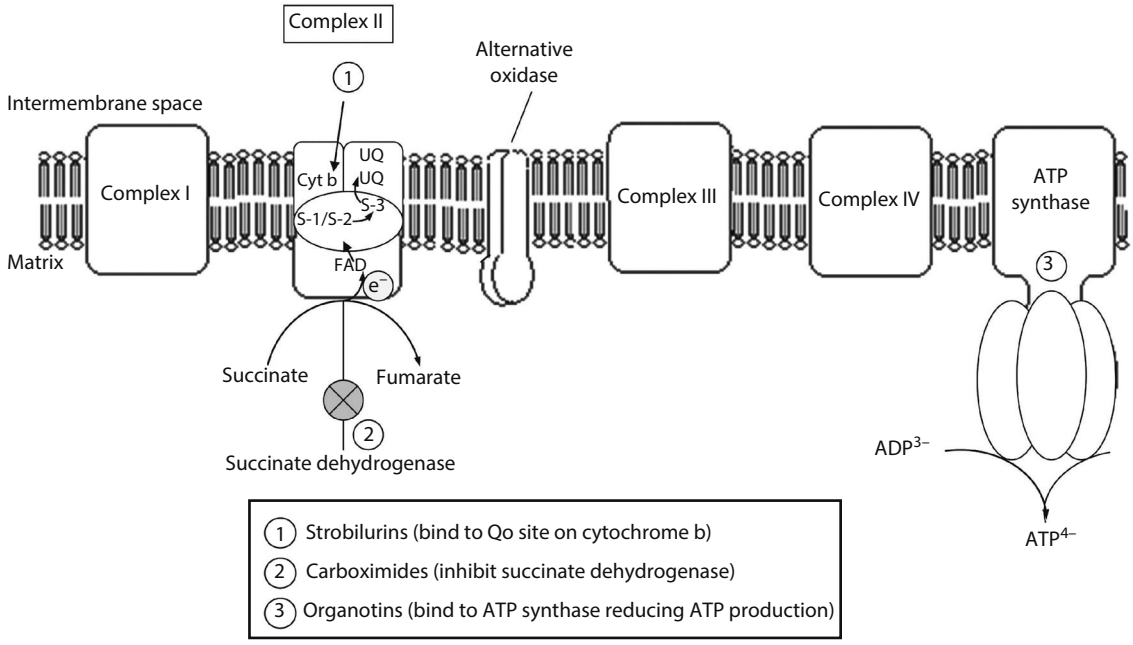


FIGURE 16.2 Mitochondrial respiration.

and amisulbrom inhibit Qi (ubiquinone reducing site) of cytochrome bc1 instead of the Qo (ubiquinol oxidizing site) affected by the strobilurins.[37] Cyazofamid and amisulbrom are both used in both foliar and soil applications to treat *Phytophthora infestans*, downy mildews, and other pathogens.[37,52,53] Amisulbrom shows some evidence suggestive of carcinogenic potential, but no additional studies are currently required by the EPA for its intended uses.[53] Structures, uses, and hazard profiles for these two fungicides appear in Table 16.10.

Organotins (FRAC Code C6)

The organometallic fungicides, which are represented here by triphenyltin, are limited in their spectrum for disease control, but are effective as protective, curative, and antisporulants in the treatment of early and late blight, scab, leaf blotch, and powdery mildew.[37] Trialkyltins affect mitochondrial respiration by (1) disrupting the membrane potential by exchanging halide ions for hydroxyl ions across the membrane; (2) binding to ATP synthase thereby reducing ATP production; and (3) causing mitochondrial swelling, especially for the more lipophilic compounds. They are also capable of causing cellular lysis in vitro[54] and in aquatic organisms.[55] Of these effects, the effect on ATP synthase is the most specific (see Figure 16.2).

ATP synthases are enzymes that make up two rotary motors. The membrane-embedded F_0 motor converts energy from a transmembrane electrochemical (Na^+) gradient into torque, which is transmitted through a common shaft to the water-exposed F_1 motor where it drives the synthesis of ATP from ADP and phosphate.[56,57] More is known about the operation of the F_1 motor than about the membrane-bound F_0 motor, the presumptive site of action of the organotin compounds.[58–60] However, interference with the production of ATP by organotin compounds would have significant toxicological consequences if those compounds reach and bind to ATP synthase.

The structure and mammalian toxicity profile of triphenyltin (Fentin) is presented in Table 16.11. Triphenyltin hydroxide is moderately acutely toxic (oral LD_{50} = 140 mg/kg/day), but is highly toxic following longer-term administration (ADI = 0.0005 mg/kg/day). It has been classified by the EPA as B2, a probable human carcinogen, based on mouse liver, tumors, as well as rat pituitary and testicular tumors. It has not been possible to predict toxicity across species based upon structure alone, because some substituted organotin compounds are more toxic to some species than to others (Table 16.11).[54,55]

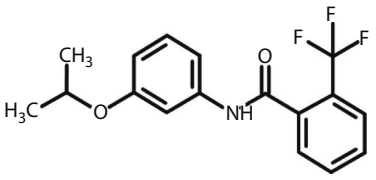
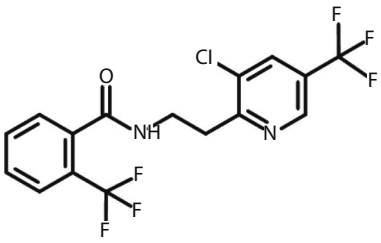
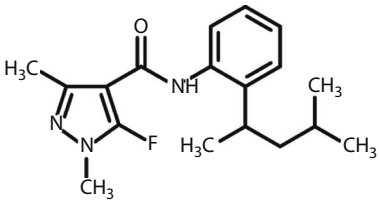
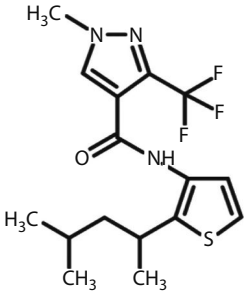
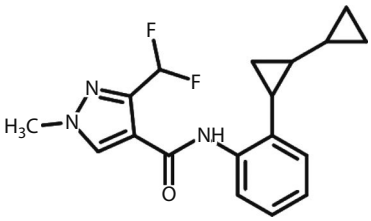
Triazolopyrimidines (FRAC Code C8)

The triazolopyrimidines include the fungicide ametoctradin, which inhibits mitochondrial respiration via complex III inhibition.[5,37] Ametoctradin shows no signs of mammalian toxicity at tested doses and is unlikely to be carcinogenic or mutagenic.[61] The structure, uses, and hazard profile for ametoctradin appear in Table 16.12.

Anilinopyrimidines (FRAC Code D1)

The spectrum of activity for anilinopyrimidines, which are represented here by cyprodinil and pyrimethanil, is limited to ascomycetes and deuteromycetes.[37] The anilinopyrimidines are used to control gray mold on vines, fruit, vegetables, and

TABLE 16.8
Structures, Uses, and Hazard Profiles for the Carboxamide (Flutolanil) Fungicides (FRAC Code C1)

Fungicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Flutolanil (Folistar®, Moncut®)[37,280,284]		Used on peanuts, rice, cereals, sugar beets, fruits, and vegetables to control *Rhizoctonia* spp.	300–1000 (peanuts) 2500–10,000 (soil) 1500–3000 g/kg (seed)
Fluopyram (Luna™)[37,280]		Controls gray mold, powdery mildew, and various fungal diseases in vines, grapes, pome fruits, stone fruits, vegetables, and field crops	—
Penflufen (Evergol™)[37,280]		Unknown	—
Penthiopyrad (Fontelis™)[37,280]		Controls rusts, *Rhizoctonia*, gray mold, powdery mildew, and apple scab	—
Sedaxane (Vibrance™)[37,280]		—	—

	Irritation		LD_{50} (mg/kg)				
Fungicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Flutolanil	Slight irritant	Nonirritant	>10,000	>5000	>6.0	Negative	Caution
Fluopyram	Minimal irritant	Nonirritant	>2000	>2000	>5.1	Negative	Caution
Penflufen	Minimal irritant	Nonirritant	>5000	>2000	>2.02	Negative	Caution
Penthiopyrad	Slight irritant	Nonirritant	>2000	>2000	>5.6	Negative	Caution
Sedaxane	Mild irritant	Nonirritant	5000	>5000	>5.64	Negative	Caution

(*continued*)

TABLE 16.8 (continued)
Structures, Uses, and Hazard Profiles for the Carboxamide (Flutolanil) Fungicides (FRAC Code C1)

Fungicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Flutolanil[284]	Rat/2-year oral	87	Mutagenicity	Not mutagenic
	Dog/1-year oral	50	Developmental	Not teratogenic
	Mouse/18-month oral	735	Reproductive	Not a reproductive toxin
	RfD (NOEL = 64 in rat reproduction study; UF = 300)	0.2	Oncogenicity	E (Not oncogenic)
Fluopyram[285]	Rat/2-year oral	8.6	Mutagenicity	Not mutagenic
	Dog/1-year oral	13.2	Developmental	Not teratogenic
	Mouse/18-month oral	4.2	Reproductive	Not a reproductive toxin
	RfD (ADI)	0.01	Oncogenicity	Unlikely (rat liver and mouse thyroid tumors)
Penflufen[286,287]	Rat/2-year oral	79.0	Mutagenicity	Not mutagenic
	Dog/1-year oral	32.0	Developmental	Not teratogenic
	Mouse/18-month oral	880	Reproductive	Not a reproductive toxin
	cRfD	0.38	Oncogenicity	Suggestive (rat sarcomas and ovarian tumors)
Penthiopyrad[288]	Rat/2-year oral	27.0	Mutagenicity	Not mutagenic
	Dog/1-year oral	54.4	Developmental	Not teratogenic
	Mouse/18-month oral	60.0	Reproductive	Not a reproductive toxin
	cRfD	0.10	Oncogenicity	Unlikely (thyroid tumors in male rat)
Sedaxane[289]	Rat/2-year oral	11.0	Mutagenicity	Not mutagenic
	Dog/1-year oral	56.0	Developmental	Not teratogenic
	Mouse/18-month oral	157	Reproductive	Not a reproductive toxin
	cRfD	0.11	Oncogenicity	Likely (liver, thyroid, and uterine tumors)

ornamentals, as well as leaf scab on pome fruit. The anilinopyrimidine fungicides inhibit methionine biosynthesis,[62] but probably not through an action of cystathionine β-lyase as originally proposed.[63–65] Cystathionine γ-synthase and methionine synthase are other possible targets of the anilinopyrimidine fungicides, but the actual target in plants is not likely relevant to humans. Methionine is one of four *essential* sulfur-containing amino acids in the aspartate pathway, which also include lysine, threonine, and isoleucine, that humans and other monogastric animals cannot synthesize *de novo*.[66,67]

The structures, uses, and hazard profiles of the anilinopyrimidine fungicides cyprodinil and pyrimethanil are presented in Table 16.13. The hazard profiles for these chemicals suggest that there are minimal risks to humans.

Quinolines (FRAC Code E1)

Quinoline fungicides, which act as growth signal disruptors, include the economically important quinoxyfen.[5,37] These are used to control powdery mildew in grapes, hops, and vegetables.[37] There is some evidence of slight dermal sensitization in humans.[68] Quinoxyfen's structure, uses, and hazard profile are illustrated in Table 16.14.

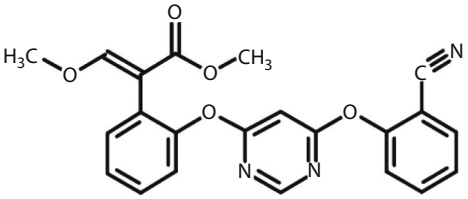
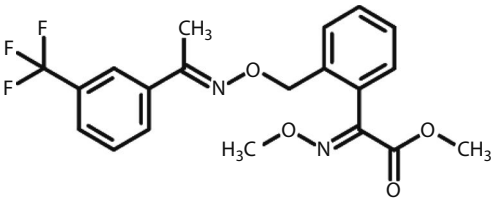
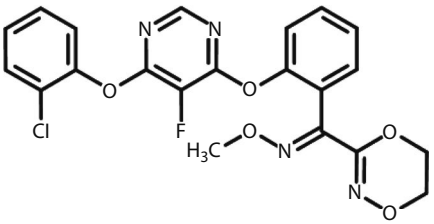
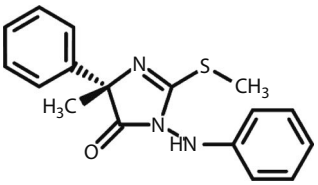
Phenylpyrroles (FRAC Code E2)

It has been proposed that the phenylpyrrole fungicides, represented here by fludioxonil, interfere with the two-component MAP kinase–mediated osmotic signal transduction pathway shown in Figure 16.3.[69] *Neurospora crassa*, incubated with fludioxonil, responds by accumulating glycerol, which causes the fungus to rupture.[70,71] Os-2 mutants do not accumulate glycerol when treated with phenylpyrroles,[69,72] indicating that if the activation of the osmotic signal transduction pathway is blocked, then the fungicidal action of the phenylpyrroles is prevented.

Although the exact molecular target of the phenylpyrrole fungicides within the osmotic signal transduction pathway has not been convincingly demonstrated,[72,73] there is evidence that at least one of the two-component pathway (Figure 16.3) is conserved in vertebrate cells.[50,74,75] Therefore, it could be conservatively assumed that the phenylpyrroles may affect this signaling pathway in mammals.[76,77]

The structure, uses, and hazard profile of fludioxonil are presented in Table 16.15. Fludioxonil is not acutely toxic, and it does not exhibit a remarkable repeat-dose toxicity profile. Fludioxonil has been classified as a category D carcinogen (nonclassifiable in regard to carcinogenicity).

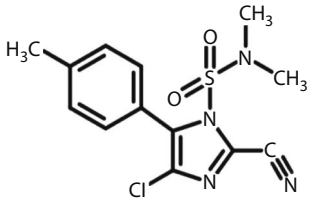
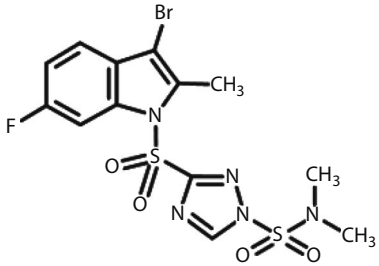
TABLE 16.9
Structures, Uses, and Hazard Profiles for the Methoxy-Acrylate, Oximinoacetate, Dihydro-Dioxanine, and Imidazoline Fungicides (FRAC Code C3)

Fungicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Azoxystrobin (Abound®, Heritage®, Quadris®)[37,280]		Used on vine crops, apples, cereals, cucurbits, tomatoes, pecans, coffee, potatoes, peanuts, peaches, citrus, rice, and turf	100–375
Trifloxystrobin (Flint®, Stratego™)[37,280]		Used on cucurbits, fruiting vegetables, pome fruits, stone fruits, grapes, hops, and pistachio	50–550
Fluoxastrobin (DISARM®, EVITO®)[37,280]		Used on cereals, vines, peanuts, bananas, and various vegetables; also used as a seed treatment in wheat	—
Fenamidone (Censor®, Consento®)[37,280]		Used as a foliar fungicide in grapes and in vegetables; also as a seed treatment or soil drench for *Pythium*	—

	Irritation		LD_{50} (mg/kg)				
Fungicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Azoxystrobin	Slight irritant	Slight irritant	>5000	>2000	>0.7	No positive	Caution
Trifloxystrobin	Mild irritant	Mild irritant	>5000	>2000	4.65	Strong	Caution
Fluoxastrobin	Moderate irritant	Nonirritant	>2000	>2000	>2.0	Negative	Caution
Fenamidone	Moderate irritant	Nonirritant	2028	>2000	2.1	Negative	Caution

Fungicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Azoxystrobin[290]	Rat/2 years	18	Mutagenicity	Not mutagenic
	Dog/52 weeks	25	Developmental	Not teratogenic
	Mouse/18 months	381	Reproductive	Not a reproductive toxin
	RfD	0.18	Oncogenicity	E (No evidence)
Trifloxystrobin[291]	Rat/2 years	9.8	Mutagenicity	Not mutagenic
	Dog/52 weeks	5	Developmental	Not teratogenic
	Mouse/18 months	39.4	Reproductive	Not a reproductive toxin
	RfD	0.038	Oncogenicity	Not likely to be a human carcinogen
Fluoxastrobin[292]	Rat/2 years	181.3	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.5	Developmental	Not teratogenic
	Mouse/18 months	775.6	Reproductive	Not a reproductive toxin
	RfD	0.015	Oncogenicity	No evidence of carcinogenicity in rats or mice
Fenamidone[293,294]	Rat/2 years	2.83	Mutagenicity	Not mutagenic
	Dog/52 weeks	100	Developmental	Not teratogenic
	Mouse/18 months	47.5	Reproductive	Not a reproductive toxin
	RfD	—	Oncogenicity	Not likely to be a human carcinogen

TABLE 16.10
Structures, Uses, and Hazard Profiles for the Cyano-Imidazole (Cyazofamid, Amisulbrom) Fungicides (FRAC Code C4)

Fungicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Cyazofamid (Ranman®)[37,280]		Used to control late blight and downy mildews on potatoes, tomatoes, and cucumbers	60–100
Amisulbrom (Shinkon®)[37,280]		Used to control *Phytophthora* and *Pseudoperonospora* in potatoes, tomatoes, and cucumbers	100–120

	Irritation		LD_{50} (mg/kg)				
Fungicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Cyazofamid	Nonirritant	Nonirritant	>5000	>2000	>5.5	Weak sensitizer	Caution
Amisulbrom	Nonirritant	Nonirritant	>5000	>5000	>2.85	Negative	Caution

Fungicide	**Species/Study**	**NOEL (mg/kg/day)**	**Toxicity Study**	**Hazard Indicator**
Cyazofamid[52]	Rat/2 years	171	Mutagenicity	Not mutagenic
	Dog/52 weeks	200	Developmental	Not teratogenic
	Mouse/18 months	94.8	Reproductive	Not a reproductive toxin
	cRfD	0.95	Oncogenicity	Not likely a human carcinogen
Amisulbrom[53]	Rat/2 years		Mutagenicity	Not mutagenic
	Dog/52 weeks	11.1	Developmental	Not teratogenic
	Mouse/18 months	10.0	Reproductive	Not a reproductive toxin
	cRfD	11.6	Oncogenicity	Suggestive (liver and for stomach tumors in rat)

Dicarboximides (FRAC Code E3)

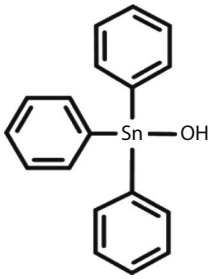
It is proposed that the dicarboximides cause lipid peroxidation in membranes by inhibiting NADH cytochrome C reductase, which is part of the mitochondrial respiratory chain (Figures 16.1 and 16.2) present in all eukaryotic cells.[78] The dicarboximides may also inhibit spore germination, and because there are reports of resistance development for the phenylpyrroles and the dicarboximides, it has been suggested that the dicarboximides may also have effects on the osmotic signal transduction pathway discussed for the phenylpyrroles.[73,79–81]

The dicarboximides have a narrow spectrum of activity limited to *Botrytis*, *Sclerotinia*, *Monilinia*, and *Alternaria*. The dicarboximides are used to treat diseases in turf, strawberries, stone fruit, peanuts, and vines. The structures, uses, and hazard profiles of the dicarboximide fungicides iprodione and vinclozolin are presented in Table 16.16.

Iprodione interferes with androgen synthesis[82] and causes an elevated incidence of interstitial cell tumors in male rats at a concentration of 1600 ppm in the diet. Vinclozolin is metabolized in animals to the antiandrogenic metabolites, 2-1[(3,5-dichlorophenyl) carbamoyl] oxyl-2-methyl-3-butenoic acid and 3,5′-dichloro-2-hydroxy-2 methylbutyl-3-enanilide. These metabolites are presumed to cause infertility in male rats.[83] It has been proposed that this effect is due to a feminization of the outer genital organs of males exposed to the metabolites during development.[84]

The EPA has not established a common mechanism of toxicity group for the dicarboximide fungicides based upon their antiandrogenic potential. For cancer risk assessment, however, the EPA has applied a linear low-dose method for evaluating the cumulative risk resulting from exposure to the metabolite, 3,5-dichloroaniline, which is common to the dicarboximide fungicides, iprodione, procymidone,

TABLE 16.11
Structure, Uses, and Hazard Profile for the Organotin Fungicide Triphenyltin (FRAC Code C6)

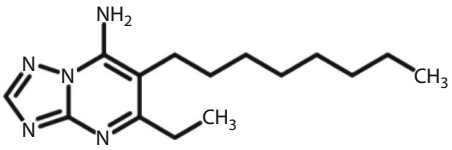
Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Used on potatoes, celery, onions, sugar beets, peanuts, beans, wheat, coffee, and pecans	200–300

	Irritation		LD_{50} (mg/kg)				
Fungicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Triphenyltin acetate	Severe irritant	Nonirritant	140	450	0.044	Positive	Danger
Triphenyltin hydroxide	Severe irritant	Slight irritant	110	1600	0.060	Negative	Danger

Fungicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Triphenyltin hydroxide[37,280,295]	Rat/2 years	<0.3	Mutagenicity	No evidence
	Dog/52 weeks	0.2	Developmental	Not teratogenic
	Mouse/18 months	1.4	Reproductive	No evidence
	ADI	0.0005	Oncogenicity	B2 (liver tumors in mice; pituitary and testicular tumors in rats)

Source: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.

TABLE 16.12
Structure, Uses, and Hazard Profile for the Triazolopyrimidine Fungicide Ametoctradin (Initium®) (FRAC Code C8)

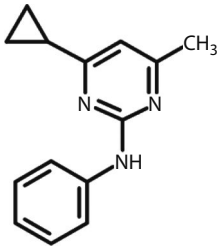
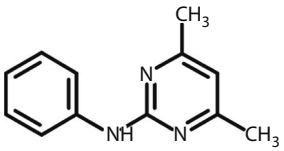
Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Used to control downy mildew on grapes and late blights in various vegetables	—

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Slight irritant	Slight irritant	>2000	>2000	>5.5	Negative	Caution

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	871	Mutagenicity	Not mutagenic
Dog/52 weeks	848	Developmental	Not teratogenic
Mouse/18 months	1099	Reproductive	Not a reproductive toxin
RfD (based on the 6-month dog study)	8.71	Oncogenicity	Not likely to be carcinogenic in humans

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; U.S. EPA, BAS 650 F (Ametoctradin): Human health risk assessment for the proposed new fungicide active ingredient, U.S. Environmental Protection Agency, Washington, DC, 2012; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.

TABLE 16.13
Structures, Uses, and Hazard Profiles for the Anilinopyrimidine Fungicides (FRAC Code D1)

Fungicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Cyprodinil (Vangard®)[37,280]		Used on cereals, grapes, pome fruit, stone fruit, almonds, strawberries, vegetables, and field crops, as well as a seed dressing	150–1500
Pyrimethanil (Mythos®, Scala®)[37,280]		Used on pome fruit, vine crops, vegetables, and ornamentals	80–1000

	Irritation		LD_{50} (mg/kg)				
Fungicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Cyprodinil	Minimal irritant	Slight irritant	>2000	>2000	>1.2	Positive	Caution
Pyrimethanil	Slight irritant	Nonirritant	>4149	>5000	>1.98	Negative	Caution

Fungicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Cyprodinil[296]	Rat/2 years	3.75	Mutagenicity	Not mutagenic
	Dog/52 weeks	65.6	Developmental	Not teratogenic
	Mouse/18 months	16.1	Reproductive	Not a reproductive toxin
	RfD	0.038	Oncogenicity	E (No evidence)
Pyrimethanil[297]	Rat/2 years	20	Mutagenicity	Not mutagenic
	Dog/52 weeks	30	Developmental	Not teratogenic
	Mouse/18 months	211	Reproductive	Not a reproductive toxin
	RfD	0.2	Oncogenicity	C with RfD (thyroid tumors in rats)

and vinclozolin. The EPA has assumed that this terminal, plant, animal, and environmental metabolite is mutagenic and carcinogenic because of a predicted structure–activity relationship with p-chloroaniline,[85] which caused an increased incidence of sarcoma in the spleen of male Fisher 344 rats.[86]

Demethylase Inhibitors (FRAC Code G1)

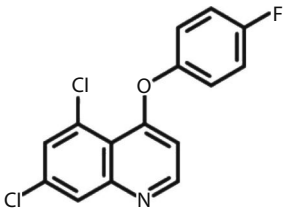
The sterol biosynthesis inhibitors or, more precisely, the sterol demethylase inhibitor (DMI) group is comprised of imidazole, piperazine, pyridine, pyrimidine, and triazole fungicides. DMI inhibitors affect fungi by inhibiting the synthesis of ergosterol.[5] Ergosterol synthesis inhibition results in an accumulation of methylated ergosterol derivatives, which due to their bulkier structure cannot be packed correctly into the lipid bilayer of the fungal membrane. These membrane alterations hinder the uptake and storage of nutrients resulting in cell death. Effects of DMIs on fungal lipid, nucleic acid, and protein synthesis are likely secondary to their effect on cell membranes.

DMI fungicides also inhibit 14-α-demethylase (P450, CYP51) in mammalian cells, where 14-α-demethylase catalyzes the conversion of lanosterol to zymosterol, a precursor to cholesterol and all the mammalian sex steroids. In addition to inhibiting 14-α-demethylase, the DMI fungicides have the potential to inhibit the P450, CYP 19 enzyme, aromatase, which catalyzes the conversion of testosterone to 17β-estrodiol and the conversion of androstenedione to estrone (Figure 16.4). EPA has not determined if there is a common mechanism grouping of the DMI fungicides based on these potential common molecular targets although it has conducted an aggregate dietary risk assessment for three DMI fungicides based upon the toxicity of the common metabolite, 1,2,4-triazole.[87]

The use and structures of the most prominent DMI fungicides are provided in Table 16.17 and their hazard profiles appear in Table 16.18.

High doses of the DMI fungicides cause a treatment-related increased incidence of mouse liver tumors for the majority of these chemicals (myclobutanil is an exception). These effects are apparently not mediated through a direct

TABLE 16.14
Structure, Uses, and Hazard Profile for the Quinoline Fungicide Quinoxyfen (Quintec™) (FRAC Code E1)

Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Used to control powdery mildews in cereals, grapes, hops, sugar beets, and vegetables	50–250

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Mild irritant	Nonirritant	>5000	>2000	>3.38	Positive	Caution

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	20	Mutagenicity	Not mutagenic
Dog/52 weeks	20	Developmental	Not teratogenic
Mouse/18 months	80	Reproductive	Not a reproductive toxin
RfD	0.2	Oncogenicity	No evidence of treatment-related tumorigenicity in rats or mice

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Joint Meeting of the F. A. O. Panel of Experts on Pesticide Residues in Food and the Environment, W. H. O. Expert Group on Pesticide Residues, F. A. O. Panel of Experts on Pesticide Residues in Food, Quinoxyfen, Pesticide residues in food: Evaluations 2006: Part II: Toxicological: Joint FAO/WHO Meeting on Pesticide Residues: World Health Organization: Food and Agriculture Organization of the United Nations, 2006, pp. 367–402; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.

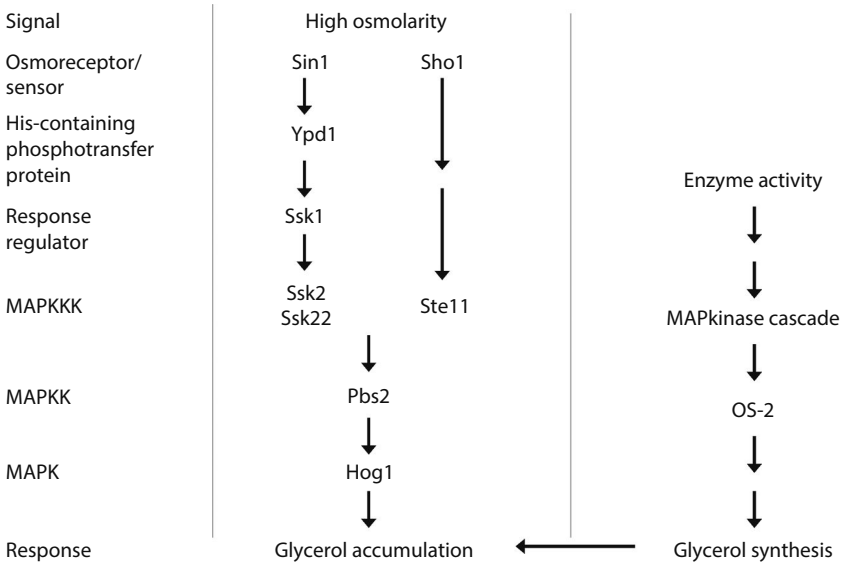
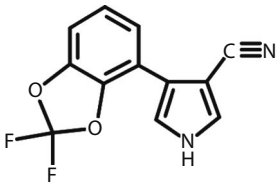


FIGURE 16.3 Osmotic signal transduction.

TABLE 16.15
Structure, Uses, and Hazard Profile for the Phenylpyrrole Fungicide Fludioxinil (CELEST®) (FRAC Code E2)

Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Potato seed dressing; used for seed application on rice, as well as on grapes, stone fruit, vegetables, field crops, turf, and ornamentals	250–800 2.5–10 g a.i./100 kg (seed)

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Slight irritant	Nonirritant	>5000	>2000	>2.6	Negative	Caution

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	50	Mutagenicity	Clastogenic (in vitro)
Dog/52 weeks	3.3	Developmental	Not teratogenic
Mouse/18 months	143	Reproductive	Not a reproductive toxin
RfD	0.03	Oncogenicity	D with RfD

Source: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.; U.S. EPA, *Fed. Regist.*, 69(188), 58084, 2004.

genotoxicity mechanism. It has been suggested that a phenobarbital-like induction of P-450 enzymes is responsible for the liver tumor response seen in mice and that a similar response is unlikely to occur in humans.[88] In addition to the carcinogenic effect, evidence of developmental and/or reproductive system effects has been noted for several of the DMI fungicides including cyproconazole[89] and triadimenol.[90] The mechanism of action has not been established, but it has been postulated that some of these effects may be secondary to the effects of the azole fungicides on steroid biosynthesis.[91]

Hydroxyanilides (FRAC Code G3)

The hydroxyanilide fungicide group includes fenhexamid. This product affects sterol biosynthesis via 3-keto-reductase during C4-demethylation to hinder germ tube elongation and growth in the mycelium.[37] Fenhexamid is not likely to be carcinogenic in humans, based on rat and mouse oncogenicity and genotoxicity studies.[92] The structure, uses, and hazard profile for fenhexamid are shown in Table 16.19.

Peptidyl Pyrimidine Nucleosides (FRAC Code H4)

The peptidyl pyrimidine nucleoside category counts polyoxin as its most important member. These fungicides interfere with cell wall growth by inhibiting chitin biosynthesis.[5,37] The structure, uses, and hazard profile for polyoxin are shown in Table 16.20.

Mandelic Acid Amides (FRAC Code H5)

Mandipropamid is representative of the mandelic acid amide category, which is believed to affect phospholipid biosynthesis and cell wall development.[5,37] Mandipropamid is a minimal ocular irritant, and it is classified as a skin sensitizer, but it is not a skin irritant, and it has very low acute toxicity.[93] The structure, uses, and hazard profile for mandipropamid appear in Table 16.21.

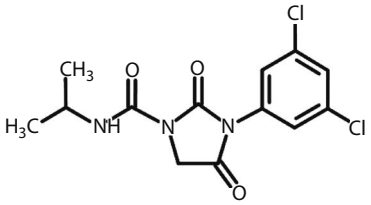
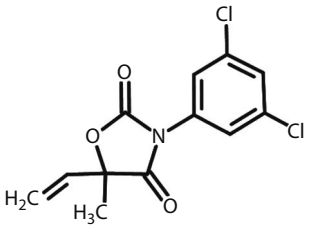
Phenylacetamides (FRAC Code U6)

Cyflufenamid is an important member of the phenylacetamides, which act as preventative or even curative fungicides, though specifics regarding the biochemistry of these products remain unknown.[5,37] Despite the potential for carcinogenicity at high doses, it has low acute toxicity, and it is not an irritant or skin sensitizer.[94] The structure, uses, and hazard profile for fenhexamid are shown in Table 16.22.

Benzophenones (FRAC Code U8)

The benzophenone herbicide category includes the important fungicide metrafenone, which is used to prevent and cure fungal pathogen attacks and also to hinder sporulation.[5,37] Metrafenone demonstrates a low risk of carcinogenicity or oral toxicity and is not neurotoxic.[95] The structure, uses, and hazard profile for metrafenone are shown in Table 16.23.

TABLE 16.16
Structures, Uses, and Hazard Profiles for the Dicarboximide Fungicides (FRAC Code F1)

Fungicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Iprodione (Rovral®)[37,280]		Sunflowers, cereals, fruit trees, berries, oilseed rape, rice, cotton, vegetables, vines, turf, and seed application	500–12,000
Vinclozolin (Ronilan®, Flotilla®)[37,280]		Pome and stone fruit, oilseed rape, vegetables, vines, turf, and ornamentals	300–430

	Irritation		LD_{50} (mg/kg)				
Fungicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Iprodione	Mild irritant	Nonirritant	>2000	>2000	>5.2	Negative	Caution
Vinclozolin	Minimal irritant	Minimal irritant	>15,000	>5000	29.1	Positive	Caution

Fungicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Iprodione[82,299,300]	Rat/2 years	6.0	Mutagenicity	No evidence
	Dog/52 weeks	4.2	Developmental	Not teratogenic
	Mouse/18 months	1870	Reproductive	Not a reproductive toxin
	RfD (UF = 300)	0.04	Oncogenicity	B2 (liver, testes)
Vinclozolin[83–85,301,302]	Rat/2 years	1.2	Mutagenicity	Not mutagenic
	Dog/52 weeks	2.4	Developmental	Not teratogenic
	Mouse/18 months	21	Reproductive	Antiandrogenic metabolite
	RfD	0.012	Oncogenicity	B2 with RfD (multiple benign tumors in rats)

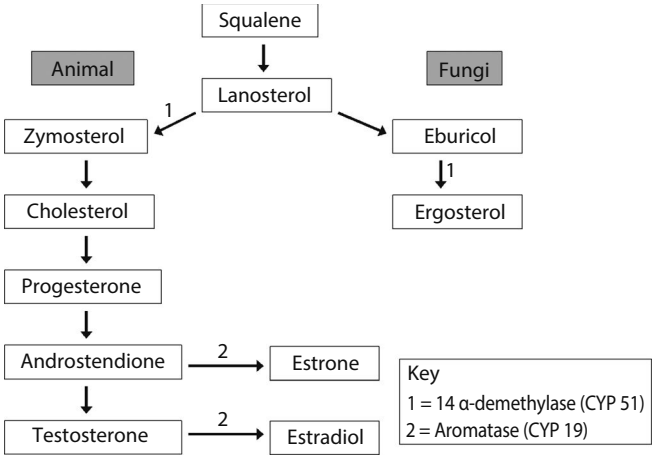


FIGURE 16.4 Steroid biosynthesis in fungi and animals.

Guanidines (FRAC Code U12)

Guanidine herbicides include dodine, a local systemic foliar fungicide that acts to both prevent and cure scab, leaf spot diseases, and other diseases.[5,37] Dodine has moderate acute oral, dermal, and inhalation toxicity, and it is known to be an irritant, but it is not a skin sensitizer.[96] The structure, uses, and hazard profile for dodine are shown in Table 16.24.

Inorganic Fungicides (FRAC Codes M1 and M2)

Inorganic chemicals such as sulfur were used before 1000 BC,[1] and elemental sulfur and copper (hydroxide, oxychloride, and sulfate) are still used as fungicides today. The MOA of the inorganic fungicides is protective or preventative, and they exert their effects by the inhibition of mitochondrial respiration.[37] The inorganic fungicides are

TABLE 16.17
Structures and Use Profiles for Demethylase Inhibitors (FRAC Code G1)

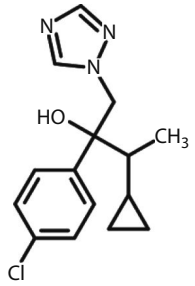
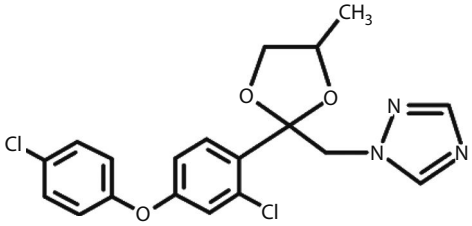
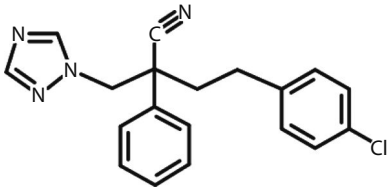
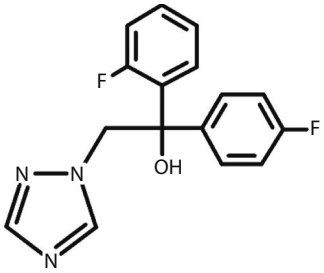
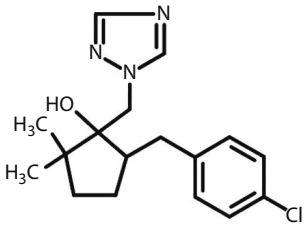
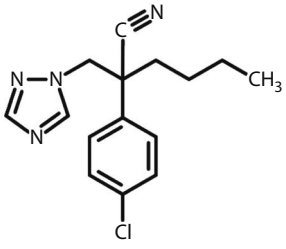
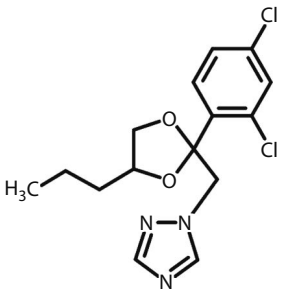
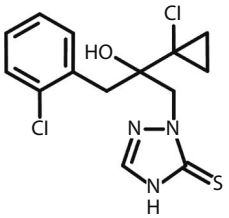
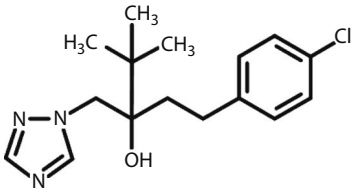
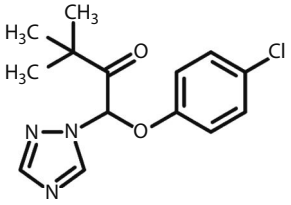
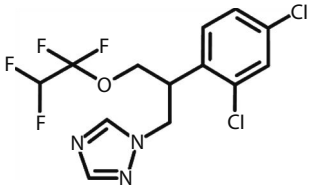
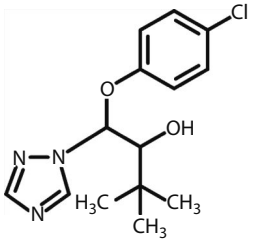
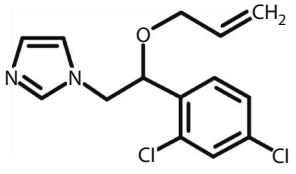
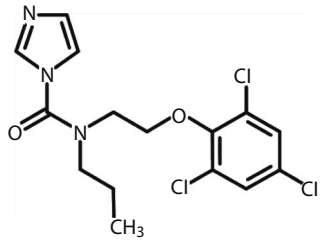
Fungicide	Structure	Principal Use/Crop
Cyproconazole (Alto®)[37]		Cereal, sugar beets, fruit trees, vines, coffee, turf, bananas, and vegetables for the application of rust, powdery mildew, *Septoria*, *Venturia*, and others; application rates are 60–100 g/ha
Difenoconazole (Dividend®)[37,280]		Seed application, grapes, fruit trees, potatoes, sugar beets, oilseed rape, banana, ornamentals, and vegetables for treating a variety of fungal diseases; application rates are 30–125 g/ha
Fenbuconazole (Indar®)[37,280]		Cereals, fruit trees, vines, beans, sugar beets, rice, bananas, ornamentals, tree nuts, and vegetables; application rates are 30–75 g/ha
Flutriafol (Armour®)[37,280]		Controls many ear and leaf diseases in cereals at 125 g/ha; also used as a seed treatment
Metconazole (Caramba®)[37,280]		*Fusarium*, *Septoria*, rusts, and other foliar diseases on a variety of crops, including cereals, at 90 g/ha
Myclobutanil (Rally®, Nova®)[37,280]		Seed application, grapes, fruit trees, rice, cotton, barley, wheat, maize, grass seed, ornamentals, and vegetables for treating a variety of fungal diseases; application rates are typically 30–60 g/ha

TABLE 16.17 (continued)
Structures and Use Profiles for Demethylase Inhibitors (FRAC Code G1)

Fungicide	Structure	Principal Use/Crop
Propiconazole (Tilt®)[37,280]		Wheat, rice, coffee, bananas, peanuts, stone fruit, maize, and turf for treating a variety of fungal diseases; application rates are roughly 24–110 g/ha
Prothioconazole (Input®)[37,280]		Used to control various blights, blotches, mildews, rusts, and eyespot in cereals and other crops; also used as a seed treatment
Tebuconazole (Folicur®)[37,280]		Seed application, cereals, coffee, fruit trees, grapes, grass seed, oilseed rape, soybeans, sugar beets, bananas, ornamentals, turf, and vegetables for treating a variety of fungal diseases; application rates range from 1 to 7.5 g/dt for seed and 200 to 375 g/ha (or 7.5–18.8 g/100 L) for spray
Triadimefon (Bayleton®)[37,280]		Cereals, corn, fruit trees, vines, berries, sugarcane, tobacco, and vegetables for treating a variety of fungal diseases; application rates range from 0.0025% to 0.0125% for fruits/vegetables and 125 to 500 g/ha for other crops
Tetraconazole (Domark®)[37,280]		Foliar treatment for mildews, rusts, leaf spots in sugar beets, cereals, various fruits, and vegetables at rates from 25 to 125 g/ha; also used as a seed treatment at rates from 3 to 12 g/100 kg
Triadimenol (Baytan®)[37,280]		Seed application, cereals, fruit trees, hops, vines, and vegetables for treating a variety of fungal diseases; rates range from 20 to 60 g/100 kg for seed, 0.0025% to 0.0125% for fruits/vegetables, and 100 to 500 g/ha for sprays
Imazalil (Fungaflor®)[37,280]		Seed, fruit trees, potatoes, bananas, vegetables, ornamentals, and cereals for treating a variety of fungal diseases; application rates range from 4 to 5 g/100 kg for seed, 5 to 30 g/ha for ornamentals/vegetables, and 2 to 4 g/t for fruit

(*continued*)

TABLE 16.17 (continued)
Structures and Use Profiles for Demethylase Inhibitors (FRAC Code G1)

Fungicide	Structure	Principal Use/Crop
Prochloraz (Sportak®)[37,280]		Citrus, tropical fruit (dip), beets, oilseed rape, mushrooms, ornamentals, and cereals (seed application); application rates range from 400 to 600 g/ha or 0.5/0.7 g/L for dip treatment

relatively ineffective since they must be applied at high use rates ranging from 1000 to 10,000 g/ha. Severe eye irritation is seen with copper hydroxide,[37] whereas copper oxychloride and copper sulfate are not eye irritants.[37] Elemental sulfur is considered practically nontoxic to humans and animals.[37]

DITHIOCARBAMATES AND ETHYLENEBISDITHIOCARBAMATES (FRAC CODE M3)

The dithiocarbamates are broad-spectrum protective fungicides having multiple sites of action.[37] They are used to control scab on pome fruit, blue mold on tobacco, rust on ornamentals, and numerous diseases on vegetables. These agents interfere with oxygen uptake and may bind to sulfur-containing enzymes. The dithiocarbamates are applied at rates of 500 to over 10,000 g/ha. Ferbam, thiram, and ziram are the commercially important chemicals in this group.

The ethylenebisdithiocarbamate fungicides (mancozeb, maneb, and zineb) have a broad spectrum of activity although their fungicidal MOA is primarily protective. Their mechanism of action is to form cyanide, which reacts with thiol compounds within cells.[37]

EPA has considered including the dithiocarbamates into a common mechanism group[97,98] based upon their potential (1) to generate carbon disulfide (potential to cause distal peripheral neuropathy)[98]; (2) to form the common metabolite ethylenethiourea (ETU), which has potential carcinogenic effects; (3) to chelate physiologically important polyvalent cations such as copper, zinc, lead, or cadmium (potential neurotoxicity resulting from nervous system sequestration of heavy metals); or (4) to inhibit acetylcholinesterase.[97] In their final decision, the EPA concluded that the available evidence suggests that neuropathology induced by the treatment of rats with the dithiocarbamates could not be linked with the formation of carbon disulfide. A common mechanism grouping for mancozeb, maneb, and metiram was supported based on their ability to form the common metabolite, ETU. It was also concluded that two dithiocarbamate pesticides, ziram and metam sodium, share a common mechanism for acetylcholinesterase inhibition.[98]

The structures, uses, and hazard profiles for the dithiocarbamates are given in Table 16.25. Ferbam, thiram, and ziram have significant acute toxicity, especially by inhalation. Both ferbam and ziram affect spermatozoa in mice, and thiram is teratogenic at high doses. There is no evidence that any of these fungicides is carcinogenic, although positive mutagenic studies were reported for thiram.

The structures, uses, and hazard profiles for mancozeb, maneb, and zineb and their common metabolite ETU are provided in Table 16.26. Both mancozeb and maneb are classified as B2, probable human carcinogens,[99] based on the formation of mouse liver tumors and/or thyroid follicular cell tumors in rats. Although zineb was not found to be oncogenic in the rat or mouse, it was observed to produce non-neoplastic hyperplasia of the follicular cells of the thyroid in rats.[100] All three fungicides are transformed in animals to the metabolite, ETU. ETU inhibits thyroid peroxidase and causes progressive lesions in the thyroid follicular cells, often leading to tumor formation.[101–103] The EPA has regulated the risk associated with exposure to ETU using a cancer slope factor (Q_1*) of 0.06 $(mg/kg/day)^{-1}$.[104]

Phthalimides (FRAC Code M4)

The phthalimide fungicides represent a relatively old group of synthetic chemicals, of which only captan remains in use. Captan has a broad spectrum of activity, which is attributed to the formation of thiophosgene.[105] Thiophosgene has the potential to react with thiol groups, most likely at the point of contact with the mucosa of the stomach, as shown in Figure 16.5. Folpet, another member of this class of fungicides, is also capable of producing thiophosgene.

The structure, uses, and hazard profile for captan are given in Table 16.27. Captan has been shown to bind to DNA in vitro but not in vivo. Captan has been classified by the EPA

TABLE 16.18
Hazard Profiles for Demethylase Inhibitors (FRAC Code G1)

	Irritation		LD_{50} (mg/kg)				
Fungicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Cyproconazole	Nonirritant	Nonirritant	>1020	>2000	5.7	Negative	Caution
Difenoconazole	Moderate irritant	Slight irritant	1453	>2000	3.3	Negative	Caution
Fenbuconazole	Nonirritant	Nonirritant	>2000	>5000	>2.1	Negative	Caution
Flutriafol	Mild irritant	Nonirritant	1140	>1000	>3.5	Negative	Warning
Metconazole	Slight irritant	Nonirritant	660	>2000	>5.6	Negative	Caution
Myclobutanil	Irritant	Nonirritant	>1600	>5000	>5.0	Positive	Danger

Fungicide	**Species/Study**	**NOEL (mg/kg/day)**	**Toxicity Study**	**Hazard Indicator**
Cyproconazole[89]	Rat/2 years	2.2	Mutagenicity	Clastogenic (CHO)
	Dog/52 weeks	1.0	Developmental	Teratogenic in rabbit
	Mouse/18 months	1.8	Reproductive	Not a reproductive toxin
	RfD	0.01	Oncogenicity	B2 (mouse liver tumors in both sexes)
Difenoconazole[302]	Rat/2 years	1.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	3.4	Developmental	Not teratogenic
	Mouse/18 months	4.7	Reproductive	Not a reproductive toxin
	RfD	0.01	Oncogenicity	C with RfD (mouse liver tumors in both sexes)
Fenbuconazole[303]	Rat/2 years	3.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	3.8	Developmental	Not teratogenic
	Mouse/18 months	1.4	Reproductive	E (No evidence)
	RfD	0.03	Oncogenicity	C with RfD (Mouse liver tumors—both sexes/thyroid tumors—male rats)
Flutriafol[304]	Rat/2 years	10.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	5.0	Developmental	Not teratogenic
	Mouse/18 months	5.9	Reproductive	Not a reproductive toxin
	RfD	0.05	Oncogenicity	Not likely to be carcinogenic in humans
Metconazole[305]	Rat/2 years	4.8	Mutagenicity	Not mutagenic
	Dog/52 weeks	11.1	Developmental	Not teratogenic
	Mouse/18 months	—	Reproductive	Not a reproductive toxin
	RfD	0.04	Oncogenicity	Not likely to be carcinogenic in humans
Myclobutanil[306]	Rat/2 years	2.5	Mutagenicity	Not mutagenic
	Dog/52 weeks	3.1	Developmental	Not teratogenic
	Mouse/18 months	13.7	Reproductive	Testicular atrophy
	RfD	0.025	Oncogenicity	E (No evidence)

	Irritation		LD_{50} (mg/kg)				
Fungicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Propiconazole	Mild irritant	Slight irritant	1517	>6000	>5.8	Negative	Caution
Prothioconazole	Nonirritant	Nonirritant	>6200	>2000	>4990 (mg/m^3)	Negative	Caution
Tebuconazole	Mild irritant	Nonirritant	>3933	>5000	>0.37	Negative	Caution
Triadimefon	Nonirritant	Nonirritant	>363	>2000	>3.6	Positive	Warning
Tetraconazole	Slight irritant	Nonirritant	1031	>2000	>3.66	Negative	Caution
Triadimenol	Nonirritant	Nonirritant	>1100	>5000	>0.9	NA	Caution
Imazalil	Nonirritant	Mild irritant	>227	4200	16	Negative	Warning
Prochloraz	Irritant	Mild irritant	1600	3000	0.42	Negative	Caution

(*continued*)

TABLE 16.18 (continued)
Hazard Profiles for Demethylase Inhibitors (FRAC Code G1)

Fungicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Propiconazole[37,87,308]	Rat/2 years	3.6	Mutagenicity	No evidence
	Dog/26-week oral	1.3	Developmental	Not teratogenic
	Mouse/18 months	15	Reproductive	Not a reproductive toxin
	RfD	0.013	Oncogenicity	C with RfD (mouse liver tumors in males)
Prothioconazole[309]	Rat/2 years	5	Mutagenicity	Not mutagenic
	Dog/26-week oral	1.6 (desthio)	Developmental	Not teratogenic
	Mouse/18 months	3.1	Reproductive	Not a reproductive toxin
	RfD	0.05	Oncogenicity	Not carcinogenic
Tebuconazole[310]	Rat/2 years	7.4	Mutagenicity	No evidence
	Dog/52 weeks	3.0	Developmental	Teratogenic in rat
	Mouse/18 months	2.9	Reproductive	Not a reproductive toxin
	RfD	0.03	Oncogenicity	C with RfD (mouse liver tumors in both sexes)
Triadimefon[87,311,312]	Rat/2 years	16.4	Mutagenicity	Not mutagenic
	Dog/2 year oral	11.4	Developmental	Not teratogenic
	Mouse/18 months	40	Reproductive	Not a reproductive toxin
	RfD (52-week dog study with UF = 300)	0.04	Oncogenicity	C with RfD (mouse liver tumors in both sexes)
Tetraconazole[313]	Rat/2 years	80	Mutagenicity	Not mutagenic
	Dog/1 year	0.7	Developmental	Not teratogenic
	Mouse/18 months	1.4	Reproductive	Not a reproductive toxin
	cRfD	0.0073	Oncogenicity	Likely to be carcinogenic to humans
Triadimenol[87,90]	Rat/2 years	7.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	3.75	Developmental	Teratogenic in rat
	Mouse/18 months	30	Reproductive	Not a reproductive toxin
	ADI	0.038	Oncogenicity	C with RfD (liver tumors in female mice)
Imazalil[314]	Rat/2 years	5.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	2.5	Developmental	Not teratogenic
	Mouse/18 months	40	Reproductive	Not a reproductive toxin
	ADI	0.025	Oncogenicity	C-Q* (mouse liver)
Prochloraz[315]	Rat/2 years	1.9	Mutagenicity	Not mutagenic
	Dog/52 weeks	0.9	Developmental	Not teratogenic
	Mouse/18 months	11.7	Reproductive	Decreased litter size
	ADI	0.009	Oncogenicity	C-Q* (mouse liver tumors in both sexes)

as a category B2 carcinogen (probable human carcinogen) based on gastrointestinal tract tumors in the mouse.[99]

Chloronitriles (FRAC Code M5)

The chloronitrile fungicides, represented here by chlorothalonil, have a broad spectrum of fungicidal activity and are considered protective. Chlorothalonil controls fungal infection by binding to sulfur-containing enzymes.[37] The structure, uses, and hazard profile of chlorothalonil are given in Table 16.28.

Chlorothalonil is a severe eye irritant, a moderate skin irritant, and a potential skin sensitizer. The EPA has classified chlorothalonil as a likely human carcinogen based on kidney and forestomach tumors in both rats and mice.[106] The occurrence of kidney tumors was preceded by a pronounced hyperplasia in the proximal tubules. It has been proposed that these preneoplastic changes are due to the formation of nephrotoxic thiol metabolites of chlorothalonil.[107] It has also been suggested that β-lyase catalyzes the conversion of the cysteine conjugate to the ultimate toxiphore (see Figure 16.6). Because the activity of glutathione-S-transferase and β-lyase in the human kidney is about 10% of the rat kidney, it is likely that humans are less susceptible to chlorothalonil than are rats.

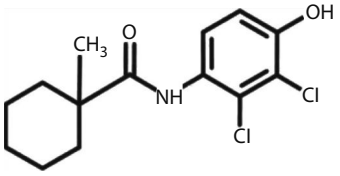
INSECTICIDES

Table 16.29 provides the list of insecticides reviewed in this chapter, grouped into categories according to the MOA[7] as defined by the Insecticide Resistance Action Committee (IRAC). The majority of the insecticidal MOA described later are relevant to humans, although a few exceptions are noted in Table 16.29 and discussed in the appropriate section provided in the following text.

Carbamates: AChE Inhibitors (IRAC Code 1A)

Acetylcholine is an excitatory neurotransmitter substance that is released from synaptic vesicles that are found within boutons of the presynaptic neuron (Figure 16.7).

TABLE 16.19
Structure, Uses, and Hazard Profile for the Hydroxanilide Fungicide Fenhexamid (Elevate®) (FRAC Code G3)

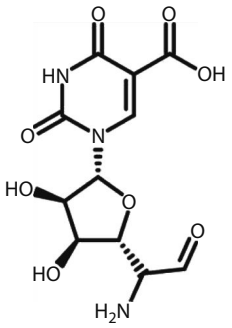
Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Control of *Botrytis*, *Monilia*, and other molds in grapes, berries, various fruits, vegetables, and ornamentals	500–1000

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Nonirritant	Nonirritant	>5000	>5000	>5057	Negative	Caution

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	28.0	Mutagenicity	Not mutagenic
Dog/52 weeks	17.0	Developmental	Not teratogenic
Mouse/18 months	247	Reproductive	Not a reproductive toxin (increased pup sensitivity)
RfD	0.17	Oncogenicity	Not oncogenic

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; U.S. EPA, Fenhexamid: Human health risk assessment for a proposed section 3 registration for use on Asparagus, U.S. Environmental Protection Agency, Washington, DC, 2007; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.; U.S. EPA, *Fed. Regist.*, 69(188), 58084, 2004.

TABLE 16.20
Structure, Uses, and Hazard Profile for the Peptidyl Pyrimidine Fungicide Polyoxin (ENDORSE®) (FRAC Code H4)

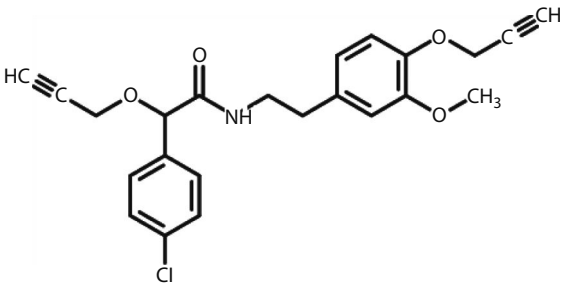
Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Used to control *Alternaria*, *Botrytis*, mildews, molds, blights, rots, and brown spot in apples, pears, flowers, melons, strawberries, tomatoes, and other vegetables	100–1400

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Nonirritant	Nonirritant	>9600	>750	2.44 males; 2.17 females	Mild[315] sensitizer	Caution

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	2470	Mutagenicity	Not mutagenic
Dog/52 weeks	Not required	Developmental	Not teratogenic
Mouse/18 months	3591	Reproductive	Not a reproductive toxin
RfD	Exempted	Oncogenicity	No evidence of treatment-related tumorigenicity in rats or mice

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.; U.S. EPA, *Fed. Regist.*, 69(188), 58084, 2004; U.S. EPA, Science review in support of a tolerance exemption petition for polyoxin D zinc salt; [beta.-D-Allofuranuronic acid, 5-((2-amino-5-0-(aminocarbonyl)-2-deoxy-Lxylonoyl) amino)-I-(5-carboxy-3, 4-dihydro-2, 4-dioxo-l (2H)-pyrimidinyl)-1, 5-dideoxy-, zinc salt (1: 1)]. First food use. Exemption from the requirement of a tolerance-final rule, U.S. Environmental Protection Agency, Washington, DC, August 18, 2008, Available from: https://www.federalregister.gov/articles/2008/11/19/E8-27485/polyoxin-d-zinc-salt-exemption-from-the-requirement-of-a-tolerance#h-13.

TABLE 16.21
Structure, Uses, and Hazard Profile for the Mandelic Acid Amide Fungicide Mandipropamid (REVUS®) (FRAC Code H5)

Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Prevents foliar fungus germination, growth, and sporulation in grapes, potatoes, tomatoes, and cucurbits	100–150

Irritation		LD_{50} (mg/kg)				
Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Nonirritant	Nonirritant	>5000	>5050	>5	Negative	Caution

Species/Study	NOEL (mg/kg/bw/day)	Toxicity Study	Hazard Indicator
Rat/2 years	15	Mutagenicity	Not mutagenic
Dog/52 weeks	5	Developmental	Not teratogenic
Mouse/18 months	—	Reproductive	Not a reproductive toxin
cRfD	0.05	Oncogenicity	Not oncogenic

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; U.S. EPA, Mandipropamid: Pesticide fact sheet, U.S. Environmental Protection Agency, Washington, DC, 2008; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.; U.S. EPA, *Fed. Regist.*, 69(188), 58084, 2004.

Neurotransmitter release is triggered by voltage-dependent calcium ion influx across the presynaptic membrane in response to an action potential-driven depolarization (sodium current) of the membrane. Once the neurotransmitter is released, it diffuses into the synaptic cleft where it binds to a postsynaptic receptor, triggering an excitatory postsynaptic potential (EPSP).

The carbamate insecticides inhibit the enzyme, acetylcholinesterase (AChE), which is found in the postsynaptic cleft and which catalyzes the cleavage of acetylcholine, into its constituent components acetyl and choline, thereby inactivating the neurotransmitter. Cholinesterase inhibition, which is defined as the percent reduction in AChE activity, results in a prolonged stimulation of the cholinergic receptors leading to a hyperexcitatory state downstream from the site of action. This translates into an intense activation of the autonomic nervous system, which depending on the severity of AChE inhibition results in piloerection, salivation, tremor, convulsion, respiratory arrest, and death.

Carbamates affect neurotransmission in an almost identical fashion to the organophosphorus insecticides (OPs) except that carbamate insecticides carbamylate AChE whereas the OPs phosphorylate AChE as shown in Figure 16.8.

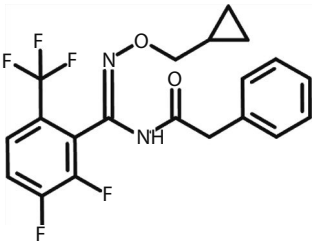
Carbamate insecticides exist as esters of carbamic acid, typically having an aryl (ring) substituent as the leaving group. Carbamates react with the serine group on acetylcholinesterase to yield a carbamylation of the serine hydroxyl group. A hydroxylated leaving group is generated. The carbamylation of AChE is reversible, unlike the phosphorylation of the AChE by organophosphate insecticides. The carbamylated complex will typically hydrolyze in minutes.

The OP reacts with acetylcholinesterase at a serine hydroxyl group within the enzyme's active site. In this reaction, the hydroxyl group is phosphorylated, yielding a leaving group. Reactivation of the enzyme can take many hours or even days.

The pesticidal MOA of the carbamate and organophosphorus insecticides are preserved in mammals. EPA has determined that a separate common mechanism grouping exists for the N-methyl carbamate and the OPs[108] based upon the differences shown in Figure 16.8.

The structure and use of some representative carbamate insecticides are given in Table 16.30 and their hazard profiles appear in Table 16.31. Aldicarb is the most acutely toxic of the carbamates selected for inclusion in this chapter, with an oral LD_{50} below 1 mg/kg and a dermal LD_{50} of 20 mg/kg. Aldicarb, carbofuran, methomyl,

TABLE 16.22
Structure, Uses, and Hazard Profile for the Phenylacetamide Fungicide Cyflufenamid (MILTREX®) (FRAC Code U6)

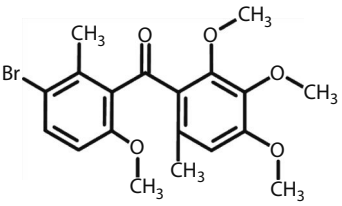
Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Control of powdery mildew in wheat, fruit, vegetables, and cereals	25

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Mild irritant	Nonirritant	>5000	>2000	>4.76	Negative	Caution

Species/Study	NOEL (mg/kg/bw/day)	Toxicity Study	Hazard Indicator
Rat/2 years	4.4	Mutagenicity	Not mutagenic
Dog/52 weeks	17.29	Developmental	Not teratogenic
Mouse/18 months	63	Reproductive	Not a reproductive toxin
RfD	0.044	Oncogenicity	Likely to be carcinogenic to humans

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; U.S. EPA, Cyflufenamid: Human health risk assessment for proposed uses on cucurbit vegetables (Crop Group 9), pome fruit (Crop Group 11), small fruit vine climbing except fuzzy kiwifruit (Subgroup 13-07F), and low growing berry (Subgroup 13-07G), except cranberry, U.S. Environmental Protection Agency, Washington, DC, 2010; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.; U.S. EPA, *Fed. Regist.*, 69(188), 58084, 2004.

TABLE 16.23
Structure, Uses, and Hazard Profile for the Benzophenone Fungicide Metrafenone (Flexity®) (FRAC Code U8)

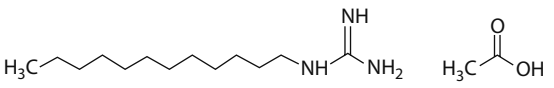
Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Eyespot and powdery mildew control in wheat, barley, and grapes	—

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Nonirritant	Nonirritant	>5000	>5000	>5.0	Negative	—

Species/Study	NOEL (mg/kg/bw/day)	Toxicity Study	Hazard Indicator
Rat/2 years	24.9	Mutagenicity	Not mutagenic
Dog/52 weeks	500	Developmental	Not teratogenic
Mouse/18 months	156	Reproductive	Not a reproductive toxin
cRfD	0.25	Oncogenicity	Suggestive evidence of carcinogenicity (mouse liver tumors)

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; U.S. EPA, Metrafenone: Human health risk assessment for proposed use on grapes, U.S. Environmental Protection Agency, Washington, DC, 2006; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.; U.S. EPA, *Fed. Regist.*, 69(188), 58084, 2004.

TABLE 16.24
Structure, Uses, and Hazard Profile for the Guanidine Fungicide Dodine (SYLLIT®) (FRAC Code U12)

Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Uses include control of scab, leaf spot, and other foliar diseases on fruit trees, nut trees, strawberries, vegetables, ornamentals, and shade trees	250–1500

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
—	Irritating	>1000	>6000 (rats)	1.05	Negative	Danger

Species/Study	NOEL (mg/kg/bw/day)	Toxicity Study	Hazard Indicator
Rat/2 years	20	Mutagenicity	Not mutagenic
Dog/52 weeks	2	Developmental	Not teratogenic
Mouse/18 months	110	Reproductive	Not a reproductive toxin
RfD	0.02	Oncogenicity	No evidence of carcinogenicity in rats or mice

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; U.S. EPA, Dodine: Human health risk assessment for proposed use in bananas and peanuts, U.S. Environmental Protection Agency, Washington, DC, 2008; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com; U.S. EPA, *Fed. Regist.*, 69(188), 58084, 2004.

and propoxur have been classified as category C (possible human carcinogens) or D (aldicarb) by the EPA.[99]

Organophosphorus Insecticides: AChE Inhibitors (IRAC Code 1B)

OPs vary tremendously in chemical structure and properties. The OPs are classified into the following groups depending on the positioning of the central phosphorus atom: phosphates, phosphonates, phosphorothionates, phosphorodithioates, and phosphoroamidothioates. Examples of OPs within these different subgroups are presented in Table 16.32. The toxicological profiles for the OPs are presented in Table 16.33. Of the OPs reviewed here, azinphos-methyl is the most acutely toxic, and malathion is the least toxic. Acephate causes liver tumors in mice, and delayed neurotoxicity was seen in studies on dichlorvos. Monocrotophos has the lowest chronic reference dose.

Cyclodiene Organochlorines: GABA Antagonists (IRAC Codes 2A and 2B)

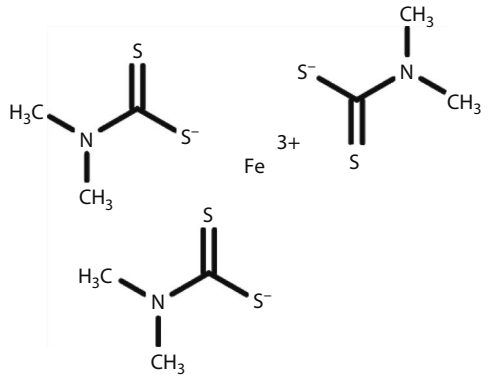
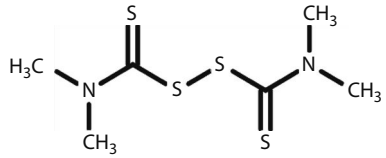
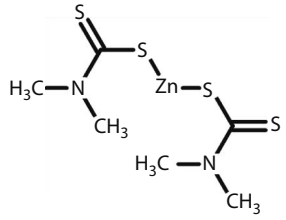
In both insects and mammals, chloride channel–blocking insecticides cause hyperexcitability, convulsions, and death.[109] Overstimulation of neuronal pathways in the central nervous system (CNS) results from blocking the action of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). Normally, when GABA is released from the presynaptic nerve terminal, it binds to a postsynaptic receptor protein containing an intrinsic chloride ion channel. When GABA binds to its receptor, the chloride channel is opened and chloride ions flow across the postsynaptic membrane. This increase in chloride permeability hyperpolarizes (makes more negative) the membrane, resulting in an inhibitory postsynaptic potential, which has a dampening effect on neuronal excitation, making it less likely that a postsynaptic action potential will occur. Attenuation of GABA-mediated neuronal inhibition leads to hyperexcitation of downstream neuronal pathway because GABA neuronal pathways are inhibitory.[110]

The structures, uses, and hazard profiles for the cyclodiene organochlorines, represented here by endosulfan and fipronil, are given in Table 16.34. They are moderately acutely toxic and have relatively low chronic reference doses. They are neither mutagens, developmental toxins, or reproductive toxins, nor carcinogens.

Pyrethroids: Sodium Channel Modulators (IRAC Code 3A)

The pyrethroid insecticides, typically esters of chrysanthemic acid, were isolated from the flowers of chrysanthemums, and three of these comprise the pyrethrum extract approved for organic farming methods.[37,111,112] Synthetic pyrethroid chemistry and insecticidal effects for type I pyrethroids are rather broadly defined, and these include pyrethroids containing descyano-3-phenoxybenzyl or other alcohols.[74] Many of the older nonphenoxybenzyl type I compounds (e.g., pyrethrins, allethrin, and tetramethrin) are unstable in the environment, and this characteristic prevented their use in row crops. Introduction of the phenoxybenzyl (e.g., permethrin) or halogenated alcohols (e.g., tefluthrin) improved chemical stability and allowed the use of pyrethroids on these crops. The characteristic clinical signs seen

TABLE 16.25
Structures, Uses, and Hazard Profiles for the Dithiocarbamate Fungicides (FRAC Code M3)

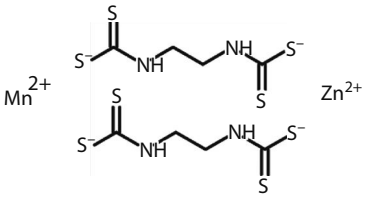
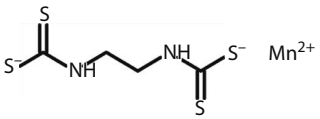
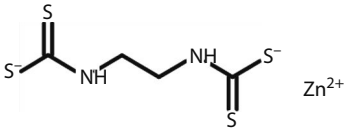
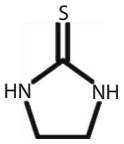
Fungicide	Structure	Principal Use/Crop	Application Rate
Ferbam		Pome fruit, peaches, and tobacco	0.1%–0.25% a. i.
Thiram		Seed dressing	50–200 g/100 kg (seed)
Ziram		Pome fruit, stone fruit, nuts, vines, vegetables, ornamentals, and seeds	50–200 g/100 kg (seed)

	Irritation		LD_{50} (mg/kg)				
Fungicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Ferbam	Mild irritant	Slight irritant	>4000	>4000	0.4	Weak positive	Warning
Thiram	Slight irritant	Irritant	>1800	>2000	>0.1	Positive	Warning
Ziram	Severe irritant	Nonirritant	270	>2000	0.06	Positive	Danger

Fungicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Ferbam[317,318]	Rat/2 years	12.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	5.0	Developmental	Not teratogenic
	Mouse/18 months	—	Reproductive	Effects on sperm in mice
	ADI	0.003 (interim)	Oncogenicity	No evidence of tumorigenicity in rats or mice
Thiram[283,319]	Rat/2 years	1.2	Mutagenicity	Positive Ames and SCE
	Dog/2-year oral	0.84	Developmental	Teratogenic in mice and hamsters at high doses
	Mouse/18 months	3.0	Reproductive	Not a reproductive toxin
	ADI	0.008	Oncogenicity	No evidence of tumorigenicity in rats or mice
Ziram[320,321]	Rat/2 years	<2.5	Mutagenicity	Clastogenic
	Dog/52 weeks	1.6	Developmental	Not teratogenic
	Mouse/18 months	3.0	Reproductive	Effects on sperm in mice
	ADI (UF = 1000)	0.003	Oncogenicity	No evidence of tumorigenicity in rats or mice

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com.

TABLE 16.26
Hazard Profiles for Dithiocarbamates Fungicides Mancozeb, Maneb, and Zineb (FRAC Code M3)

Fungicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Mancozeb (Dithane®, Manzate®)[37,280]		Potatoes, tomatoes, fruits, vegetables, cereals, vines, ornamentals, and tobacco	1500–2000
Maneb (Kypman®)[37,280]		Potatoes, tomatoes, vegetables, apples, pears, cereals, ornamentals, vines, and tobacco	450–3600
Zineb (Kypzin®)[37,280]		Oilseed rape, berries, apples, pears, stone fruit, citrus fruit, bananas, currants, olives, celery, vegetables, and vines	2250
ETU (common metabolite)[37,97,98,104,280]		—	—

	Irritation		LD_{50} (mg/kg)					
Fungicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L)	Inhalation	Sensitization Potential	Signal Word
Mancozeb	Severe irritant	Slight irritant	>5000	>5000	5.14		Positive	Danger
Maneb	Moderate irritant	Slight irritant	6750	>5000	7.38		Positive	Warning
Zineb	Mild irritant	Slight irritant	>5200	>6000	NA		Negative	Caution

Fungicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Mancozeb[101,104,322]	Rat/2 years	4.8	Mutagenicity	Equivocal evidence
	Dog/52 weeks	7.0	Developmental	Teratogenic at high doses
	Mouse/18 months	17	Reproductive	Not a reproductive toxin
	ADI	0.03[a]	Oncogenicity	B2 (thyroid tumors in rats of both sexes)
Maneb[102,104,323,324]	Rat/2 years	5.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	6.4	Developmental	Not teratogenic
	Mouse/18 months	11	Reproductive	Not a reproductive toxin
	ADI	0.03[a]	Oncogenicity	B2 (Liver tumors in mice of both sexes; thyroid tumors in rats)
Zineb[103]	Rat/2 years	<25	Mutagenicity	No evidence
	Dog/52 weeks	50	Developmental	Not teratogenic
	Mouse/18 months	No adequate study	Reproductive	Not a reproductive toxin
	ADI	0.03[a]	Oncogenicity	No evidence

[a] ADI based on ETU as a metabolite common to mancozeb, maneb, metiram, and zineb.[97]

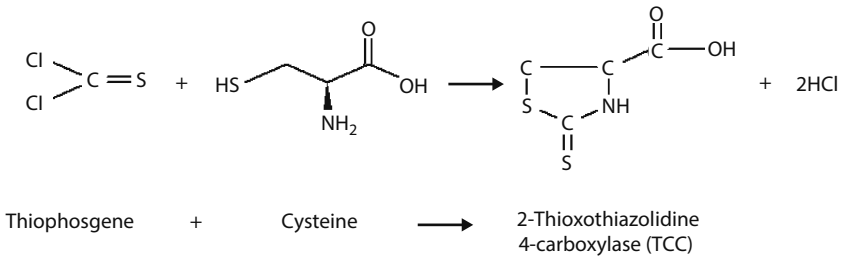
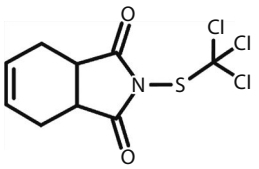


FIGURE 16.5 Proposed toxiphore for captan.

TABLE 16.27
Structure, Uses, and Hazard Profile for the Multisite Contact Phthalimide Fungicide Captan (Alpha™) (FRAC Code M4)

Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Used on stone fruit, citrus, almonds, vegetables, potatoes, tomatoes, oilseed rape, berries, and ornamentals; also used as a seed treatment	350–825

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Corrosive	Mild irritant	9000	>4500	5.8	Positive	Danger

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	25	Mutagenicity	Positive in vitro
Dog/66-week oral	60	Developmental	Positive in monkeys and hamsters
Mouse/18 months	—	Reproductive	No evidence
RfD (based on the rat reproduction study)	0.13	Oncogenicity	B2 (GI tract tumors in mice; kidney tumors in rats)

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com; Joint Meeting of the F. A. O. Panel of Experts on Pesticide Residues in Food and the Environment, W. H. O. Expert Group on Pesticide Residues, F. A. O. Panel of Experts on Pesticide Residues in Food, eds. Captan. Pesticide Residues in Food—1995: Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues: Geneva, Switzerland, September 16–27, 1995, FAO, Geneva, Switzerland, 1996; U.S. EPA, Captan: Amendment to the 1999 captan RED, U.S. Environmental Protection Agency, Washington, DC, 2004.

in mammals following exposure to type I pyrethroids[113] include the occurrence of fine tremors, hyperexcitability, and myoclonus (T-syndrome).

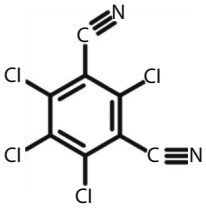
The type II pyrethroids are more narrowly defined in terms of their chemical structure. They contain an α-cyano-3-phenoxybenzyl alcohol, which increases insecticidal activity about 10-fold.[114] Clinical signs seen in mammals following exposure to type II pyrethroids[113] include sinuous writhing (choreoathetosis), salivation, hyperactivity, and clonic/tonic convulsions (CS-syndrome).

The distinction between types I and II pyrethroids was confirmed in a recent comparative acute neurotoxicity study aimed at providing a detailed description of clinical signs at the time of peak effect, using a modern functional observatory battery (FOB) of tests in rats treated with minimally effective or maximally tolerated doses of type I (bifenthrin, S-bioallethrin, permethrin, pyrethrin, resmethrin, and tefluthrin) and type II (β-cyfluthrin, cypermethrin, deltamethrin, esfenvalerate, fenpropathrin, and λ-cyhalothrin) pyrethroids. The FOB data, which were subjected to a principal components/factor analysis, confirmed that two major factors (T and CS) accounted for more than 90% of the variability in the group means. Dose responsiveness was observed, with marginally effective doses clustering with the control groups near the origin, whereas groups receiving larger doses tended to be deployed along the T or CS axes (Figure 16.9).

Despite clear differences in the profile of mammalian clinical signs between type I and type II pyrethroids, it is generally believed that the pyrethroids exert their effects by modifying the kinetic characteristics of the sodium channel function, largely based upon results from studies conducted ex vivo[115] and by the observation that the development of knockdown resistance (kdr-associated gene mutations) confers decreased sensitivity of insects to both DDT and the pyrethroid insecticides.[116,117]

In a comparative study where the $NA_V1.8$ mammalian sodium channel was expressed in xenopus oocytes,[118] the kinetics of the sodium channel response to electrical stimulation differed among 11 pyrethroids. Multidimensional scaling was used to quantify and to display the magnitude of differences between chemicals with respect to the kinetics of activation, fast inactivation, and tail current decay parameters (see Figure 16.10). The results show that in general, the pyrethroids cluster (e.g., are similar) with respect to sodium channel kinetic parameters along the type I/type II classification. Exceptions were noted for bifenthrin (type I), as well as for fenvalerate and fenpropathrin (type II), which appear intermediated between the predominant type I/type II groupings. Similar results have been seen in comparative

TABLE 16.28
Structure, Uses, and Hazard Profile for the Contact Chloronitrile Fungicide Chlorothalonil (FRAC Code M5)

Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Used on pome fruit, stone fruit, citrus, cane fruit, vegetables, corn, ornamentals, mushrooms, tobacco, soy, and turf	1000–2500

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Severe irritant	Mild irritant	>10,000	>10,000	0.093	Negative	Danger

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	2.0	Mutagenicity	No evidence
Dog/52 weeks	150	Developmental	Not teratogenic
Mouse/18 months	5.35	Reproductive	No evidence
ADI[2]	0.03 (JMPR)	Oncogenicity	Likely (forestomach tumors in mice; kidney tumors in rats) $Q_1^* = 7.6 \times 10^{-2}$ (mg/kg/day)$^{-1}$
RfD (Noncancer)	0.02		
RfD (Cancer)	0.015		

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; U.S. EPA, Chlorothalonil: Reregistration Eligibility Decision (RED), EPA-738-R-99-004, U.S. Environmental Protection Agency, Washington, DC, 1999; Wilkinson, C.F. and Killeen, J.C., *Regul. Toxicol. Pharmacol*. [10.1006/rtph.1996.0065], 24(1 Pt 1), 69, 1996; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com; Joint Meeting of the F. A. O. Panel of Experts on Pesticide Residues in Food and the Environment, W. H. O., Expert Group on Pesticide Residues, F. A. O. Panel of Experts on Pesticide Residues in Food, eds. Chlorothalonil. Pesticide Residues in Food—1992: Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues: Rome, September 21–30, 1992, FAO, Rome, Italy, 1993.

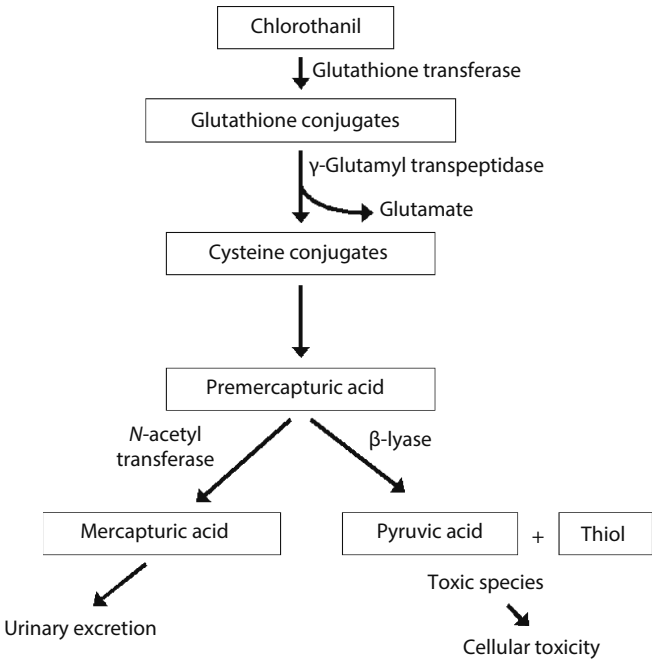


FIGURE 16.6 Proposed mechanism of action for chlorothalonil.

studies of the effects of pyrethroid insecticides on functional characteristics of the calcium[119] and chloride[120] channels (data not shown).

The voltage-gated sodium channel is formed by transmembrane proteins,[116] which in insects is comprised of an α-subunit that has four repeated-homologous domains (I–IV) each having six members (S1–S6) spanning the membrane, and these are connected to each other by intracellular and extracellular loops of amino acids (Figure 16.11). The S5 and S6 sections of each domain line the ion channel pore with S4 being the voltage sensor element.[121]

Nine sodium channel isoforms have been identified in mammals,[122] as compared to only one found in insects.[116] There is evidence of structural and functional homology between mammalian sodium channel isoforms and the insect sodium channel,[123] although there are exceptions. For example, Usherwood et al. showed that specific mutations that result in resistance to the pyrethroids do not confer resistance to DDT, contrary to expectation.[124] Gilles et al. reported that α-like toxin from the venom of scorpion, which inhibits sodium current inactivation in insects, is bound to receptor site three of the insect sodium channel, but did not bind to rat brain synaptosomes.[125] Gordon et al. reported that scorpion

TABLE 16.29
Insecticides Listed according to IRAC Classification System

Chemical Group	Common Name	IRAC Code	IRAC MOA	Biochemical Target Conserved in Mammals?	Other Biochemical Target(s) in Mammals?
Carbamates	Aldicarb Carbaryl Carbofuran Methomyl Propoxur (Tables 16.30 and 16.31)	1A	Inhibit acetylcholine esterase (AChE)	Yes	Common MOA grouping based upon AChE inhibition
Organophosphates	Acephate Azinphos-methyl Chlorpyrifos Diazinon Dichlorvos Malathion (Tables 16.32 and 16.33)	1B			
Cyclodiene organochlorines	Endosulfan	2A	Block inhibitory circuits (GABA) leading to hyperexcitation	Likely	Possible
Phenylpyrazoles	Fipronil (Table 16.34)	2B			
Pyrethroids Pyrethrins	Bioallethrin Cyfluthrin Cypermethrin Deltamethrin Lambda-cyhalothrin Permethrin Resmethrin Tefluthrin (Tables 16.35 through 16.38)	3A	Delay sodium ion channel inactivation leading to hyperexcitation	Likely	Common MOA grouping yet to be determined
Organochlorines	Methoxychlor (Table 16.39)	3B			
Neonicotinoids	Imidacloprid Thiamethoxam	4A	Activate nicotinic receptors leading to hyperexcitation	Likely	Common MOA grouping to be determined
Sulfoxaflor	Sulfoxaflor (Table 16.40)	4C	Activate nicotinic receptors leading to hyperexcitation	Yes	
Spinosyns	Spinosad Spinetoram	5	Likely activate nicotinic receptors leading to hyperexcitation	Likely	Common MOA grouping yet to be determined
Avermectins Milbemycin	Abamectin Emamectin benzoate Milbemycin (Table 16.41)	6	Simulates a GABA-like activation of the chloride channel leading to hyperexcitation	Likely	Common MOA grouping yet to be determined
Sesquiterpenoids	Methoprene	7A	Mimics JH3; interferes with molting	Possible	None known
Carbamates	Fenoxycarb	7B			
Alkoxypyrimidine	Pyriproxyfen	7C			
Azomethine pyridines	Pymetrozine (Table 16.42)	9B	Selective homopteran feeding blockers affects feeding behavior	Unlikely	None known
Phenyltetrazines	Clofentezine	10	Inhibits mite growth	Unknown	None known
Carboxamides	Hexythiazox (Table 16.43)				
Bacillus thuringiensis toxin	*B.t.* sp. Aizawai *B.t.* sp. Kurstaki (Table 16.44)	11A	Produce toxins that bind to protein receptors in the midgut of insects and subsequently form pores in the insect midgut epithelium[172]	Unlikely	None known

(*continued*)

TABLE 16.29 (continued)
Insecticides Listed according to IRAC Classification System

Chemical Group	Common Name	IRAC Code	IRAC MOA	Biochemical Target Conserved in Mammals?	Other Biochemical Target(s) in Mammals?
Pyrroles	Chlorfenapyr (propesticide requiring oxidative N-dealkylation to the NH derivative) (Tables 16.48 and 16.49)	13	Disrupt proton gradient by transporting protons across the mitochondrial membrane	Yes	None known
Benzylureas	Diflubenzuron (Table 16.45)		Blocks chitin biosynthesis; interferes with molt	Unlikely	None known
Aminotriazines	Cyromazine (Table 16.43)	17	Dipteran molting disruptor	Unlikely	None known
Diacylhydrazine	Tebufenozide (Table 16.46)	18	Ecdysone receptor agonist	Possible	None known
Formamidine	Amitraz (Table 16.47)	19	Octopaminergic receptor agonist	Yes—Homologous receptor is the α_2 adrenergic receptor	Inhibit synthesis of monoamine oxidase and prostaglandin E2[185]
Hydramethylnon	Hydramethylnon	20A	Block electron transport in mitochondrial complex III	Yes	Complex III, major site of superoxide radical formation; inhibition is detrimental to developing cardiac cells[328]
Amidinohydrazones	Pyridaben	21A	Block electron transport in mitochondrial complex I	Yes	Induces autophagic and cell death mediated by reactive oxygen species[329]
Pyridazinones	Rotenone	21B			
Semicarbazones	Metaflumizone (Tables 16.48 and 16.49)	22B	Blocks sodium channels by binding selectively to the slow-inactivated state	Yes	None known
Diamides	Chlorantraniliprole Cyantraniliprole Flubendiamide (Table 16.50)	28	Ryanodine receptor modulators	Yes	Ryanodine causes release of calcium from stores in the sarcoplasmic reticulum leading to massive muscular contractions; this is true for mammals and insects

Source: IRAC. Mode of action classification, Version 7.1, 2012.

toxin binds to homologous but not identical receptor sites in rat brain and insect sodium channels.[126]

In recent years, there has been evidence of a biological basis for a common mechanism of toxicity for type I and type II pyrethroids in terms of their effects on the sodium, calcium, and GABA-gated chloride channels.[127–129] In 2009, the EPA concluded that although there is support for the separation of type I and type II subgroups based on α-cyano group absence or presence, the in vivo evidence for the distinction is less apparent, based on functional observational batteries.[130]

The structures and use of some of economically important noncyano (type I) pyrethroids are given in Table 16.35 and their hazard profiles appear in Table 16.36. Comparable information for type II (cyano-substituted) pyrethroids is given in Tables 16.37 and 16.38.

The synthetic pyrethroids are generally effective as insecticides in the low grams per hectare range. In general, the mammalian acute toxicity of the type II pyrethroids is greater than type I pyrethroids. Because the pyrethroids are highly lipophilic, the acute oral LD_{50} can differ by up to 40-fold depending on the lipophilic characteristics and the volume of the vehicle used to administer the chemical. For example, deltamethrin's LD_{50} is reported to be in the range from 128 mg/kg to greater than 5000 mg/kg.[131] Since pyrethroids are 500–4500 times less toxic to mammals than to insects, they have been safely used with relatively few reports

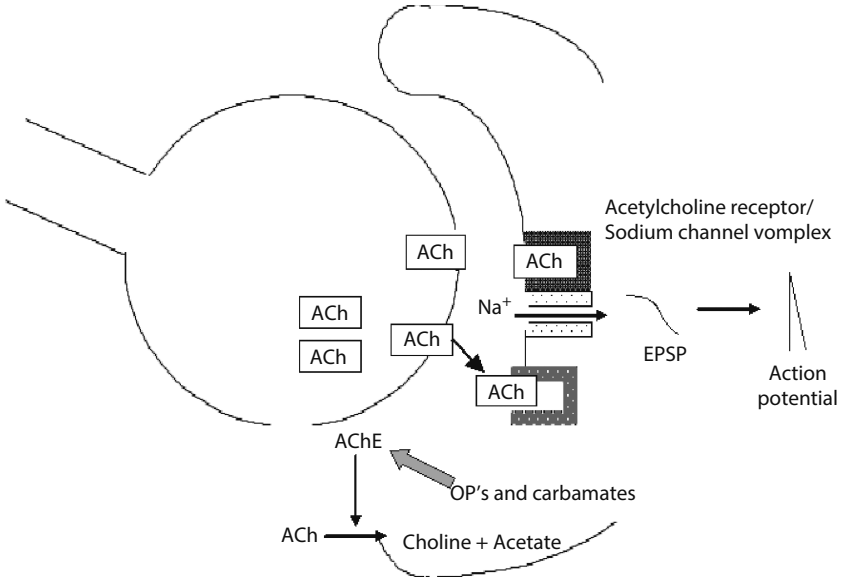


FIGURE 16.7 Pre-/postsynaptic events associated with acetylcholine-mediated neurotransmission.

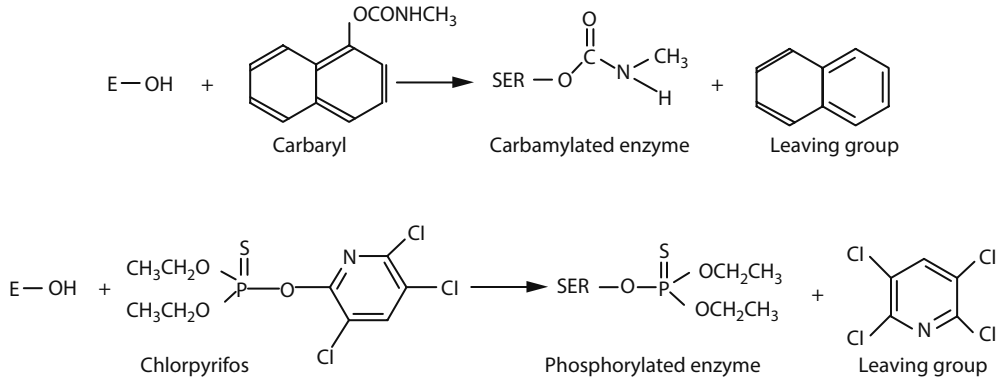


FIGURE 16.8 Interaction of organophosphate and carbamate insecticides with AChE.

of human poisoning.[132] Aside from acute neurotoxicity, the hazard profile for the pyrethroids is unremarkable, although a treatment-related increase in the incidence of lung and liver tumors has been reported for permethrin.[133]

Organochlorine: Sodium Channel Modulators (IRAC Code 3B)

The organochlorines are one of the oldest groups of synthetic insecticides, dating back to the early 1940s.[134] These lipophilic compounds are environmentally stable and persistent, and many like dieldrin, endrin, and DDT have been banned in the United States. However, more biodegradable materials like endosulfan still have limited use today in some countries. Fipronil, which is an arylheterocycle with a similar MOA, has improved selective toxicity toward insects.

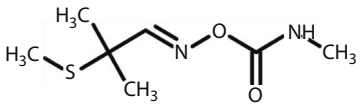
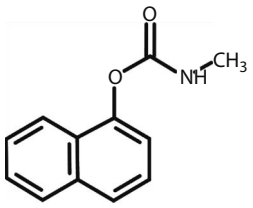
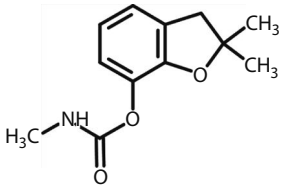
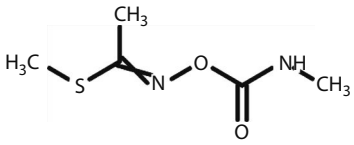
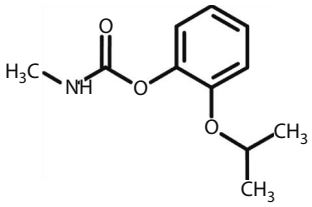
The organochlorine insecticides induce repetitive action potentials by slowing the kinetics of sodium channel activation and inactivation (closing), resulting in prolonged tail currents that cause a state of hyperstimulation of the CNS.[135] Because organochlorines are highly lipophilic, and because of the relatively small size of insects and their lower body temperatures, they more readily reach their target (the sodium channel in the nervous system), and they have a greater effect (approximately 500–4500 times greater) in insects than in mammals.[135]

The structure, uses, and hazard profile for methoxychlor are provided in Table 16.39. Methoxychlor induces weak estrogenic activity.[136]

Neonicotinoids and Sulfoxaflor–Acetylcholine Receptor Agonists (IRAC Codes 4A and 4C)

Nicotine has been used as a contact insecticide since the middle of the eighteenth century.[37] Nicotine mimics the action of acetylcholine, which is a major excitatory neurotransmitter in the CNS. Nicotine, which acts as a ligand to the postsynaptic nicotinic acetylcholine receptor (nAchR), activates an intrinsic cation channel resulting in the depolarization of the postsynaptic cell due to an influx of sodium. The resulting EPSP triggers an action potential if there is sufficient degree

TABLE 16.30
Structures and Use Profiles for Carbamate AChE-Inhibiting Insecticides (IRAC Code 1A)

Insecticide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Aldicarb (Temik®)[37,280]		Controls chewing and sucking insects in vegetables and crops	500–3400
Carbaryl (Sevin®)[37,280]		Controls chewing and sucking insects in vegetables and various crops	250–2000
Carbofuran (Furadan®)[37,280]		Controls soil dwelling and foliar feeding insects in food crops	260–2050
Methomyl (LanoxC®)[37,280]		Controls chewing and sucking insects in vegetables, food crops, and turf	120–2000
Propoxur (Aprocarb®)[37,280]		Controls cockroaches, flies, fleas, ants, and mosquitoes	1200

of membrane depolarization. Persistent activation of the nicotinic acetylcholine receptors result in an overstimulation of the cholinergic neurotransmission system, resulting in hyperexcitation, convulsions, paralysis, and death.

The neonicotinoids, represented here by imidacloprid and thiamethoxam, are nicotine-like agonists that are used as insecticides. These chemicals, which are absorbed by plants either following foliar application or applied as seed treatment, are effective in controlling piercing and sucking insects such as aphids, leafhoppers, and whiteflies.[137] While the neonicotinoids act as ligands on homologous receptors in insects and vertebrates, the affinity of the neonicotinoid for the insect nicotinic acetylcholine receptor is reported to be 5- to 3500-fold greater than that observed in vertebrates.[137,138] The molecular basis for differences in affinity has been proposed,[137,139] and molecular design aimed at achieving greater selectivity for insects has been discussed.[140] Differences in absorption, distribution, metabolism, and elimination are also expected to play a role in insect selectivity. A common mechanism grouping for neonicotinoid insecticides has not yet been proposed, but appears to be supported by what is known about their MOA.

The structures, uses, and hazard profiles for imidacloprid, thiamethoxam, and sulfoxaflor are presented in Table 16.40. Imidacloprid and thiamethoxam are moderately acutely toxic by the oral route and are much less toxic to mammals than nicotine. Imidacloprid is not a developmental toxin or a carcinogen. High doses of thiamethoxam caused testicular effects in the multigeneration reproduction study and caused liver tumors in the mouse. A MOA underlying the occurrence of the mouse liver tumors has been described,[141,142] and the EPA has classified thiamethoxam as not likely a human carcinogen.

Spinosyns: Acetylcholine Receptor Agonists (IRAC Code 5)

Two of the most important spinosyns are spinosad and spinetoram. Spinosad, a fermentation-produced macrolide, was initially derived from the soil actinomycete *Saccharopolyspora spinosa* and is comprised of spinosyns A and D (Figure 16.12). It is highly toxic to *Lepidopteran*, *Dipteran*, and some *Coleopteran* insects.[143] Symptoms seen in insects include CNS hyperexcitation, involuntary muscle

TABLE 16.31
Hazard Profiles for Carbamate AChE-Inhibiting Insecticides (IRAC Code 1A)

	Irritation		LD_{50} (mg/kg)				
Insecticide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Aldicarb	Nonirritant	Nonirritant	0.93	20	0.2	Negative	Danger
Carbaryl	Nonirritant	Nonirritant	500	>4000	206	Negative	Caution
Carbofuran	Mild irritant	Mild irritant	8	>3000	0.075	Negative	Danger
Methomyl	Irritant	Nonirritant	17	>5000	0.3	NA	Danger
Propoxur	Slight irritant	Nonirritant	50	>5000	0.5	Negative	Warning

Insecticide	**Species/Study**	**NOEL (mg/kg/day)**	**Toxicity Study**	**Hazard Indicator**
Aldicarb[330,331]	Rat/2 years	0.3	Mutagenicity	Not mutagenic
	Dog/104 weeks	0.1	Developmental	Not teratogenic
	Mouse/18 months	0.3	Reproductive	Not a reproductive toxin
	ADI	0.003	Oncogenicity	D
	RfD	0.001		
Carbaryl[332–334]	Rat/2 years	200	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.43	Developmental	Not teratogenic
	Mouse/18 months	—	Reproductive	Not a reproductive toxin
	Human	0.01	Oncogenicity	E (No evidence)
	RfD	0.01		
Carbofuran[335,336]	Rat/2 years	20	Mutagenicity	Not mutagenic
	Dog/2-year oral	10	Developmental	Not teratogenic
	Mouse/18 months	20	Reproductive	Not a reproductive toxin
	RfD	0.002	Oncogenicity	C with RfD (mouse liver tumors in both sexes)
Methomyl[125,337,338]	Rat/2 years	200	Mutagenicity	Not mutagenic
	Dog/52 weeks	200	Developmental	Teratogenic in mice
	Mouse/18 months	500	Reproductive	Not a reproductive toxin
	ADI	0.02	Oncogenicity	C with RfD (liver tumors—female mice)
Propoxur[339]	Rat/2 years	5.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.25	Developmental	Not teratogenic
	Mouse/18 months	40	Reproductive	Not a reproductive toxin
	ADI	0.01	Oncogenicity	C with RfD

contraction, and tremors, which ultimately result in neuromuscular fatigue and paralysis.[143,144] Spinosyns are believed to be nicotinic acetylcholine receptor (nAchR) agonists, although the experimental evidence supporting this proposed MOA remains inconclusive.[145] In addition to the proposed effects mediated by binding to nAchR, the spinosyns caused a dose-responsive reduction in the response to GABA rundown in isolated small-diameter cockroach neurons,[146] an effect that is probably mediated through the chloride channel/current.

The hazard profile for spinosad, which is shown in Table 16.41, suggests that the MOA is highly selective for insects. Spinosad is not very acutely toxic to mammals (Oral LD_{50} = 3738 mg/kg), and it is not neurotoxic.[147] Spinosad is listed at the Organic Materials Research Institute (OMRI) as approved for certified organic production under the USDA's National Organic Program §[206.207](e).[111,112]

Spinetoram is more active than spinosad in terms of its effects on insects, and it is created synthetically via 3′-*O*-ethylation of the rhamnose sugar moiety found in naturally occurring spinosyns and then modified further by 5,6-hydrogenation of the tetracyclic ring system.[37] The hazard profile for spinetoram is also included in Table 16.41.

Avermectins and Milbemycin: Chloride Channel Activators (IRAC Code 6)

The avermectins, along with milbemycin, comprise a group of closely related 16-membered macrocyclic lactones, isolated from *Streptomyces avermitilis* and *Streptomyces hygroscopicus*.[148] The chemical structures for abamectin, emamectin benzoate, and milbemycin are shown in Figure 16.13. These potent acaracides cause signs of ataxia, paralysis, and death, but the hyperexcitation typically found with most other insecticides is absent.[149] While a number of pharmacologic effects of ivermectin have been described,[150] it is generally agreed that the principal MOA of this class is an activation of chloride ion current by a GABA-like opening of the chloride channel.[109,151] In addition, opening of a glutamate-gated chloride channel has also been implicated.[152,153]

TABLE 16.32
Structures and Use Profiles for the Organophosphate AChE-Inhibiting Insecticides (IRAC Code 1B)

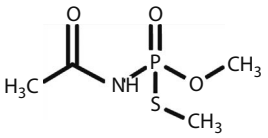
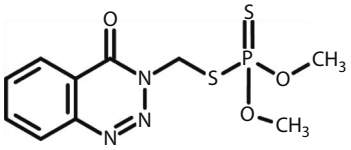
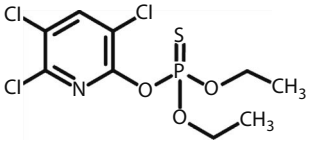
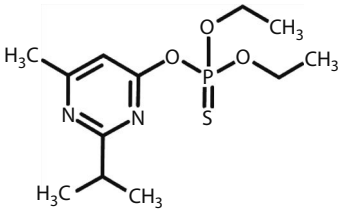
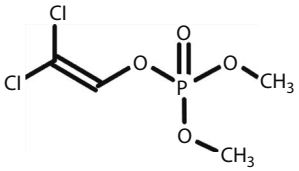
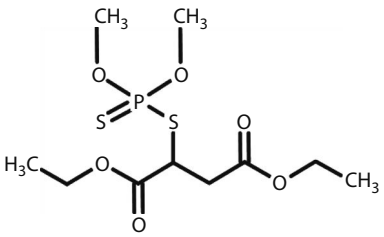
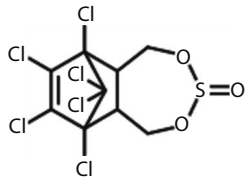
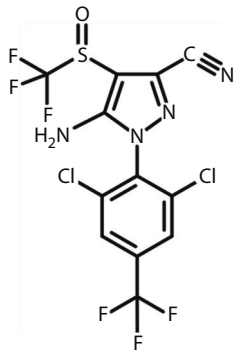
Insecticide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Acephate (Amithene®)[37,280]		Control of sucking and chewing insects	500–1000
Azinphos-methyl (Guthion®)[37,280]		Control of sucking and chewing insects	—
Chlorpyrifos (Lorsban®)[37,280]		Control of sucking, chewing, and boring insects	300–600
Diazinon (Spectracide®)[37,280]		Control of sucking and chewing insects, as well as mites	300–600
Dichlorvos (Vapona®)[37,280]		Control of sucking insects, chewing insects, and spider mites in household sprays, etc.	100
Malathion (Acimal®)[37,280]		Control of sucking and chewing insects	500–1250

TABLE 16.33
Hazard Profiles for the Organophosphate AChE-Inhibiting Insecticides (IRAC Code 1B)

	Irritation		LD_{50} (mg/kg)				
Insecticide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Acephate	—	Nonirritant	866	>2000	>15	Negative	Caution
Azinphos-methyl	Mild irritant	Nonirritant	6–19	150	0.15	Positive	Danger
Chlorpyrifos	Nonirritant	Nonirritant	2680	>2000	>0.67	Negative	Caution
Diazinon	Nonirritant	Nonirritant	1250	>2150	2.33	Negative	Caution
Dichlorvos	Irritant	Irritant	50	90	0.34	Negative	Danger
Malathion	NA	NA	1000E	4100	>5.2	NA	Caution

Insecticide	**Species/Study**	**NOEL (mg/kg/day)**	**Toxicity Study**	**Hazard Indicator**
Acephate[340,341]	Rat/2 years	0.5	Mutagenicity	Not mutagenic
	Dog/26-week oral	0.75	Developmental	Not teratogenic
	Mouse/18 months	—	Reproductive	Not a reproductive toxin
	Human	0.3	Oncogenicity	C (Mouse liver tumor)
	ADI (Human; UF = 10)	0.03		
Azinphos-methyl[342,343]	Rat/2 years	0.86	Mutagenicity	Effects in vitro; not in vivo
	Dog/52-week oral	0.74	Developmental	Not teratogenic
	Mouse/18 months	0.88	Reproductive	Effects on fertility
	Human	0.005	Oncogenicity	E (No evidence)
	ADI	0.005		
Chlorpyrifos[344,345]	Rat/2 years	0.1	Mutagenicity	Not mutagenic
	Dog/13-week oral	10	Developmental	Not teratogenic
	Mouse/18 months	3.9	Reproductive	Not a reproductive toxin
	Human	0.1	Oncogenicity	No evidence
	ADI (UF = 10)[1]	0.01		
Diazinon[346,347]	Rat/2 years	0.07	Mutagenicity	Not mutagenic
	Dog/2-year oral	0.02	Developmental	Not teratogenic
	Mouse/18 months	—	Reproductive	Not a reproductive toxin
	Human	0.025	Oncogenicity	No evidence
	ADI (UF = 10)[1]	0.002		
Dichlorvos[348,349]	Rat/2 years	2.4	Mutagenicity	May be mutagenic
	Dog/52 weeks	—	Developmental	Not teratogenic
	Mouse/18 months	10	Reproductive	Not a reproductive toxin
	Human/21 days	0.04	Oncogenicity	No evidence
	ADI (UF = 10)[1]	0.004		
Malathion[350–352]	Rat/2 years	3.0	Mutagenicity	No evidence
	Dog/52 weeks	<62.5	Developmental	Not teratogenic
	Mouse/18 months	143	Reproductive	Effects on litter size
	Human/56 days	0.34		
	ADI		Oncogenicity	Suggestive

TABLE 16.34
Structures, Uses, and Hazard Profiles for the Organochlorine GABA-Gated Chloride Channel Antagonists (IRAC Codes 2A and 2B)

Insecticide	Structure	Principal Use	Application Rate (g a.i./ha)
Endosulfan (Thiodan®)[37,280]		Used to control sucking, chewing, and boring insects in an variety of crops including fruit, vines, vegetables, cotton, and cereal	800–2500 L/ha
Fipronil (Regent®)[37,280]		Used to control thrips, corn root worms, and termites	10–200

	Irritation		LD_{50} (mg/kg)				
Insecticide	Eye	Skin	Oral	Potential	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Endosulfan	Nonirritant	Nonirritant	70	359	>0.034	Negative	Danger
Fipronil	Nonirritant	Nonirritant	97	>2000	0.68	Negative	Warning

Insecticide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Endosulfan[351,352]	Rat/2 years	0.60	Mutagenicity	Not mutagenic
	Dog/52 weeks	0.57	Developmental	Not teratogenic
	Mouse/18 months	0.84	Reproductive	Not a reproductive toxin
	ADI	0.006	Oncogenicity	No treatment-related evidence for tumorigenicity in rats or mice
Fipronil[353,354]	Rat/2 years	0.20	Mutagenicity	Not mutagenic
	Dog/52 weeks	0.30	Developmental	Not teratogenic
	Mouse/18 months	0.50	Reproductive	Not a reproductive toxin
	RfD	0.0002	Oncogenicity	No treatment-related evidence for tumorigenicity in rats or mice

The toxicity profiles for abamectin, emamectin benzoate, and milbemycin are given in Table 16.41. Abamectin and emamectin are neurotoxic in mammals, which exhibit hyperexcitability, tremors, incoordination, ataxia, and coma-like sedation.[154] Much of the early hazard evaluation for abamectin and emamectin was conducted in the wild-type CF-1 mouse,[154,155] which has been found to be heterozygous for p-glycoprotein.[156–159] The toxic effects of abamectin were reduced in studies using animals having a fully intact p-glycoprotein blood–brain barrier, supporting the idea that differential expression of p-glycoprotein, which is a substrate for the avermectins, might account for differences in selectivity among species.[160] The importance of an intact blood–brain barrier in humans has been considered.[161]

Milbemycin is less acutely toxic than the avermectins, and it is not a developmental toxin in animal bioassays. Its toxicity profile is also found in Table 16.41.

Juvenile Hormone Mimics and Selective Feeding Blockers (IRAC Codes 7A, 7B, 7C, and 9B)

Juvenile hormones modulate an extraordinarily broad range of morphological and physiological processes during larval development and metamorphosis,[162] in addition to having effects on various aspects of adult reproduction and behavior.[163] Juvenile hormones are terpenoid-based compounds (Figure 16.14) that from an evolutionary point of view could be precursors to steroids and retinoids, which are also terpenoid derivatives.[164] The failure to identify classical nuclear

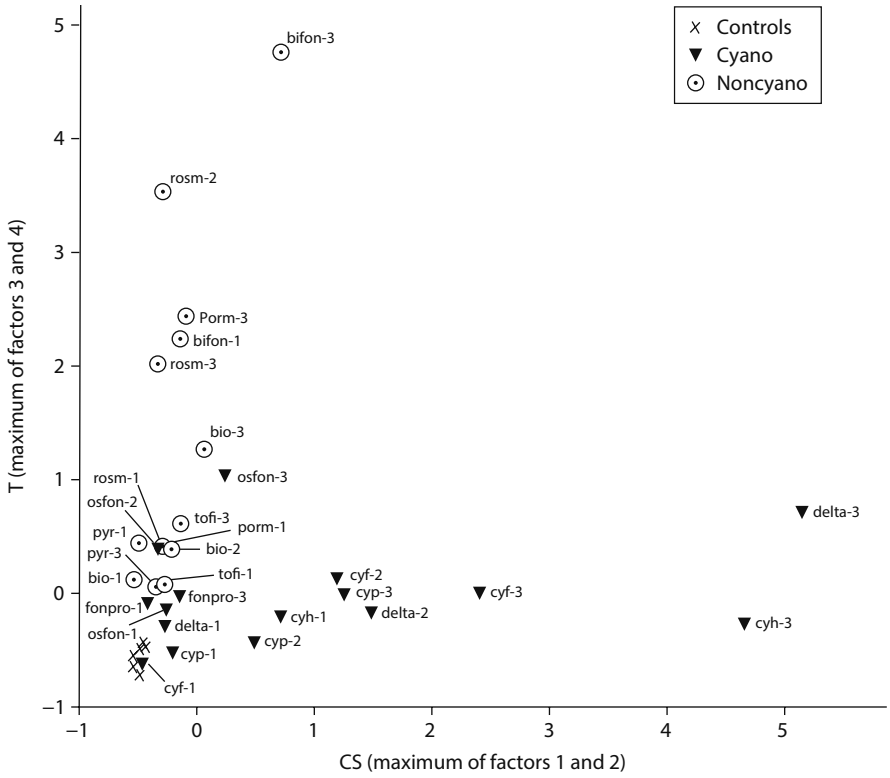


FIGURE 16.9 Results from a principal components/factor analysis of FOB data.

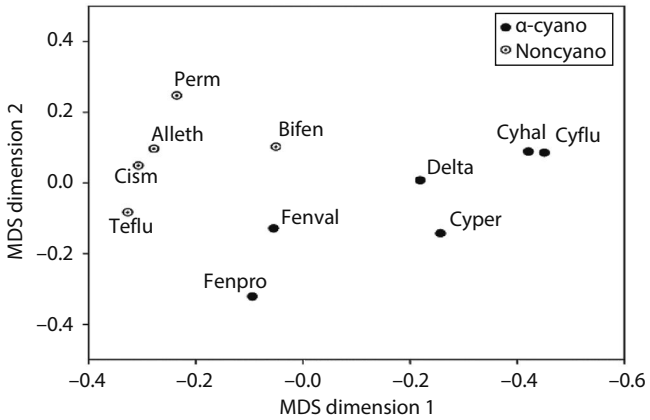


FIGURE 16.10 Multidimensional scaling of sodium channel kinetic parameters.

receptors for juvenile hormones, despite decades of effort,[165] may be attributed to the possibility that juvenile hormones signal through membrane receptors.[166] Because juvenile hormones are capable of binding to a large number of proteins, they may exert their effects by binding to G-coupled membrane receptors, which could in turn trigger a cascade of intracellular MAP kinase signaling molecules, such as those that have been described for gonadotropin-releasing hormone in mammals.[167]

The juvenile hormone mimics (JHMs) are compounds that bear a structural resemblance to the juvenile hormones

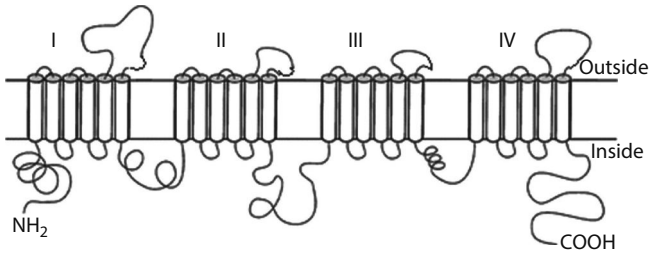


FIGURE 16.11 Schematic of the transmembrane voltage-gated sodium channel.

in insects, which are lipophilic sesquiterpenoids containing an epoxide and methyl ester groups (see Figure 16.14). These chemicals mimic the action of juvenile hormones affecting a number of physiological processes, such as molting and reproduction. Exposure to JHMs at molting cause death by producing mixed larval/pupal or larval/adult morphologies. The efficacy of these compounds is greatest when normal juvenile hormone titers are low, namely, in the last larval or early pupal stages.[162,165,168]

The structures, uses, and hazard profiles of three JHMs (methoprene, fenoxycarb, and pyriproxyfen) and the feeding inhibitor pymetrozine[169] are presented in Table 16.42. These chemicals are not acutely toxic, and no endocrinological effects have been reported for methoprene.[170]

High doses of pymetrozine caused liver tumors in male and female mice, as well as in female rats, and fenoxycarb induced

TABLE 16.35
Structure and Use Profiles for the Noncyano Pyrethroid Insecticides (IRAC Code 3)

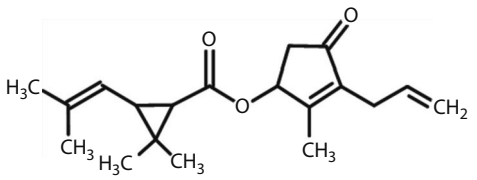
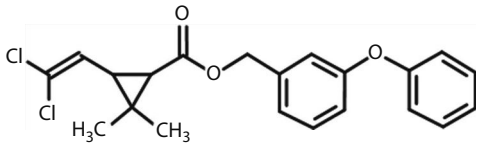
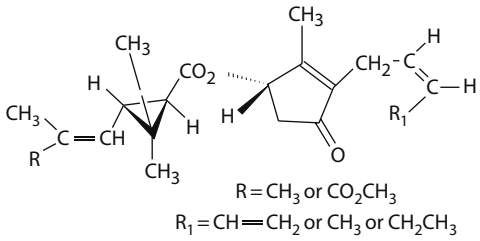
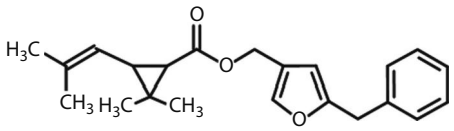
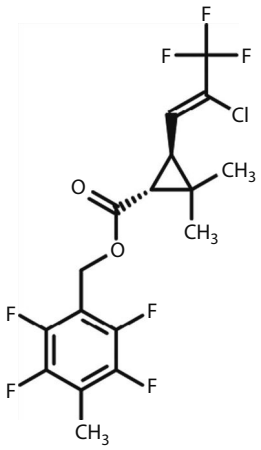
Insecticide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Bioallethrin[37,280]		Contact, nonsystemic, nonresidual with rapid knockdown used against household insects	—
Permethrin (Ambush®)[37,280]		Controls leaf- and fruit-eating *Lepidoptera* and *Coleoptera* in cotton	25–200
Pyrethrins (Pyrethrum)[37,280]		Contact, nonsystemic used to control a wide range of insects and mites in public health, stored products, animal houses, domestic animals, and farm animals	1400–2800
Resmethrin (Crossfire®)[37,280]		Contact, nonsystemic used to control insecticides on agricultural, horticultural, household, and public health pests	4–10
Tefluthrin (Force™)[37,280]		Contact nonsystemic insecticide used to control soil pests of corn, sugar beet, wheat, and other crops	50–200

TABLE 16.36
Hazard Profiles for Noncyano Pyrethroids (IRAC Code 3)

	Irritation		LD_{50} (mg/kg)				
Insecticide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Bioallethrin	Nonirritant	Nonirritant	53.8	>2000	2.5	Negative	Warning
Permethrin	Nonirritant	Nonirritant	430	>2000	>0.68	Moderate	Warning
Pyrethrin	—	—	1030	>1500	3.4	Sensitizer	Warning
Resmethrin	Nonirritant	Nonirritant	>2500	>3000	9.49	Negative	Caution
Tefluthrin	—	Slight irritant	21.8	316	0.037	Sensitizer	Danger

Insecticide	**Species/Study**	**NOEL (mg/kg/day)**	**Toxicity Study**	**Hazard Indicator**
Bioallethrin[127,357]	Rat/2 years	5	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.5	Developmental	Not teratogenic
	Mouse/18 months	2.5	Reproductive	Not a reproductive toxin
	ADI	0.04	Oncogenicity	Not carcinogenic
	Acute NOEL	1		
Permethrin[133,358]	Rat/2 years	5.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	5.0	Developmental	Not teratogenic
	Mouse/18 months	7.1	Reproductive	Not a reproductive toxin
	RfD	0.05	Oncogenicity	C with RfD (lung and liver tumors in female mice)
Pyrethrins[359]	Rat/2 years	25	Mutagenicity	Not mutagenic
	Dog/52 weeks	—	Developmental	Not teratogenic
	Mouse/18 months	143	Reproductive	Not reproductive toxin
	ADI	0.125	Oncogenicity	Not carcinogenic
	Acute NOEL	—		
Resmethrin[360,361]	Rat/2 years	<25	Mutagenicity	Not mutagenic
	Dog/6-month oral	10	Developmental	Not teratogenic
	Mouse/18 months	50	Reproductive	Not a developmental toxin
	ADI	0.1	Oncogenicity	Not carcinogenic
	Acute NOEL	—		
Tefluthrin[362]	Rat/2 years	4.6	Mutagenicity	Not mutagenic
	Dog/52 weeks	0.5	Developmental	Delayed development
	Mouse/18 months	3.4	Reproductive	Not a reproductive toxin
	ADI	0.005	Oncogenicity	Not carcinogenic
	Acute NOEL	0.5		

lung tumors in mice, presumably through its metabolite ethyl carbamate, which is known to cause murine lung tumors.[171] Difolatan, a structural analogue of fenoxycarb, which does not have the ethyl carbamate moiety, does not cause lung tumors in mice. There does not appear to be a basis for creating a common mechanism grouping for this diverse group of chemicals, because the juvenile hormone is unique to insects.

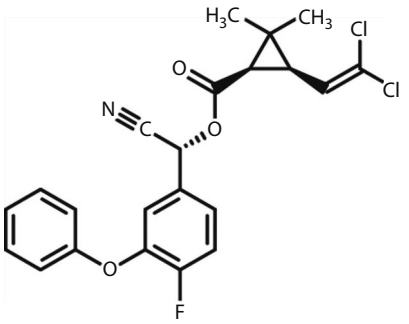
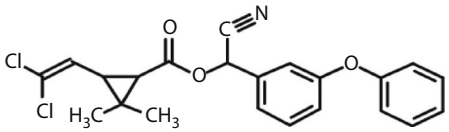
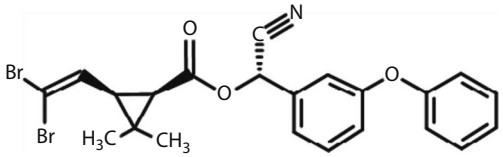
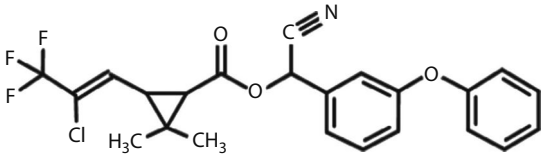
Phenyltetrazines and Aminotriazines: Larvicides/Molt Disruptors (IRAC Codes 10A and 17)

The structures, uses, and hazard profiles for a diverse group of insecticides that are larvicidal (cyromazine) and/or molting disruptors (clofentezine and hexythiazox) through unknown or nonspecific MOA are presented in Table 16.43. These insecticides are not acutely toxic and do not have effects on development. Clofentezine causes thyroid tumors in male rats, and hexythiazox causes liver tumors.

Delta-Endotoxins Derived from *Bacillus thuringiensis* (IRAC Code 11A)

Bacillus thuringiensis (Bt) forms a crystalline inclusion body during sporulation that contains a number of insecticidal proteins.[37] When consumed by an insect, the inclusion body is dissolved in the midgut, and it subsequently releases delta-endotoxins. Mixtures of different delta-endotoxins are usually present in the inclusion body, and individual toxin proteins are designated with the prefix cry. These proteins contain anywhere from a few hundred to over a thousand amino acids. After they are ingested, delta-endotoxins are cleaved to an active form by proteases within the midgut. The active toxins bind specifically to the membranes of the midgut epithelia and alter their ion permeability by forming a cation channel or pore. Ion movements through this pore disrupt potassium and the pH gradients and lead to lysis of the epithelium, gut paralysis, and eventual death.[3,172,173]

TABLE 16.37
Structure and Use Profiles for the α-Cyano Pyrethroid Insecticides (IRAC Code 3)

Insecticide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Cyfluthrin (Baythroid®)[37,280]		Contact, nonsystemic insecticide used on cereals, cotton, fruit, and vegetables	15–40
Cypermethrin (Ammo™)[37,280]		Contact, nonsystemic insecticide used on fruits, vines, coffee, cereals, and ornamentals	10–100
Deltamethrin (Crackdown™)[37,280]		Contact, nonsystemic insecticide used on cereals, citrus, cotton, grapes, cotton, maize, oilseed rape, and soybeans	2.5–21
Lambda-cyhalothrin (Karate®)[37,280]		Controls a broad spectrum of chewing and piercing insects on cereals, hops, ornamentals, vegetables, and cotton	2–5

Delta-endotoxins derived from Bt have been used as insecticides for over 30 years, and more recently, plants have been genetically engineered to express delta-endotoxins. Considering that these bacterial strains of Bt are found in nature and that there is no evidence of adverse effects on human health resulting from the use of delta-endotoxin as an insecticide,[174–177] the EPA requires a limited toxicological assessment of new Bt products. Acute oral toxicity, in vitro digestibility, and infectivity/pathogenicity studies are required, along with evaluations of amino acid homologies.[177] Bt is also listed at OMRI for use in organic crop protection methods.[111,112] The structures, uses, and hazard profiles for delta-endotoxins derived from Bt subspecies are shown in Table 16.44.

Benzoylureas: Chitin Synthesis Inhibitors (IRAC Code 15)

The benzoylureas, represented here by diflubenzuron, block molting in insects by preventing the formation of a new cuticle exoskeleton, which is comprised of about 50% chitin. Chitin is a polysaccharide comprised of *N*-acetylglucosamine. It has been proposed that polymerization is blocked by the benzoylureas by (1) the inhibition of chitin synthase or its biosynthesis, (2) the inhibition of proteases or their biosynthesis, or (3) the inhibition of a membrane-transport step involving UDP-*N*-acetylglucosamine.[178] These biochemical pathways are not present in mammals.[179]

Diflubenzuron is not toxic to mammals. It is highly lipophilic, and it therefore tends to bioconcentrate in fat, which has led to the expression of delayed toxicity in animal studies for some members of this class (including lufenuron), apparently due to tissue bioaccumulation. The structure, uses, and hazard profile of diflubenzuron are presented in Table 16.45.

Diacylhydrazine Ecdysone Agonists (IRAC Code 18A)

The hormone 20-hydroxyecdysone (20E) is synthesized by insects either from cholesterol or from phytosteroids, which they obtain from their diets, as insects do not possess the biochemical pathways needed to synthesize the steroid nucleus.[180] Ecdysone levels, titered in hemolymph, rise progressively up until the time of molting, and then they fall precipitously. Ablation of the eyestalk, the source of this hormone in crustaceans, results in a failure of molting behavior, with no change in ecdysteroid concentration in hemolymph.[181] The ecdysteroid hormone, 20E, apparently mediates this effect on molting and reproductive function by binding to the nuclear ecdysteroid receptor, EcR.[182] The ecdysone agonists,

TABLE 16.38
Hazard Profiles for α-Cyano Pyrethroids (IRAC Code 3)

	Irritation		LD_{50} (mg/kg)				
Insecticide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Cyfluthrin	Mild irritant	Nonirritant	500	>5000	0.5	Negative	Warning
Cypermethrin	Irritant	Irritant	247	>4920	2.5	Weak sensitizer	Warning
Deltamethrin	Mild irritant	Nonirritant	>5000	>2000	2.2	Negative	Caution
Lambda-cyhalothrin	Nonirritant	Mild irritant	56	632	0.60	Negative	Danger

Insecticide	**Species/Study**	**NOEL (mg/kg/day)**	**Toxicity Study**	**Hazard Indicator**
Cyfluthrin[363]	Rat/2 years	50	Mutagenicity	Not mutagenic
	Dog/52 weeks	5	Developmental	Not teratogenic
	Mouse/18 months	200	Reproductive	Not a reproductive toxin
	ADI	0.02	Oncogenicity	Not carcinogenic
	Acute NOEL	20		
Cypermethrin[364,365]	Rat/2 years	7.5	Mutagenicity	Not mutagenic
	Dog/52 weeks	5	Developmental	Not teratogenic
	Mouse/18 months	14	Reproductive	Not a reproductive toxin
	ADI	0.05	Oncogenicity	Not carcinogenic
	Acute NOEL	5		
Deltamethrin[127,131,366]	Rat/2 years	1	Mutagenicity	Not mutagenic
	Dog/2-year oral	1	Developmental	Not teratogenic
	Mouse/18 months	12	Reproductive	Not a reproductive toxin
	ADI	0.01	Oncogenicity	Not carcinogenic
	Acute NOEL	1		
Lambda-cyhalothrin[367]	Rat/2 years	2.5	Mutagenicity	No evidence
	Dog/52 weeks	0.1	Developmental	Not teratogenic
	Mouse/18 months	14.2	Reproductive	No evidence
	ADI	0.001	Oncogenicity	D (not classifiable)
	Acute NOEL	0.5		

represented here by tebufenozide, are lethal to lepidopteran pests by inducing premature molting.[162] The toxicity of tebufenozide (RH-5992) to insect larvae is proportional to its binding affinity to EcR proteins (Figure 16.15).[183]

The EcR receptor is comprised of at least two proteins, which are gene products of the EcR and USP (ultraspiracle) genes. These genes are members of the steroid hormone receptor superfamily and are the insect homologues of the vertebrate retinoid X-receptor gene.[184] As such, it is possible that ecdysone agonists could have affinity for vertebrate receptor proteins.

The structure, uses, and hazard profile of tebufenozide are presented in Table 16.46. It is neither acutely toxic nor a developmental or reproductive toxin.

Octopaminergic Agonists and Monoamine Oxidase Inhibitors (IRAC Code 19)

Octopamine is an excitatory neurotransmitter in insects. The octopaminergic agonist amitraz, a member of the formamidine class of insecticides, is selective for parasitic mites and ticks, as well as some *Lepidoptera* and *Homoptera* species.[185] Recent studies in vertebrates have shown that amitraz causes sympathomimetic effects,[186] apparently by binding to α-2 adrenergic receptors.[187,188] It has been suggested that amines like the octopamines could signal through G-coupled protein receptors.[189] Human poisoning associated with amitraz exposure has been reported.[190]

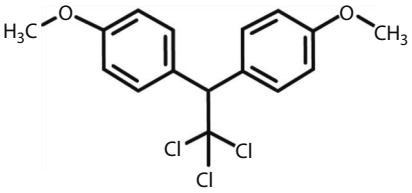
Piperonyl butoxide inhibits methyl farnesoate epoxidase, which catalyzes the synthesis of juvenile hormone III, thereby affecting molting behavior and reproductive function in insects.[48] Piperonyl butoxide also inhibits P450 monooxygenases, thereby retarding the metabolism of the pyrethroid insecticides and serving to prolong their period of effective action.

The structures, uses, and hazard profiles for amitraz and piperonyl butoxide are provided in Table 16.47. Amitraz is moderately acutely neurotoxic and has effects on the development and reproductive function. It is also an animal carcinogen. Piperonyl butoxide neither is acutely toxic nor has reproductive or developmental effects.[191]

Respiratory Inhibitors and Uncouplers (IRAC Codes 13, 20A, 21A, 21B, and 22B)

Compounds that disrupt energy metabolism have been identified from both natural and synthetic sources. An important natural product is rotenone, which is derived from roots of

TABLE 16.39
Structure, Uses, and Hazard Profile for the Organochlorine Sodium Channel Modulator Methoxychlor (IRAC Code 3)

Structure	Principal Use	Application Rate (g a.i./ha)
	Last registered use cancelled in the United States in 2003	—

Irritation		LD_{50} (mg/kg)				
Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitizing Potential	Signal Word
NA	NA	3460	NA	NA	NA	NA

Species/Study	NOEL (mg/kg/day)	Toxicity Studies	Hazard Indicator
Rat/2 years	20	Mutagenicity	Not mutagenic
Dog/52 weeks	21	Developmental	Not teratogenic
Mouse/18 months	28	Reproductive	Weak estrogenic properties
RfD[2]	NA	Oncogenicity	Probably not carcinogenic

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com; Joint Meeting of the F. A. O. Panel of Experts on Pesticide Residues in Food and the Environment, W. H. O. Expert Group on Pesticide Residues, F. A. O. Panel of Experts on Pesticide Residues in Food. Methoxychlor. Pesticide Residues in Food: 1977 Evaluations: Food and Agriculture Organization of the United Nations, 1977; WHO, Methoxychlor: Evaluation of the toxicity of pesticide residues in food. World Health Organization, Geneva, Switzerland, 1965.

Derris and *Lonchocarpus*,[192] as well as the leaves of some species of *Tephrosia*.[193] The synthetic compounds in this structurally diverse group include the pyrrole known as chlorfenapyr, as well as the amidinohydrazones, including hydramethylnon and pyridaben.

Disruption of energy metabolism occurs in the mitochondria and usually takes the form of either an inhibition of the electron transport system or an uncoupling of the transport system from ATP production. Inhibition of the electron transport system blocks the production of ATP and causes a decrease in oxygen consumption by the mitochondria. These uncouplers act on either coenzyme Q oxidoreductase in the electron transport chain or the cytochrome b–Ci complex.[194] The electron transport system functions normally, but the production of ATP is uncoupled from the electron transport process due to a dissipation of the proton gradient across the inner mitochondrial membrane (see Figure 16.2). In the presence of uncouplers, oxygen consumption increases, but no ATP is produced.[37] The disruption of energy metabolism and the subsequent loss of ATP result in a slowly developing toxicity, and the effects of all these insecticides include inactivity, paralysis, and death. It is expected that this MOA would be preserved in mammalian systems if the chemical reached its enzyme target. Thus, chemicals that interfere with mitochondrial respiration are expected to have similar effects in mammalian species.

The structures, uses, and hazard profiles for these insecticides that inhibit mitochondrial respiration (chlorfenapyr, hydramethylnon, pyridaben, rotenone, and metaflumizone) are given in Tables 16.48 and 16.49.

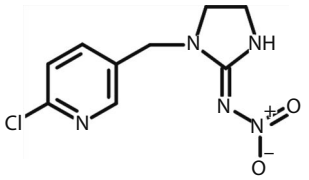
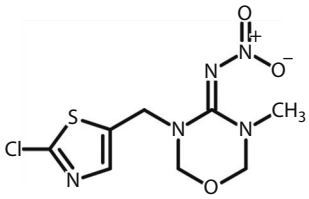
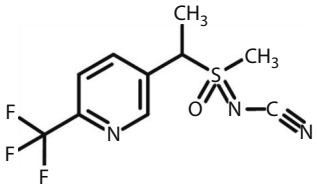
Diamides (IRAC Code 28)

The diamide insecticide group includes chlorantraniliprole, cyantraniliprole, and flubendiamide, which represent the three most important products commercially. When *Lepidoptera* and other pests ingest diamides, their ryanodine receptors are activated, causing an uncontrollable loss of calcium stores.[37] The insects quickly become lethargic, show signs of muscle paralysis, stop feeding, and then die.[37] The structures, uses, and hazard profiles for the representative diamides are found in Table 16.50.

Pheromones

Pheromones are chemical attractants secreted by special glands of insects to assist them in identifying or locating members of the opposite gender.[195] EPA has defined pheromones as chemicals produced by arthropods (insects, arachnids, or crustaceans) that modify the behavior of other individuals of the same species.[196] The EPA has registered 17 arthropod pheromones as active ingredients, 11 of which are lepidopteran pheromones.[196] The information submitted covered

TABLE 16.40
Hazard Profiles for Neonicotinoid Insecticides (IRAC Codes 4A and 4C)

Insecticide	Structure	Principal Use	Application Rate (g a.i./ha)
Imidacloprid (Admire®, Provado®)[37,280]		Used to control sucking insects including aphids, thrips, and whiteflies; also used as a seed treatment	25–100 (foliar) 50–700 g/100 kg (seed)
Thiamethoxam (Actara®, Cruiser®, Platinum®)[37,280]		Used to control sucking insects including ricehoppers, aphids, thrips, and whiteflies	10–200
Sulfoxaflor (Transform™)[37,280,370]		Used to control sap-feeding insects	—

Insecticide	Irritation: Eye	Irritation: Skin	LD_{50} (mg/kg): Oral	LD_{50} (mg/kg): Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Imidacloprid	Nonirritant	Nonirritant	424	>5000	0.07	Negative	Warning
Thiamethoxam	Nonirritant	Nonirritant	1563	>2000	>3.72	Negative	Caution
Sulfoxaflor	Nonirritant	Nonirritant[370]	1000	>5000	>2.09	Negative	Caution

Insecticide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Imidacloprid[371,372]	Rat/2 years	5.7	Mutagenicity	Not mutagenic
	Dog/2-year oral	41	Developmental	Not teratogenic
	Mouse/18 months	208	Reproductive	No evidence
	RfD	0.057	Oncogenicity	E (No evidence)
Thiamethoxam[141,142,373,374]	Rat/2 years	21.0	Mutagenicity	No evidence
	Dog/52 weeks	4.05	Developmental	Not teratogenic
	Mouse/18 months	2.63	Reproductive	Testicular effects
	RfD[2]	0.0006	Oncogenicity	Unlikely (mouse liver tumors)
Sulfoxaflor[370]	Rat/2 years	4.24	Mutagenicity	No evidence
	Dog/52 weeks	6.0	Developmental	Not teratogenic
	Mouse/18 months	10.4	Reproductive	No evidence
	RfD	0.05	Oncogenicity	Unlikely (rat and mouse liver tumors)

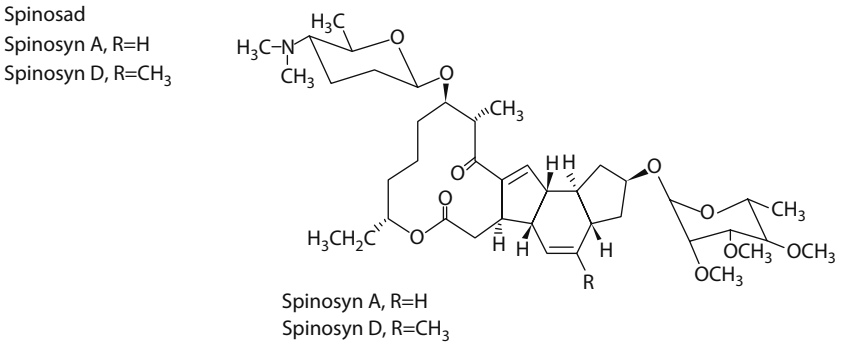


FIGURE 16.12 Chemical structure of spinosad.

TABLE 16.41
Hazard Profiles for the Spinosyns, the Avermectins, and Milbemycin (IRAC Codes 5 and 6)

	Irritation		LD_{50} (mg/kg)				
Insecticide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Spinosad[37,280]	Nonirritant	Nonirritant	3738	>5000	>5.18	Negative	Caution
Spinetoram[37,280,375,376]	Nonirritant	Nonirritant	>5000	>5000	>5.5	Sensitizer	Caution
Abamectin[37,280]	Mild irritant	Nonirritant	13.6	>2000	5.73	Negative	Danger
Emamectin benzoate[37,280]	Severe irritant	Nonirritant	76	>2000	2.12	Negative	Danger
Milbemycin[37,280]	Mild irritant	Nonirritant	456	>5000	1.9	Negative	Caution

Insecticide	**Species/Study**	**NOEL (mg/kg/day)**	**Toxicity Study**	**Hazard Indicator**
Spinosad[143,144,147]	Rat/2 years	5.0	Mutagenicity	Not mutagenic
	Dog/26-week oral	2.7	Developmental	Not teratogenic
	Mouse/18 months	7.5	Reproductive	Not a reproductive toxin
	RfD	0.027	Oncogenicity	E (No evidence)
Spinetoram[375,376]	Rat/2 years	10.8	Mutagenicity	Not mutagenic
	Dog/52-week oral	2.49	Developmental	Not teratogenic
	Mouse/18 months	18.8	Reproductive	Dystocia
	RfD (ADI)	0.05	Oncogenicity	No evidence of treatment-related tumorigenicity in rats and mice
Abamectin[154,156]	Rat/2 years	1.5	Mutagenicity	Not mutagenic
	Dog/26-week oral	0.25	Developmental	Teratogenic (rabbit, mouse)
	Mouse/18 months	4.0	Reproductive	Not a reproductive toxin
	RfD (based on the rat reproduction study; UF = 1000)	0.00012	Oncogenicity	E (No evidence)
Emamectin benzoate[377]	Rat/2 years	0.25	Mutagenicity	Not mutagenic
	Dog/26-week oral	0.25	Developmental	Not teratogenic
	Mouse/18 months	2.5	Reproductive	Not a reproductive toxin
	RfD (based on a 15-day neurotoxicity in CF-1 rats)	0.00083		
	Mouse (UF = 900)		Oncogenicity	E (No evidence)
Milbemycin[149,160]	Rat/2 years	6.81	Mutagenicity	Not mutagenic
	Dog/26-week oral	3	Developmental	Not teratogenic
	Mouse/18 months	18.9	Reproductive	Not a reproductive toxin
	RfD	0.03	Oncogenicity	Not carcinogenic

Abamectin
(80% avermectin B1a,
20% avermectin B1b)
R (B1a=ethyl)
(B1b–methyl)

Emamectin benzoate
(80% avermectin benzoate B1a
20% avermectin benzoate B1b)
R (B1a=ethyl)
(B1b–methyl)

Milbemycin

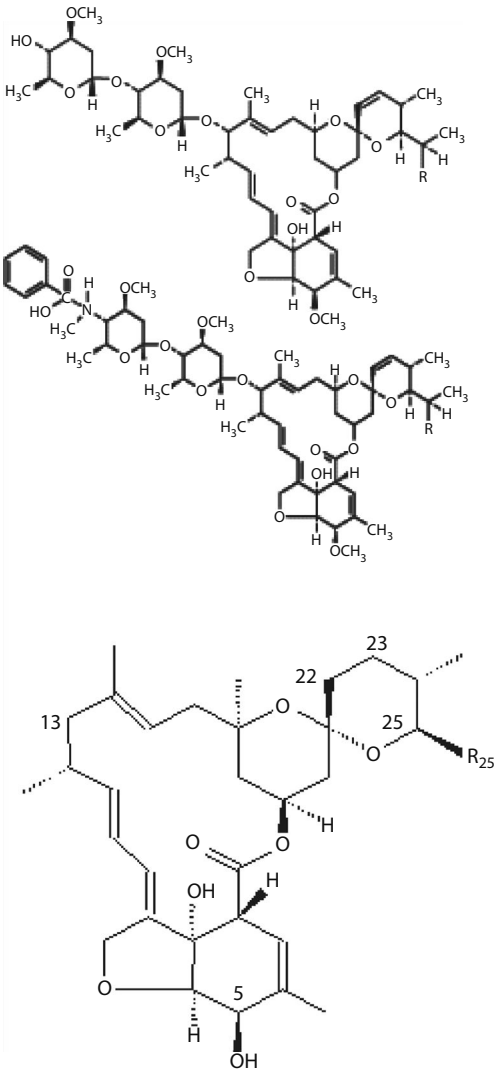


FIGURE 16.13 Chemical structures for the avermectins and milbemycin.

Methoprene

Juvenile Hormone 3

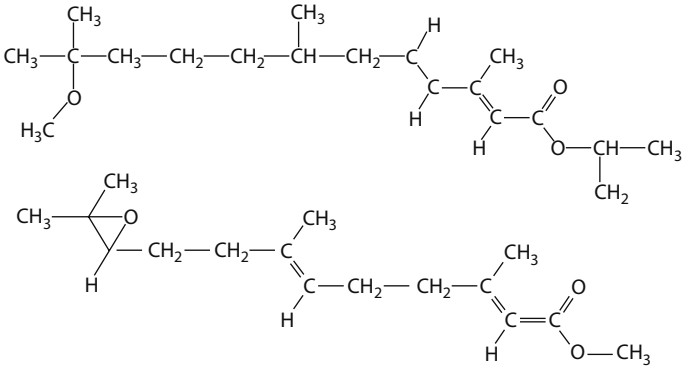
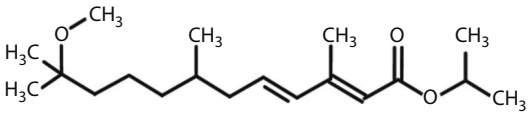
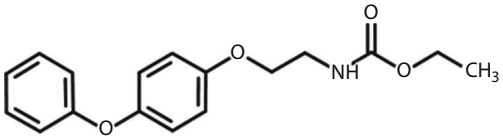
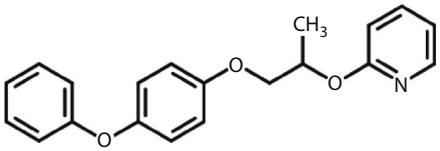
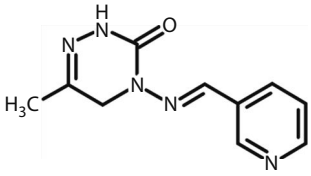


FIGURE 16.14 Comparison of the structure of methoprene to juvenile hormone 3.

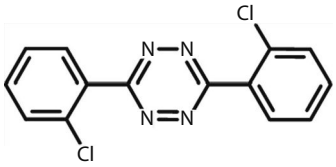
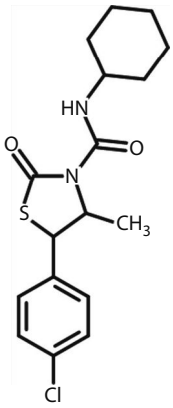
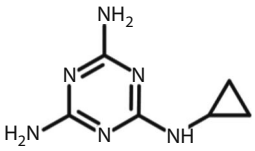
TABLE 16.42
Structures, Uses, and Hazard Profiles for Juvenile Hormone Mimics Methoprene, Fenoxycarb, and Pyriproxyfen (IRAC Code 7), as well as for the Selective Feeding Blocker Pymetrozine (IRAC Code 9)

Insecticide	Structure	Principal Use	Application Rate (g a.i./ha)
Methoprene (Apex®)[37,280]		Prevents metamorphosis to viable adults—used in public health, food-handling facilities, and mushroom houses	11,300
Fenoxycarb (INSEGAR®)[37,280]		Used for control of fire ants, other ants, and other public health insect pests	25–50
Pyriproxyfen (Knack®)[37,280]		Used to control public health insect pests	25–50
Pymetrozine (Sterling®)[37,280]		Used to control aphids and whiteflies in vegetables, ornamentals, cotton, and field crops	150–300

	Irritation		LD_{50} (mg/kg)				
Insecticide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Methoprene	Nonirritant	Nonirritant	34,600	3500	210	Negative	Caution
Fenoxycarb	Slight irritant	Nonirritant	>10,000	>2000	4.4	Negative	Caution
Pyriproxyfen	Nonirritant	Nonirritant	>5000	>2000	>3.1	Negative	Caution
Pymetrozine	Nonirritant	Nonirritant	>5820	>2000	>1.8	Negative	Caution

Insecticide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Methoprene[170]	Rat/2 years	5000	Mutagenicity	Not mutagenic
	Dog/52 weeks	—	Developmental	Not teratogenic
	Mouse/18 months	2500	Reproductive	Not a reproductive toxin
	ADI	0.1	Oncogenicity	Not carcinogenic
Fenoxycarb[168,378]	Rat/2 years	10	Mutagenicity	Not mutagenic
	Dog/52 weeks	25	Developmental	Not teratogenic
	Mouse/18 months	5	Reproductive	Not a reproductive toxin
	RfD (based on Q_1*)	0.0000007	Oncogenicity	Lung/liver in mice, C with Q_1*of 5.6×10^{-2} $(mg/kg/day)^{-1}$
Pyriproxyfen[379,380]	Rat/2 years	35	Mutagenicity	Not mutagenic
	Dog/52 weeks	100	Developmental	Not teratogenic
	Mouse/18 months	85	Reproductive	Not reproductive toxin
	RfD	0.35	Oncogenicity	E (No evidence)
Pymetrozine[169,380,381]	Rat/2 years	3.7	Mutagenicity	No evidence
	Dog/52 weeks	0.57	Developmental	Not teratogenic
	Mouse/18 months	—	Reproductive	Not a reproductive toxin
	RfD	0.0057	Oncogenicity	Not carcinogenic

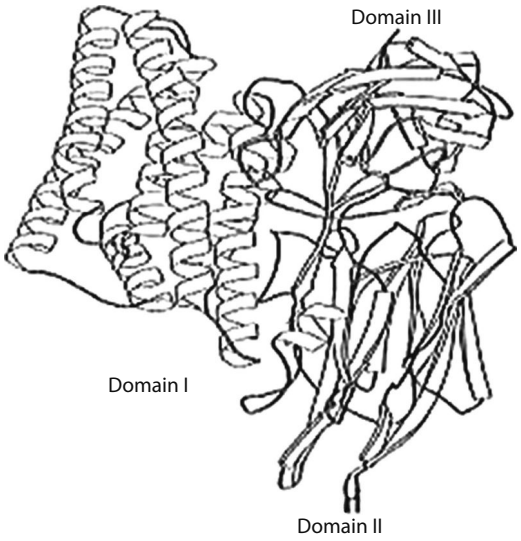
TABLE 16.43
Structures, Uses, and Hazard Profiles for the Phenyltetrazines (Clofentezine, Hexythiazox; IRAC Code 10A) and Aminotriazines (Cyromazine; IRAC Code 17): Larvicides/Growth and Molting Disruptors with an Unknown or Nonspecific MOA

Insecticide	Structure	Principal Use	Application Rate
Clofentezine (Apollo®)[37,280]		Used to control eggs and young mobile stages of mites in vegetables and fruit	100–300
Hexythiazox (Nissorun®)[37,280]		Used to control larvae and eggs of phytophagous mites in fruit, vines, cotton, and vegetables	150–300
Cyromazine (Trigard®)[37,280]		Used to control fly larvae in manure and leaf miners in vegetables	75–450

	Irritation		LD50 (mg/kg)				
Insecticide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC50 (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Clofentezine	Nonirritant	Nonirritant	>5200	>2100	>2.0	Weak positive	Caution
Hexythiazox	Mild irritant	Nonirritant	>5000	>5000	>2.0	Negative	Caution
Cyromazine	Nonirritant	Mild irritant	2029	>1370	>2.7	Negative	Caution

Insecticide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Clofentezine[383,384]	Rat/2 years	2.0	Mutagenicity	Not mutagenic
	Dog/26-week oral	1.25	Developmental	Not teratogenic
	Mouse/18 months	7.1	Reproductive	Not a reproductive toxin
	RfD	0.012	Oncogenicity	C with Q* (thyroid tumors in male rats)
Hexythiazox[385]	Rat/2 years	21.5	Mutagenicity	Not mutagenic
	Dog/26-week oral	2.5	Developmental	Not teratogenic
	Mouse/18 months	37.5	Reproductive	Not a reproductive toxin
	RfD	Q* = 0.039 $(mg/kg/day)^{-1}$	Oncogenicity	C with Q* (based on liver tumors)
Cyromazine[386]	Rat/2 years	1.8	Mutagenicity	Not mutagenic
	Dog/26-week oral	0.75	Developmental	Not teratogenic
	Mouse/18 months	6.5	Reproductive	Not a reproductive toxin
	RfD	0.008	Oncogenicity	E (No evidence)

TABLE 16.44
Structures, Uses, and Hazard Profiles for Delta-Endotoxins Derived from *Bacillus thuringiensis* Aizawai and Kurstaki Subspecies (IRAC Code 11)

Insecticide	Structure of Delta-Endotoxin Protein[3]	Principal Use	Application Rate (g a.i./ha)
Bacillus thuringiensis Aizawai subsp. Kurstaki subsp.[3,37,172,174–177,387]		Controls caterpillars of not only the *Lepidoptera* genus (butterflies and moths and corn root worm), but also mosquito larvae and the blackflies that vector river blindness in Africa	1121

	Irritation		LD_{50} (mg/kg)				
Insecticide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Aizawai subsp.	—	—	No infectivity	—	—	—	Caution
Kurstaki subsp.	No infectivity	No infectivity	No infectivity	No infectivity	5.4	—	Caution

Insecticide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Aizawai subsp.	Rat/2 years	8.4	Mutagenicity	Waived
	Dog/52 weeks	—	Developmental	Waived
	Mouse/18 months	—	Reproductive	Waived
	RfD	—	Oncogenicity	Waived
Kurstaki subsp.	Rat/2 years	—	Mutagenicity	Waived
	Dog/52 weeks	—	Developmental	Waived
	Mouse/18 months	—	Reproductive	Waived
	ADI	—	Oncogenicity	Waived

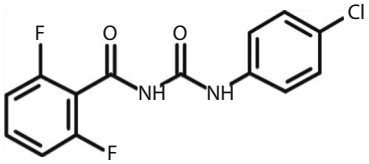
compounds that were from 6- to 16-carbon unbranched alcohols, acetates, and aldehydes, which are volatile.

There are lower data requirements for the registration of pheromones. The available data on lepidopteran and other arthropod pheromones, including several aromatic pheromones, have shown no acute mammalian toxicity at the limit dose levels tested, and these chemicals are also approved for use in organic farming methods.[112] The acute toxicity profiles generally reveal oral and dermal LD_{50} values of greater than 5000 and 2000 mg/kg, respectively.[197] Acute inhalation LC_{50} values generally are greater than 5 mg/L. Eye and skin irritation potentials fall in the mild or nonirritating range, and there is no evidence of skin sensitization potential. Since small amounts of the pheromone are present inside bait stations, there is practically no human contact, and, therefore, the full data package requirement for conventional pesticides is waived by the EPA.[196]

HERBICIDES

The MOA of herbicides used in crop protection have been classified by the Herbicide Resistance Action Committee (HRAC) into groups,[6] as presented in Table 16.51. It has been suggested that the MOA for 60% of herbicides introduced during the period from 1960 to 2000 involve biochemical pathways specific to either chloroplasts or plant-signaling hormones.[198] Whether or not any of the molecular targets of these herbicides in plants have homologous targets in animals will be considered in the following section.

TABLE 16.45
Structure, Uses, and Hazard Profile for the Benzoylurea Chitin Synthesis Inhibitor Diflubenzuron (DIMILIN®) (IRAC Code 15)

Structure	Principal Use	Application Rate (g a.i./ha)
	Used to control major insect pests in cotton, soy, citrus, tea, vegetables, and mushrooms, including larvae of flies, mosquitoes, grasshoppers, and locusts	25–150

Irritation		LD_{50} (mg/kg)				
Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Nonirritant	Nonirritant	>4640	>10,000	>35	Negative	Caution

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	2.0	Mutagenicity	Not mutagenic
Dog/52 weeks	2.0	Developmental	Not teratogenic
Mouse/18 months	2.0	Reproductive	Not a reproductive toxin
RfD	0.02	Oncogenicity	E (No evidence)

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com; U.S. EPA, Diflubenzuron: Reregistration Eligibility Decision (RED), EPA-738-R-97-008, U.S. Environmental Protection Agency, Washington, DC, 1997; U.S. EPA, *Fed. Regist*., 63(37), 9528, 1998; U.S. EPA, *Fed. Regist.*, 63(92), 26481, 1998.

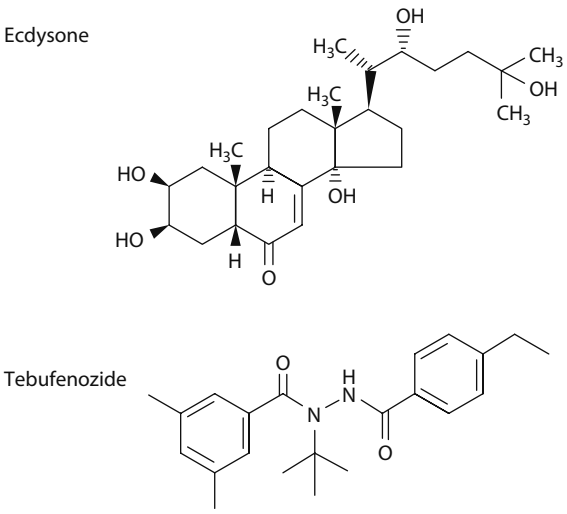


FIGURE 16.15 Comparison of the structure of ecdysone 20 to tebufenozide.

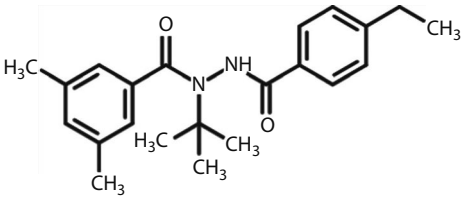
Acetyl-CoA Carboxylase Inhibitors (HRAC Code A)

The aryloxyphenoxypropionate, cyclohexanedione, and phenylpyrazoline herbicides inhibit acetyl coenzyme A (acetyl-CoA) carboxylase (ACCase), although the exact binding site of these herbicides on this enzyme has not yet been determined.[199] They block the synthesis of fatty acids essential for the production of plant lipids, which are vital to the integrity of cell membranes and to the formation of cuticle waxes during new plant growth (see Figure 16.16). Injury is slow to develop (7–10 days) and appears first on new leaves emerging from the whorl of the grass plant. The herbicide is taken up by the foliage and moves via the phloem to areas of new growth.[200]

ACCase exists in two forms in plants: the prokaryote form, which is found in broadleaf dicotyledonous plants that are 400–6000 times more tolerant to these herbicides, and the eukaryote form found in perennial and annual grasses, which are more susceptible. The prokaryotic form of ACCase is heterodimeric, comprised of four gene products, the biotin carboxyl carrier (BCC) and biotin carboxylase (BCase), as well as both the α- and β-subunits of carboxyltransferase (CTase). The eukaryotic, homodimeric form is a single 220–230 dDA polypeptide comprised of linked BCC, BCase, and CTase domains.[199,201] Differences in tolerance among the monocotyledonous plants are attributed to differences in the rate of detoxification among the subspecies.[202–204] Resistance development is attributed to the acquisition of mutations that (1) increase the expression of ACCase, (2) alter binding of the herbicide to ACCase, or (3) increase expression of enzymes involved in herbicide metabolism.[199]

Biochemical pathways for fatty acid synthesis are conserved in mammalian species (Figure 16.16). ACCase in mammals is like the eukaryotic form. There are no specific data to indicate that the AOPP or CHD herbicides alter fatty acid synthesis in animal studies, although drugs have been developed to block this pathway. For example, the rate-limiting enzyme for sterol synthesis, HMG-CoA

TABLE 16.46
Structure, Uses, and Hazard Profile for the Diacylhydrazine Ecdysone Agonist Tebufenozide (CONFIRM®) (IRAC Code 18A)

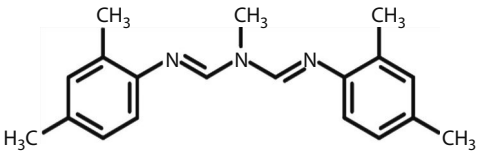
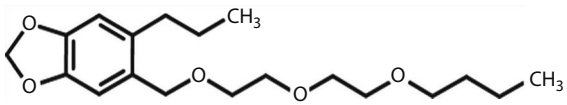
Structure	Principal Use	Application Rate (g a.i./ha)
	Used for control of *Lepidopteran* larvae on rice, fruit, row crop, nut crops, vegetables, and vines	70–560

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Nonirritant	Nonirritant	>5000	>5000	4.5	Negative	Caution

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	4.8	Mutagenicity	Not mutagenic
Dog/52 weeks	1.8	Developmental	Not teratogenic
Mouse/18 months	143	Reproductive	Not a reproductive toxin
RfD	0.018	Oncogenicity	E (No evidence)

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Valentine, B.J. et al., *Aust. J. Exp. Agric*., 36(4), 501, 1996; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com; U.S. EPA, *Fed. Regist.*, 63(160), 44439, 1998.

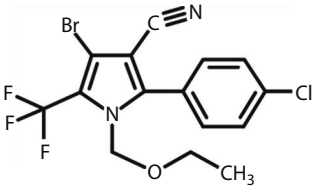
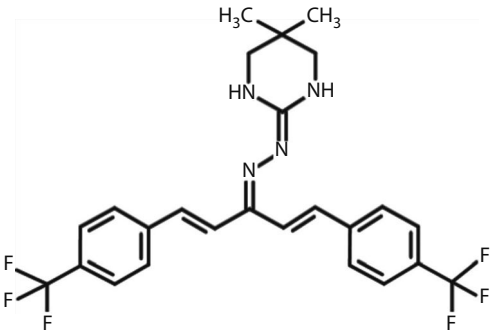
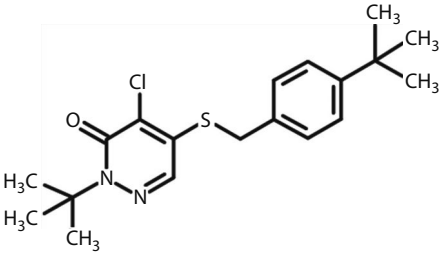
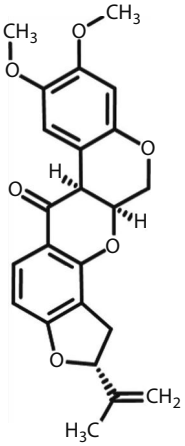
TABLE 16.47
Structures, Uses, and Hazard Profiles for the Octopaminergic Agonist Amitraz (IRAC Code 19) and the P-450 Monooxygenase Inhibitor Piperonyl Butoxide (IRAC Code 27)

Insecticide	Structure	Principal Use	Application Rate (g a.i./ha)
Amitraz (Taktic®)[37,280]		Nonsystemic, with contact and respiratory actions to expel ticks, mites, scale insects, whiteflies, aphids, and others	—
Piperonyl butoxide[37,280]		Inhibits insects MFO, increasing the efficacy of the applied insecticide	—

	Irritation		LD_{50} (mg/kg)				
Insecticide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Amitraz	Nonirritant	Nonirritant	531	>200	2.4	Negative	Danger
Piperonyl butoxide	Nonirritant	Nonirritant	4570	>2000	>5.9	Positive	Caution

Insecticide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Amitraz[186,188,190,392]	Rat/2 years	2.5	Mutagenicity	Not mutagenic
	Dog/52 weeks	0.25	Developmental	Delayed development in rabbits
	Mouse/18 months	3.75	Reproductive	Effects on fecundity
	ADI	0.003	Oncogenicity	C with Q_1*
Piperonyl butoxide[191]	Rat/2 years	30	Mutagenicity	Not mutagenic
	Dog/52 weeks	16	Developmental	Not teratogenic
	Mouse/18 months	30	Reproductive	Not a reproductive toxin
	ADI	0.2	Oncogenicity	C (ileocecal and liver tumors at high doses)

TABLE 16.48
Structures and Use Profiles for the Mitochondrial Respiration–Inhibiting Insecticides Chlorfenapyr (IRAC Code 13) and Hydramethynon (IRAC Code 20A), as well as for Pyridaben (IRAC Code 21A), Rotenone (IRAC Code 21B), and Metaflumizone (IRAC Code 22B)

Insecticide	Structure	Principal Use	Application Rate (g a.i./ha)
Chlorfenapyr (Pirate®)[37,280]		Used to control many insects and mites in cotton, vegetables, citrus, vines, and soybeans	0.125%–0.50% w/w
Hydramethylnon (Amdro®)[37,280]		Used on agricultural and household *Formicidae*	16
Pyridaben (Poseidon®)[37,280]		Used to control acarids on field crops, fruits, vegetables, and ornamentals	100–300
Rotenone (Chem Fish®)[37,280]		Used to control aphids, thrips, suckers, moths, beetles, and spider mites in fruits and vegetables; also fish control	280–420 0.005–0.250 ppm (fish)

(*continued*)

TABLE 16.48 (continued)
Structures and Use Profiles for the Mitochondrial Respiration–Inhibiting Insecticides Chlorfenapyr (IRAC Code 13) and Hydramethynon (IRAC Code 20A), as well as for Pyridaben (IRAC Code 21A), Rotenone (IRAC Code 21B), and Metaflumizone (IRAC Code 22B)

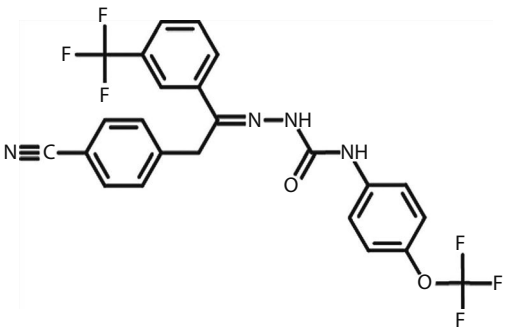
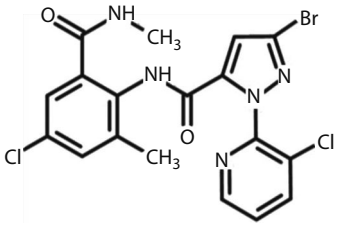
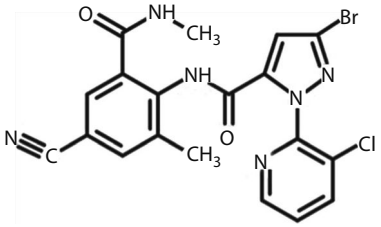
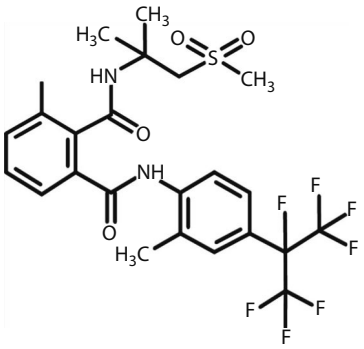
Insecticide	Structure	Principal Use	Application Rate (g a.i./ha)
Metaflumizone (ProMeris™)[37,280]		Used to control a broad range of insects in various crops and elsewhere, including tuberous, cruciferous, leafy, and fruiting vegetables	60–280

TABLE 16.49
Hazard Profiles for Mitochondrial Respiration-Inhibiting Insecticides Chlorfenapyr (IRAC Code 13), Hydramethynon (IRAC Code 20A), Pyridaben (IRAC Code 21A), Rotenone (IRAC Code 21B), and Metaflumizone (IRAC Code 22B)

	Irritation		LD_{50} (mg/kg)				
Insecticide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Chlorfenapyr	Moderate irritant	Nonirritant	441	>2000	1.9	Negative	Warning
Hydramethylnon	Mild irritant	Nonirritant	817	>2000	2.9	Negative	Caution
Pyridaben	Slight irritant	Nonirritant	820	>2000	0.66	Negative	Caution
Rotenone	—	—	39.5	—	—	—	—
Metaflumizone	Nonirritant	Nonirritant	>5000	>5000	>5.2	Negative	Caution

Insecticide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Chlorafenapyr[393]	Rat/2 years	2.9	Mutagenicity	Not mutagenic
	Dog/52 weeks	4.0	Developmental	Not teratogenic
	Mouse/18 months	2.8	Reproductive	Not a reproductive toxin
	RfD	0.03	Oncogenicity	E (No evidence)
Hydramethylnon[194,394]	Rat/2 years	50	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.0	Developmental	Not teratogenic
	Mouse/18 months	25	Reproductive	Not a reproductive toxin
	RfD	0.01	Oncogenicity	C with RfD (lung and liver tumors in mice)
Pyridaben[395]	Rat/2 years	1.13	Mutagenicity	No evidence
	Dog/52 weeks	<0.5	Developmental	Not teratogenic
	Mouse/18 months	2.78	Reproductive	No evidence
	RfD	0.005	Oncogenicity	E (No evidence)
Rotenone[193,296,396]	Rat/2 years	7.5	Mutagenicity	Not mutagenic
	Dog/52 weeks	0.4	Developmental	Not teratogenic
	Mouse/18 months	10.7	Reproductive	Not a reproductive toxin
	ADI	—	Oncogenicity	Not carcinogenic
Metaflumizone[397]	Rat/2 years	30	Mutagenicity	Not mutagenic
	Dog/52 weeks	12	Developmental	Not teratogenic
	Mouse/18 months	250	Reproductive	Not a reproductive toxin
	ADI	0.1	Oncogenicity	Unlikely to pose carcinogenic risk to humans

TABLE 16.50
Structures, Uses, and Hazard Profiles for the Diamide Fungicides Chlorantraniliprole, Cyantraniliprole, and Flubendiamide (IRAC Code 28)

Insecticide	Structure	Principal Use	Application Rate (g a.i./ha)
Chlorantraniliprole (Coragen®)[37,280]		Used against many types of chewing insect pests, such as *Lepidoptera*, in fruits, vegetables, cotton, rice, grass, sugarcane, and vines	10–100
Cyantraniliprole (Cyazypyr™)[37,280]		Used as a foliar spray and a soil-based insecticide to control various flies, beetles, leaf miners, and *Lepidoptera* in various fruits and vegetables	10–100
Flubendiamide (Belt®)[37,280]		Controls various stages of Lepidoptera in corn, cotton, tobacco, fruits, rice, lawns, and vegetables	34–180

	Irritation		LD_{50} (mg/kg)				
Insecticide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Chlorantraniliprole	Slight irritant	Nonirritant	>5000	>5000	>5.1	Negative	Caution
Cyantraniliprole	Nonirritant	Nonirritant	>5000	>5000	5.2	Negative	Caution
Flubendiamide	Slight irritant	Nonirritant	>2000	>2000	>0.0685	Negative	—

Insecticide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Chlorantraniliprole[398]	Rat/2 years	805	Mutagenicity	Not mutagenic (Ames)
	Dog/52-week oral	1164	Developmental	Not teratogenic
	Mouse/18 months	158	Reproductive	Not a reproductive toxin
	RfD	1.58	Oncogenicity	Not a likely human carcinogen
Cyantraniliprole[399]	Rat/2 years	9.34	Mutagenicity	Not mutagenic
	Dog/26-week oral	<0.96	Developmental	Not teratogenic
	Mouse/18 months	15.45	Reproductive	Not a reproductive toxin
	RfD	—	Oncogenicity	Not carcinogenic
Flubendiamide[37,400]	Rat/1 year	1.95	Mutagenicity	Not mutagenic (Ames)
	Dog/52-week oral	<2.21 (male)	Developmental	No evidence
	Mouse/18 months	<4.44 (female)	Reproductive	No evidence
	cRfD	0.024	Oncogenicity	Not a likely human carcinogen

TABLE 16.51
Herbicides Listed according to HRAC Classification System

Chemical Group	Common Name	HRAC Code	HRAC MOA	MOA Conserved in Mammals?	Other Common MOA in Mammals
Aryloxyphenoxypropionates (AOPP)	Clodinafop-propargyl Diclofop-methyl Fenoxaprop-P-ethyl Fluazifop-P-butyl Quizalofop-*p*-ethyl (Table 16.52)	A	Inhibition of acetyl-CoA carboxylase (ACCase)	Yes	Rodent peroxisomal proliferation; not likely relevant to man.
Cyclohexanediones (CHD)	Clethodim Sethoxydim				
Phenylpyrazolines (DEN)	Pinoxaden (Table 16.53)				
Sulfonylureas (SUs)	Bensulfuron-methyl Chlorimuron-ethyl Chlorsulfuron Halosulfuron Imazosulfuron Metsulfuron-methyl Nicosulfuron Primisulfuron-methyl Prosulfuron Rimsulfuron Sulfometuron-methyl Sulfosulfuron Thifensulfuron-methyl Triasulfuron Tribenuron-methyl Trifloxysulfuron-methyl (Tables 16.54 and 16.55)	B	Inhibition of acetolactate synthetase (ALS) or AHAS (acetohydroxyacid synthases) AHAS	No	Binding to sulfonylurea receptors in pancreatic cells results in the release of insulin (Add Reference) and decrease in glucose levels. SU herbicides do not appear to be effective (i.e., potent) SU receptor binders as no interference with glucose regulation has been reported for this class of herbicides.
Imidazolinones	Imazameth (Imazapic) Imazamethabenz-methyl Imazamox Imazapyr Imazaquin Imazethapyr (Table 16.56)				
Triazolopyrimidines	Cloransulam-methyl Flumetsulam				
Pyrimidinylthiobenzoates	Pyrithiobac-sodium				
Sulfonylaminocarbons	Flucarbazone-sodium Propoxycarbazone-sodium (Table 16.57)				
Triazines	Atrazine Cyanazine Propazine Simazine Ametryn Prometryn Prometon	C1	Inhibition of photosynthesis at photosystem II	No No	Chloro-S-triazines belong to a common mechanism class based on effects on the hypothalamic–pituitary gonadal axis.[219]
Triazinones	Metribuzin (Tables 16.58 and 16.59)				
Triazolinones	Flucarbazone				None known.
Uracils	Bromacil Terbacil				
Pyridazinones	Norflurazon (Table 16.60)				

TABLE 16.51 (continued)
Herbicides Listed according to HRAC Classification System

Chemical Group	Common Name	HRAC Code	HRAC MOA	MOA Conserved in Mammals?	Other Common MOA in Mammals
Ureas	Diuron	C2			
	Fluometuron				
	Linuron				
Amides	Propanil (Table 16.61)				
Nitriles	Bromoxynil	C3			
	Dichlobenil				
Benzothiadiazinones	Bentazon (Table 16.62)				
Bipyridyliums	Diquat	D	Inhibition of photosynthesis at photosystem I	No	Capacity to undergo redox recycling in specific tissues.
	Paraquat (Table 16.63)				
Diphenyl ethers	Acifluorfen	E	Inhibition of protoporphyrinogen oxidase (PPO)	Yes—Potential effects on the heme biosynthetic pathway in mammals	Peroxisome proliferation and potential binding to PPARα receptors.[204,229]
	Fomesafen				
	Lactofen				
	Oxyfluorfen (Table 16.64)				
N-Phenylphthalimides	Flumiclorac-pentyl				
	Flumioxazin				
Thiadiazoles	Fluthiacet-methyl				
Triazolinones	Carfentrazone-ethyl				
	Sulfentrazone				
Oxadiazoles	Oxadiazon (Table 16.65)				
Pyridazinones	Norflurazon	F1	Bleaching inhibition of carotenoid biosynthesis at the phytoene desaturase step (PDS)	No	None known.
	Fluridone (Table 16.66)				
Isoxazoles	Isoxaflutole	F2	Bleaching inhibition of 4-hydroxyl-phenypyruvate-dioxygenase [4-HPPD]	Yes	HPDD inhibition leads to an increase in tyrosine in animal models for some members of this class.
Triketones	Mesotrione				
	Tembotrione (Table 16.67)				
Triazoles	Amitrole	F3	Bleaching inhibition of carotenoid biosynthesis (unknown target)	Unknown	The triazole, amitrole, may have an effect on cholesterol synthesis.
Isoxazolidinones	Clomazone				
	Topramezone (Table 16.68)				
Glycines	Glyphosate	G	Inhibition of 5-enol-pyruvy-lshikimate-3-phosphate synthase (EPSP)	No	None known.
Phosphinic acids	Glufosinate-ammonium	H	Inhibition glutamine synthetase	Yes	None known.
Carbamates	Asulam (Table 16.69)	I	Inhibition of dihydropteroate synthase (DHP)	Yes	None known.
Dinitroanilines	Benfluralin	K1	Inhibition of microtubule assembly	Yes	None known, but this class has a common structural homology to aniline.
	Pendimethalin				
	Trifluralin				
Pyridines	Aminopyralid (Table 16.70)				None known.
Chloroacetamides	Alachlor	K3	Inhibit very-long-chain fatty acid (VLCFA) synthesis in cell walls	Unlikely	Alachlor and acetochlor share a common mechanism based on their ability to form a reactive Quinone imine.[249,250]
	Acetochlor				
	Metolachlor				
	S-Metolachlor				
	Dimethenamid (Table 16.71)				

(*continued*)

TABLE 16.51 (continued)
Herbicides Listed according to HRAC Classification System

Chemical Group	Common Name	HRAC Code	HRAC MOA	MOA Conserved in Mammals?	Other Common MOA in Mammals
Benzamides	Isoxaben	L	Inhibition of cellulose synthesis	No	None known.
Quinoline carboxylic acids	Quinclorac				
Thiocarbamates	Butylate (Table 16.72)	N	Inhibition of lipid synthesis—not ACCase inhibition	Yes	Inhibition of the hydrolysis of cholesterol from high-density lipoprotein in the rat testes.[401]
Phenoxycarboxylic acids	2,4-dichlorophenoxyacetic acid (2,4-D)	O	Auxin (indole acetic acid) hormone mimic	Unlikely	None known.
Benzoic acids	MCPA Dicamba				
Pyridine carboxylic acids	Fluroxypyr Picloram Triclopyr				
Quinoline carboxylic acids	Quinclorac (see Tables 16.72 and 16.73)				
Semicarbazones	Diflufenzopyr-sodium (Table 16.74)	P	Inhibition of auxin transport	Unlikely	None known.
Organoarsenicals	Monosodium methanearsonate (MSMA) (Table 16.75)	Z	Herbicides with an unknown MOA	Unknown	None known.

Source: HRAC, Classification of Herbicides according to Mode of Action, 2012.

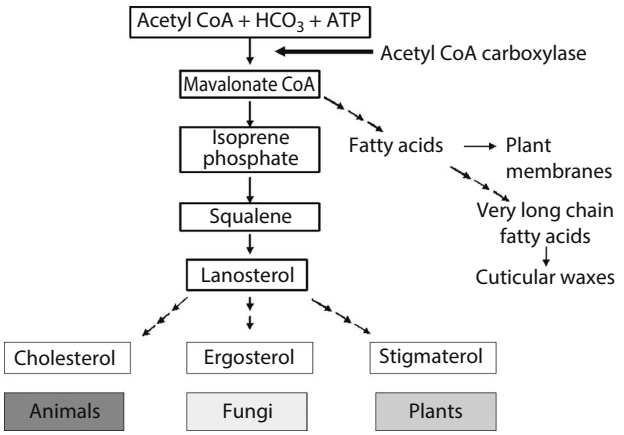


FIGURE 16.16 Fatty acid synthesis and by-products in plants, fungi, and animals.

reductase, is inhibited by simvastatin, a drug designed to reduce cholesterol biosynthesis.

Aryloxyphenoxypropionates (HRAC Code A)

The structures, uses, and hazard profiles for six aryloxyphenoxypropionate herbicides are presented in Table 16.52. These herbicides are generally not acutely toxic. Clodinafop-propargyl has been identified as a peroxisomal proliferator in rodents. The relevance of peroxisomal proliferation to humans is determined on a case-by-case basis, using a framework assessment of the weight of the evidence.[205]

Cyclohexanediones (HRAC Code A)

The structures, uses, and hazard profiles for the cyclohexanedione herbicides, clethodim and sethoxydim, are presented in Table 16.53. Both clethodim and sethoxydim have limited toxicity in acute and repeat-dose toxicity studies. They are not mutagenic, carcinogenic, developmental, or reproductive toxicants.

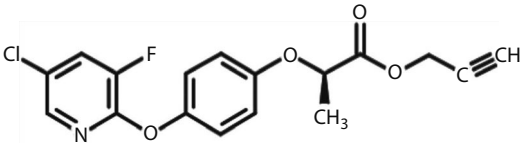
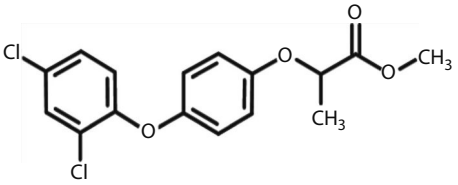
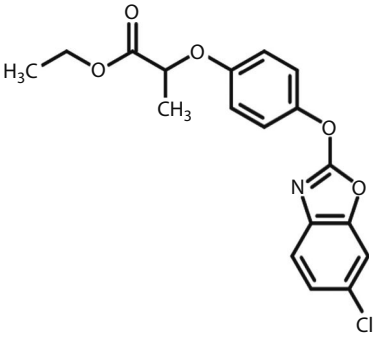
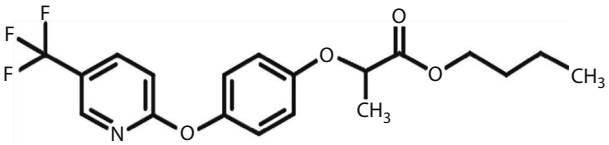
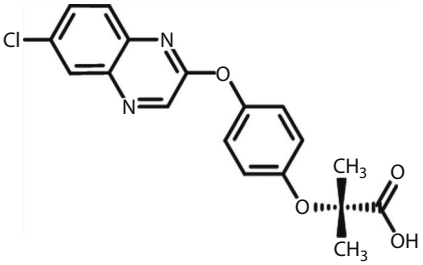
Phenylpyrazolines (HRAC Code A)

The structure, uses, and hazard profile for the phenylpyrazoline herbicide pinoxaden are presented in Table 16.53. Pinoxaden affects fatty acid synthesis when it is used as a postemergent, systemic herbicide, and it is the only phenylpyrazoline herbicide noted by the HRAC.[6,37]

Acetolactate Synthase Inhibitors (HRAC Code B)

The acetolactate synthase (ALS) inhibitors, which are comprised of the sulfonylureas (SUs), imidazolinones, triazolopyrimidines, and pyrimidinylthiobenzoates, interact with the ALS enzyme, thereby blocking the biosynthesis of

TABLE 16.52
Structures, Uses, and Hazard Profiles of Acetyl-CoA Carboxylase–Inhibiting Aryloxyphenoxypropionate (AOPP) Herbicides (HRAC Code A)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Clodinafop-propargyl (Discover®)[37,280]		Cereals for control of annual grasses	30–60
Diclofop-methyl (Hoelon®)[37,280]		Applied to cereals as a selective systemic herbicide	840–1680
Fenoxaprop-P-ethyl (Puma®)[37,280]		Cereals, soybeans, and turf for weed control	37.5–111
Fluazifop-P-butyl (Fusilade®)[37,280]		Cotton, fruit, and soybeans for postemergent weed control	175–1400
Quizalofop-P-ethyl (Assure®)[37,280]		Used for selective control of postemergent annual and perennial grass weeds in potatoes, soy, sugar beets, peanuts, and other crops	—

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Clodinafop-propargyl	Nonirritant	Nonirritant	1829	>2000	2.325	Positive	Caution
Diclofop-methyl	Nonirritant	Nonirritant	2020	>5000	>3.83	NA	Caution
Fenoxaprop-P-ethyl	Slight irritant	Slight irritant	2565	>2000	>0.511	Negative	Caution
Fluazifop-P-butyl	Mild irritant	Slight irritant	4096	>2420	>5.24	Negative	Caution
Quizalofop-P-ethyl	Nonirritant	Nonirritant	2350	>5000	5.8	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Clodinofop-propargyl[402]	Rat/2 years	0.35	Mutagenicity	Not a mutagen
	Dog/52 weeks	3.3	Developmental	Not a developmental toxin
	Mouse/18 months	1.2	Reproductive	Not a reproductive toxin
	ADI (2-year rat)	0.004	Oncogenicity	Peroxisomal proliferator (mouse liver tumors)

(*continued*)

TABLE 16.52 (continued)
Structures, Uses, and Hazard Profiles of Acetyl-CoA Carboxylase–Inhibiting Aryloxyphenoxypropionate (AOPP) Herbicides (HRAC Code A)

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Diclofop-methyl[403]	Rat/2 years	20	Mutagenicity	Not a mutagen
	Dog/15-month oral	8.0	Developmental	Not a developmental toxin
	Mouse/18 months	NA	Reproductive	Not a reproductive toxin
	ADI	0.001	Oncogenicity	NA
Fenoxaprop-P-ethyl[404]	Rat/2 years	1.5	Mutagenicity	Not a mutagen
	Dog/15-month oral	0.375	Developmental	Not a developmental toxin
	Mouse/18 months	5.7	Reproductive	Not a reproductive toxin
	RfD (rat reproduction)	0.0025	Oncogenicity	C (pending)-adrenal tumors
Fluazifop-P-butyl[405]	Rat/2 years	0.5	Mutagenicity	Negative
	Dog/15-month oral	5.0	Developmental	Delayed skeletal ossification
	Mouse/18 months	12.1	Reproductive	Not a reproductive toxin
	cRfD	0.0074	Oncogenicity	Not likely
Quizalofop-P-ethyl[37]	Rat/2 years	0.9	Mutagenicity	Not mutagenic
	Dog/15-month oral	13.4	Developmental	Not teratogenic
	Mouse/18 months	1.55	Reproductive	Not a reproductive toxin
	cRfD	0.009	Oncogenicity	D (not classifiable)

branched-chain amino acids, valine, leucine, and isoleucine, as illustrated in Figure 16.17.[206] The binding site is considered to be a vestigial quinine-binding site on the enzyme.[207] Because the biochemical pathway for the synthesis of branched-chain amino acids does not exist in monogastric animals, this herbicidal MOA is not relevant to humans. In fact, some researchers have taken advantage of this selectivity to design antituberculosis drugs.[208]

Sulfonylureas (HRAC Code B)

SU herbicides belong to a class of compounds comprised of three distinct components, as each is formed by an aryl group linked to a nitrogen-containing heterocycle via an SU bridge. SU herbicides inhibit root and shoot growth in rapidly growing plants by suppressing cell division.[79] Initial research conducted on *E. coli* and *Salmonella typhimurium*, and later confirmed in plants and yeasts, indicates that the herbicidal activity is due to the inhibition of ALS, an enzyme necessary for the biosynthesis of branched-chain amino acids in bacteria, fungi, and higher plants.

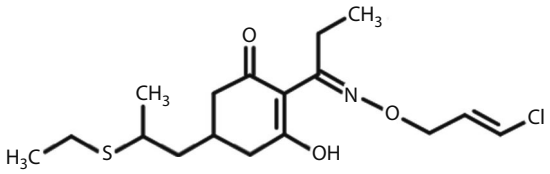
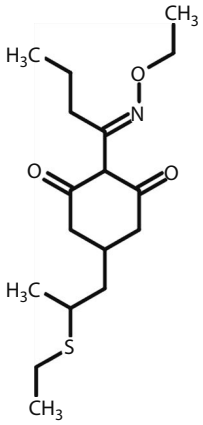
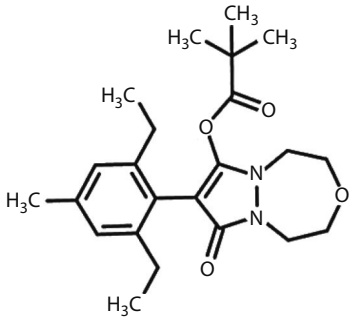
A large number of SU herbicides have been developed for commercial use in North America and Europe. The structures, uses, and application rates for the most commonly used SUs are provided in Table 16.54.

SU herbicides generally are neither acutely toxic or irritating to the skin and eye, nor mutagenic, developmentally toxic, or oncogenic. Their hazard profiles are given in Table 16.55. Various target organs have been identified at high doses in chronic studies in rodents and dogs, including bone marrows, livers, kidneys, and testes, as well as the peripheral system and CNS. Tumor incidence was elevated above control levels in the liver (primisulfuron) at doses that exceed the maximum tolerated dose. An earlier appearance of mammary tumors has also been observed in female Sprague-Dawley rats (prosulfuron and tribenuron).

A unitary MOA underlying effects of this class of chemical on mammalian systems is not discernable. The diversity of the effects observed in various target organs is attributed to specific functional groups and not to the defining characteristic of the class, the SU bridge.

An alternate MOA for the SUs is derived from the fact that an SU receptor protein in pancreatic β-cell plays an important role in glucose regulation. An ATP-sensitive potassium ion channel known as K_{ATP} has been identified in β-cells of the pancreas. The ultrastructure of the K_{ATP} channel is unique among K^+ ion channels in that it is comprised of two proteins: a sulfonylurea receptor (SUR) protein, which belongs to the family of ABC (ATP cassette) transporter proteins, and a smaller protein, Kir6.2, which belongs to a family of inward-rectifying potassium current proteins. Four Kir6.2 subunits are constitutively expressed with four SUR subunits to make up the selective K^+ pore.[209] K_{ATP} channels containing the SUR1 isoform can be blocked by SUs[210] and can be opened with diazoxide. SUR1 is thus critically involved in the regulation of K_{ATP} channel activity. It is proposed that an elevation in blood glucose concentration leads to an increased rate of glucose metabolism in pancreatic β-cells and a consequent alteration in the intracellular ratio of ATP/ADP, resulting in the inhibition of K_{ATP} channels. The subsequent depolarization of the β-cell plasma membrane activates voltage-sensitive Ca^{2+} channels, and the ensuing influx of Ca^{2+} initiates insulin secretion.[211]

TABLE 16.53
Structures, Uses, and Hazard Profiles of Acetyl-CoA Carboxylase-Inhibiting Cyclohexanedione (CHD) Herbicides and the Phenylpyrazoline Herbicide Pinoxaden (HRAC Code A)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Clethodim (Select®)[37,280]		Used to control grasses in soybeans and cotton	60–240
Sethoxydim (Nabu®)[37,280]		Used to control grasses in soybean, cotton, and peanut crops	200–500
Pinoxaden (AXIAL®)[37,280]		Control of postemergent annual grasses in wheat and barley crops	30–60

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Clethodim	NA	Nonirritant	1360	>2000	>3.9	Negative	Caution
Sethoxydim	Nonirritant	Nonirritant	2676	>5000	6.1	Negative	Caution
Pinoxaden	Irritant	Nonirritant	>5000	>2000	5.22	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Clethodim[403,406]	Rat/2 years	19	Mutagenicity	Not a mutagen
	Dog/52 weeks	1	Developmental	Not a developmental toxin
	Mouse/18 months	28	Reproductive	Not a reproductive toxin
	ADI	0.01	Oncogenicity	No evidence
Sethoxydim[202–204,403,407]	Rat/2 years	17.2	Mutagenicity	Not a mutagen
	Dog/52 weeks	8.9	Developmental	Not a developmental toxin
	Mouse/18 months	14	Reproductive	Not a reproductive toxin
	ADI	0.14	Oncogenicity	No evidence
Pinoxaden[408,409]	Rat/2 years	100	Mutagenicity	Not a mutagen
	Dog/52 weeks	125	Developmental	Not a developmental toxin
	Mouse/18 months	181	Reproductive	Not a reproductive toxin
	cRfD	0.30	Oncogenicity	Not oncogenic

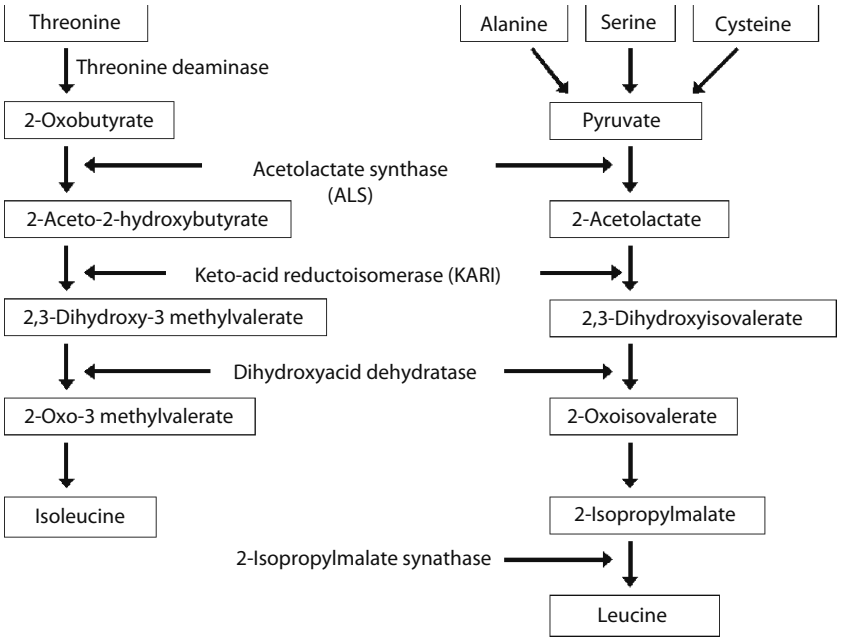


FIGURE 16.17 Branched-chain amino acid synthesis: acetolactate synthase (ALS).

It appears that the SU herbicides have rather low affinity to SUR, because there is no evidence of an effect on glucose regulation in the animal studies.

Imidazolinones (HRAC Code B)

The structures, uses, and hazard profiles for the imidazolinones imazameth and imazamethabenz-methyl are provided in Table 16.48. These ALS inhibitors are relatively nontoxic, even at high doses, with no evidence of mutagenic, developmental, or oncogenic effects.

The triazolopyrimidine ALS inhibitors include imazamox, imazapyr, imazaquin, and imazethapyr. As with the imidazolinones, these chemicals also have excellent hazard profiles (Table 16.56). No evidence of significant target organ toxicity, mutagenic, developmental, or oncogenic potential has been realized, even at doses that approximate the limit dose of 1000 mg/kg/day.

Triazolopyrimidines, Pyrimidinylthiobenzoates, and Sulfonylaminocarbons (HRAC Code B)

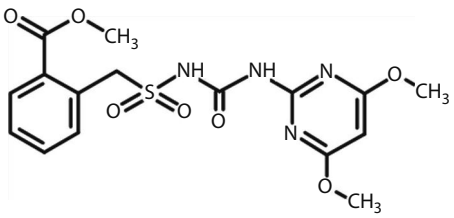
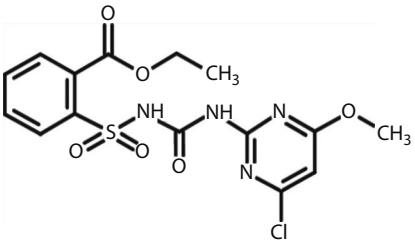
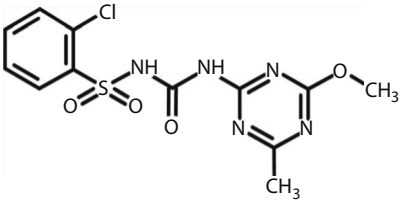
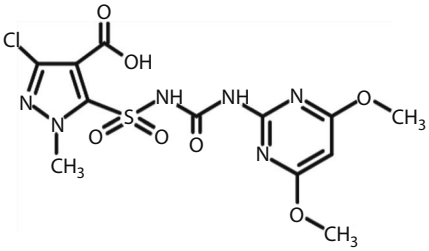
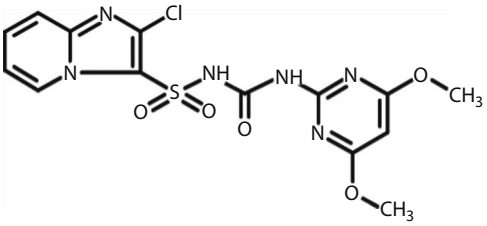
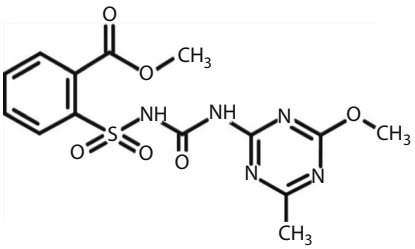
The members of this class of ALS-inhibiting herbicides, including flumetsulam (Table 16.57), are slightly less well tolerated in mammalian systems than for other ALS inhibitors, as evidenced by lower NOELs. However, the hazard profiles for these chemicals are still favorable, as no mutagenic, developmental, or oncogenic effects have been reported.

Inhibition of Photosynthetic Electron Transport (HRAC Codes C and D)

Photosynthesis is a process unique to plants whereby light energy captured by chlorophyll is converted to electrochemical energy through an electron transport chain to produce NADPH (photosystem I) or ATP (photosystem II). Herbicides that interfere with electron transport in the photosynthetic pathways have been grouped into two groups by HRAC.[6] The group D herbicides (bipyridyliums), represented here by paraquat and diquat, block photosynthesis at the photosystem I stage by capturing electrons that reduce the herbicide (Figure 16.18). The reduced form of the herbicide is then oxidized, leading to the formation of supraoxides and hydrogen peroxide and then ultimately hydroxyl radicals that damage cellular components affecting unsaturated membrane lipids, resulting in fatty acid peroxidation, losses in membrane semipermeability, desiccation, and cell death.[211] Group C1 (triazines, triazolinones, uracils, pyridazinones, and phenyl-carbamates), C2 (ureas and amides), and C3 herbicides (nitriles, benzothiadiazinone, and phenylpyridazines) all affect photosystem II (Figure 16.18). When electron transport is interrupted by Group C herbicides, and light continues to fall on the chloroplast, the energy level of chlorophyll is raised from a singlet to a triplet state, which itself damages cell membrane lipids or creates reactive oxygen species that interact with cellular lipids, proteins, and nucleic acids.[213,214]

Aside from creating reactive oxygen species, photosynthesis inhibitors also block food-producing processes in susceptible plants by limiting the availability of NADPH and ATP to enter into the so-called dark reaction (Calvin cycle), where CO_2 is fixed and carbohydrates are produced. The reduction in carbohydrate synthesis may result in a slow starvation of the plant.[215] Signs of injury include yellowing (chlorosis) of leaf tissue followed by death (necrosis) of the tissue. Preemergent- or early postemergent–applied herbicides like the triazines are taken up into the plant via the roots or foliage and are then transported via the xylem to the plant leaves. As a result, signs of injury first appear

TABLE 16.54
Structure and Use Profiles of the Acetolactate Synthase (ALS) and Acetohydroxyacid Synthase (AHAS)–Inhibiting Sulfonylurea Herbicides (HRAC Code B)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Bensulfuron-methyl (Londax®)[37,280]		Rice	46–60
Chlorimuron-ethyl (Classic®)[37,280]		Soybeans and peanuts	9–13
Chlorsulfuron (Glean®)[37,280]		Cereals and IWC	9–140
Halosulfuron-methyl (Permit®)[37,280]		Cereals, corn, sorghum, and turf	18–35
Imazosulfuron (Sibatito®, Takeoff®)[37,280]		Cereals, rice, and turf	75–1000
Metsulfuron-methyl (Ally®, Escort®)[37,280]		Cereals	4–8

(*continued*)

TABLE 16.54 (continued)
Structure and Use Profiles of the Acetolactate Synthase (ALS) and Acetohydroxyacid Synthase (AHAS)–Inhibiting Sulfonylurea Herbicides (HRAC Code B)

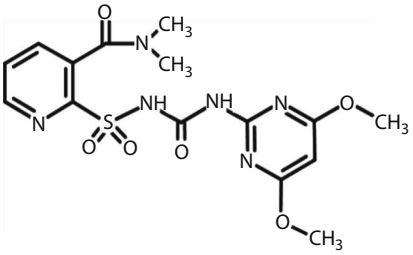
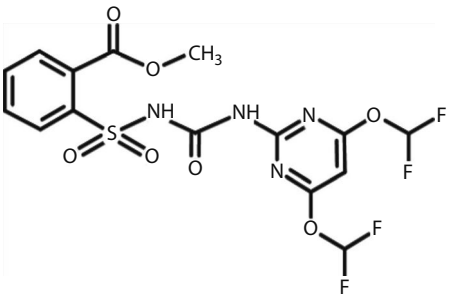
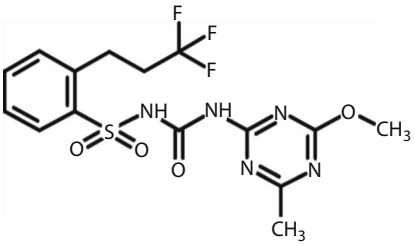
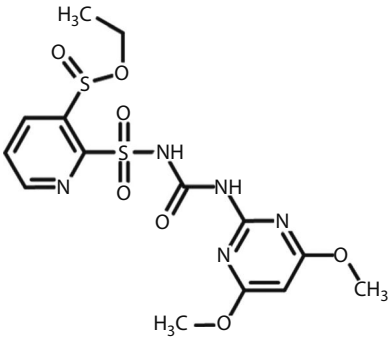
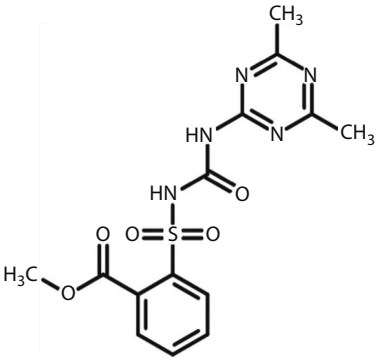
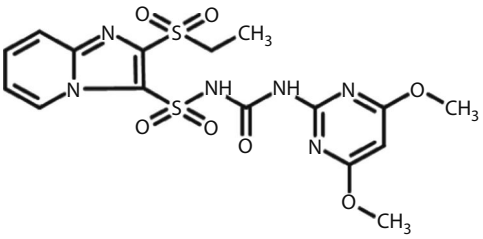
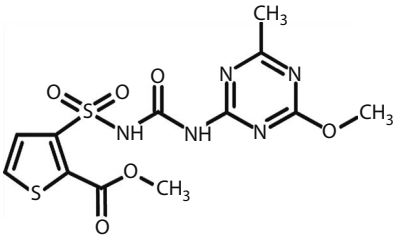
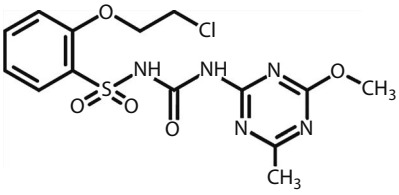
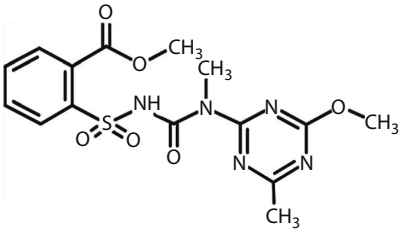
Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Nicosulfuron (Accent®)[37,280]		Corn	35–70
Primisulfuron-methyl (Beacon®)[37,280]		Corn	20–40
Prosulfuron (Peak®)[37,280]		Cereals, corn, sorghum, and pasture	12–30
Rimsulfuron (Matrix®)[37,280]		Corn, tomatoes, and potatoes	15
Sulfometuron-methyl (Oust®)[37,280]		IWC	26–420

TABLE 16.54 (continued)
Structure and Use Profiles of the Acetolactate Synthase (ALS) and Acetohydroxyacid Synthase (AHAS)–Inhibiting Sulfonylurea Herbicides (HRAC Code B)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Sulfosulfuron (Maverick®)[37,280]		Cereal (wheat) and IWC	10–35
Thifensulfuron-methyl (Pinnacle®, Harmony®)[37,280]		Cereals, corn, soybean, and pasture	9–60
Triasulfuron (Amber®, Logran®)[37,280]		Cereal (wheat) and IWC	5–10
Tribenuron-methyl (Express®)[37,280]		Cereals (wheat)	7.5–30

on older leaves or along leaf margins. Foliar-applied photosynthetic inhibitors generally remain in the foliar portion of the treated plant, with the movement from foliage to roots being negligible.[216]

Triazines and Triazinone (HRAC Code C1)

The S-triazines, phenylureas, and uracil herbicides all inhibit photosynthetic electron transport (Hill reaction) in photosystem II [662[217]] by binding to the D1 protein[218] and blocking the mobile electron carrier, plastoquinone.[213] The most common mechanism of resistance to s-triazines is a mutation of the psbA gene, which encodes the D1 protein, whereby glycine is substituted for serine at amino acid 264 in the stromal loop of the D1 protein.[218]

While the molecular targets relating to the inhibition of the Hill reaction do not exist in mammalian systems, a common MOA of toxicity for the chloro-s-triazines has been defined, based on effects on the hypothalamic–pituitary–gonadal axis.[219] The specific molecular target underlying this MOA has not been identified. However, it is likely related to the formation of a good leaving group by the chlorine atom, as indicated by reactivity with glutathione to form glutathione conjugates as part of the detoxification pathway or by reactions with sulfhydryl groups to form adducts to proteins.[220,221]

The structure and use of the symmetrical triazines, as well as the asymmetrical triazine or triazinone, metribuzin, are presented in Table 16.58. The hazard profiles are given in Table 16.59.

The triazines are generally not acutely toxic. The symmetrical chloro-s-triazines, including atrazine, propazine, and simazine, induce an earlier onset and/or an increase in the incidence of mammary tumors in lifetime feeding studies in Sprague-Dawley female rats.[222,223] The MOA underlying the occurrence of these tumors in female

TABLE 16.55
Hazard Profiles of the Acetolactate Synthase (ALS) and Acetohydroxyacid Synthase (AHAS)–Inhibiting Sulfonylurea Herbicides (HRAC Code B)

	Irritation		LD_{50} (mg/kg)				
Herbicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Bensulfuron-methyl	Nonirritant	Nonirritant	>5000	>2000	>7.5	NA	Caution
Chlorimuron-methyl	Nonirritant	Nonirritant	4102	>2000	>5.0	Negative	Caution
Chlorsulfuron	Slight irritant	Nonirritant	5545 (male)	2500	>5.9	Negative	Caution
Halosulfuron	Nonirritant	Nonirritant	8866	>2000	NA	NA	Caution
Imazosulfuron	Nonirritant	Nonirritant	>5000	>2000	>2.4	Negative	Caution
Metsulfuron	Mod. irritant	Mild irritant	>5000	>2000	>5.0	Negative	Caution
Nicosulfuron	Mod. irritant	NA	>5000	>2000	5.47	Negative	Caution
Primisulfuron	Slight irritant	Nonirritant	>5050	>2010	>4.8	Negative	Caution

Herbicide	**Species/Study**	**NOEL (mg/kg/day)**	**Toxicity Study**	**Hazard Indicator**
Bensulfuron-methyl[403]	Rat/2 years	37.5	Mutagenicity	Not mutagenic
	Dog/52 weeks	227	Developmental	Not teratogenic
	Mouse/18 months	455	Reproductive	Not a reproductive toxin
	ADI[2]	0.2	Oncogenicity	Not carcinogenic
Chlorimuron-methyl[403,410]	Rat/2 years	12.5	Mutagenicity	Not mutagenic
	Dog/52 weeks	6.25	Developmental	Not teratogenic
	Mouse/18 months	180	Reproductive	Not a reproductive toxin
	ADI[2]	0.02	Oncogenicity	Not carcinogenic
Chlorsulfuron[403,411,412]	Rat/2 years	5	Mutagenicity	Not mutagenic
	Dog/52 weeks	50	Developmental	Not teratogenic
	Mouse/18 months	71	Reproductive	Not a reproductive toxin
	RfD[2]	0.05	Oncogenicity	Not carcinogenic
Halosulfuron[413]	Rat/2 years	50	Mutagenicity	Not mutagenic
	Dog/52 weeks	10	Developmental	Not teratogenic
	Mouse/18 months	430	Reproductive	Not a reproductive toxin
	ADI	0.1	Oncogenicity	Not carcinogenic
Imazosulfuron[37]	Rat/2 years	106	Mutagenicity	Not mutagenic
	Dog/52 weeks	75	Developmental	Teratogenic in mice
	Mouse/18 months	NA	Reproductive	Not a reproductive toxin
	RfD or ADI	NA	Oncogenicity	Not carcinogenic
Metsulfuron-methyl[403]	Rat/2 years	25	Mutagenicity	Not mutagenic
	Dog/52 weeks	12.5	Developmental	Not teratogenic
	Mouse/18 months	710	Reproductive	Not a reproductive toxin
	ADI (Germany)	0.0125	Oncogenicity	Not carcinogenic
Nicosulfuron[403,414]	Rat/2 years	1000	Mutagenicity	Not mutagenic
	Dog/52 weeks	125	Developmental	Not teratogenic
	Mouse/18 months	1070	Reproductive	Not a reproductive toxin
	ADI	1.25	Oncogenicity	Not carcinogenic
Primisulfuron[403]	Rat/2 years	13	Mutagenicity	Not mutagenic
	Dog/52 weeks	25	Developmental	Not teratogenic
	Mouse/18 months	45	Reproductive	Testicular degeneration
	ADI	0.13	Oncogenicity	D (liver tumors in male mice at doses >MTD)

	Irritation		LD_{50} (mg/kg)				
Herbicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Prosulfuron	Nonirritant	Nonirritant	986	>2000	>5.0	Negative	Caution
Rimsulfuron	Mod. irritant	Nonirritant	>5000	>2000	>5.4	Negative	Caution
Sulfometuron	Slight irritant	Slight irritant	>5000	>2000	>11	Negative	Caution
Sulfosulfuron	Nonirritant	Slight irritant	>5000	>5000	NA	Negative	Caution
Thifensulfuron	Slight irritant	Nonirritant	>5000	>2000	>7.9	Negative	Caution
Triasulfuron	Slight irritant	Nonirritant	>5000	>2000	>5.1	Negative	Caution
Tribenuron-methyl	Slight irritant	Nonirritant	>5000	>2000	>5.0	Positive	Caution
Trifloxysulfuron-methyl	Slight irritant	Slight irritant	>5000	>2000	>5.03	Negative	Caution

TABLE 16.55 (continued)
Hazard Profiles of the Acetolactate Synthase (ALS) and Acetohydroxyacid Synthase (AHAS)–Inhibiting Sulfonylurea Herbicides (HRAC Code B)

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Prosulfuron[37]	Rat/2 years	8.6	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.9	Developmental	Not teratogenic
	Mouse/18 months	80	Reproductive	Not a reproductive toxin
	ADI	0.019	Oncogenicity	D (mammary tumors in female rats—early onset)
Rimsulfuron[415]	Rat/2 years	11.8	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.6	Developmental	Not teratogenic
	Mouse/18 months	351	Reproductive	Not a reproductive toxin
	RfD	0.016	Oncogenicity	Not carcinogenic
Sulfometuron[403,416]	Rat/2 years	2.5	Mutagenicity	Not mutagenic
	Dog/52 weeks	5.0	Developmental	Not teratogenic
	Mouse/18 months	140	Reproductive	Not a reproductive toxin
	ADI	0.0275	Oncogenicity	Not carcinogenic
Sulfosulfuron[417]	Rat/2 years	24.4	Mutagenicity	Not mutagenic
	Dog/52 weeks	100	Developmental	Not teratogenic
	Mouse/18 months	93.4	Reproductive	Not a developmental toxin
	ADI	0.24	Oncogenicity	Likely human carcinogen
Thifensulfuron[403]	Rat/2 years	2.6	Mutagenicity	Not mutagenic
	Dog/52 weeks	19	Developmental	Not teratogenic
	Mouse/18 months	1070	Reproductive	Not a reproductive toxin
	ADI	0.026	Oncogenicity	Not carcinogenic
Triasulfuron[403]	Rat/2 years	32.1	Mutagenicity	Not mutagenic
	Dog/52 weeks	33	Developmental	Not teratogenic
	Mouse/18 months	1.2	Reproductive	Not a reproductive toxin
	ADI	0.012	Oncogenicity	Not carcinogenic
Tribenuron[403]	Rat/2 years	1.25	Mutagenicity	Not mutagenic
	Dog/52 weeks	8.2	Developmental	Not teratogenic
	Mouse/18 months	30	Reproductive	Not a reproductive toxin
	ADI	0.011	Oncogenicity	C (Mammary tumors in female rats—early onset)
Trifloxysulfuron-methyl[291]	Rat/2 years	24	Mutagenicity	Not mutagenic
	Dog/52 weeks	15	Developmental	Not teratogenic
	Mouse/18 months	112	Reproductive	Not a reproductive toxin
	ADI	0.15	Oncogenicity	No evidence

Sprague-Dawley rats has been described, and it is not considered relevant to humans.[224] The IARC has classified atrazine and simazine as "not classifiable as to carcinogenicity to humans."[23] The EPA has also classified atrazine and simazine as "not likely to be carcinogenic to humans."[224,225]

Uracils and Pyridazinones (HRAC Code C1)

The structures, uses, and hazard profiles for two uracils (bromacil and terbacil) and the pyridazinone herbicide, norflurazon, are given in Table 16.60.

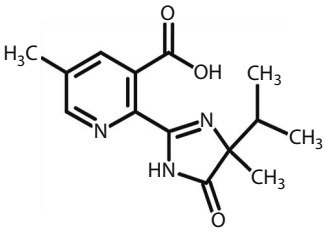
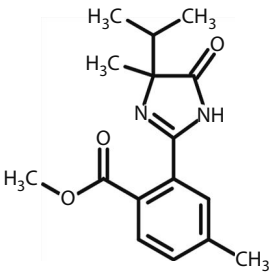
The acute toxicities of bromacil, terbacil, and norflurazon are unremarkable. These herbicides are not mutagenic, teratogenic, or reproductive toxins. However, bromacil and norflurazon have been classified as category C (possible human carcinogens), based on mouse liver tumors.

Ureas (HRAC Code C2)

The structures, uses, and toxicity for the urea class of photosynthesis-inhibiting herbicides diuron, fluometuron, and linuron are provided in Table 16.61.

Diuron, linuron, and fluometuron have limited acute toxicity. Fluometuron caused hemosiderosis in the spleen in repeat-dose studies. Diuron and linuron have been classified by the EPA as either known or likely human carcinogens as defined in the EPA 1996 classification scheme[37] or as category C (possible human carcinogens) based on an earlier scheme.[37] The cancer classification of fluometuron is

TABLE 16.56
Structures, Uses, and Hazard Profiles of the Acetolactate Synthase (ALS)–Inhibiting Imidazolinone Herbicides (HRAC Code B)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Imazameth (Cadre®)[37,280]		Soybeans, peanuts, and sugarcane	
Imazamethabenz-methyl (Assert®)[37,280]		Wheat, barley, and sunflower	250–700 post

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Imazameth	NA	Nonirritant	>5000	>5000	2.38	NA	Caution
Imazamethabenz-methyl	Slight irritant	Nonirritant	>5000	>2000	>5.8	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Imazameth (Imazapic)[418]	Rat/2 years	1029	Mutagenicity	No evidence
	Dog/52 weeks	<137	Developmental	Not teratogenic
	Mouse/18 months	1134	Reproductive	No evidence
	RfD (UF = 300)	0.5	Oncogenicity	No evidence
Imazamethabenz-methyl[403,419]	Rat/2 years	12.5	Mutagenicity	No evidence
	Dog/52 weeks	6.25	Developmental	Not teratogenic
	Mouse/18 months	19.5	Reproductive	No evidence
	ADI	0.06	Oncogenicity	No evidence

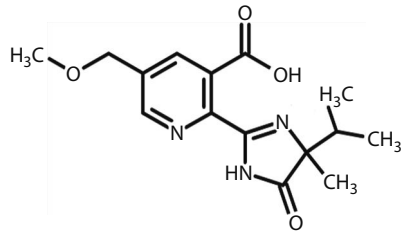
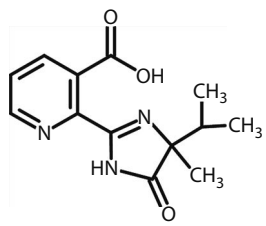
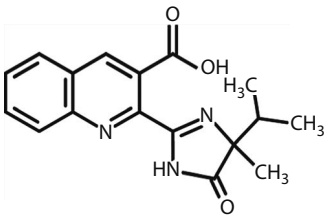
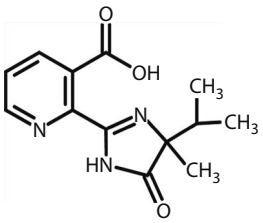
Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Imazamox (Raptor®)[37,281]		Soybeans and legumes	34–43
Imazapyr (Arsenal®)[37,280]		IWC	250–1700

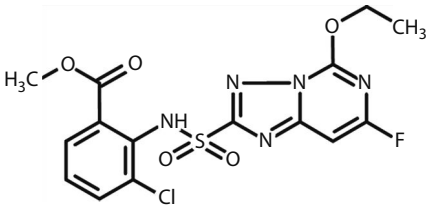
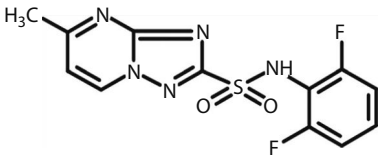
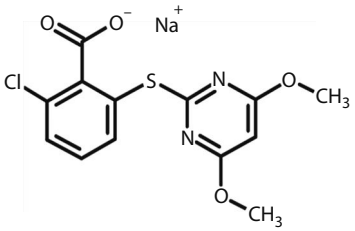
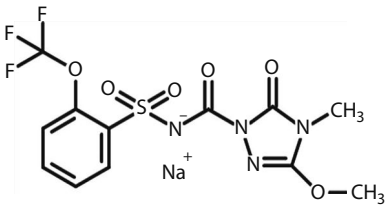
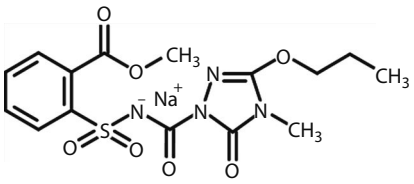
TABLE 16.56 (continued)
Structures, Uses, and Hazard Profiles of the Acetolactate Synthase (ALS)–Inhibiting Imidazolinone Herbicides (HRAC Code B)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Imazaquin (Scepter®)[37,280]		Soybeans	70–140 preplant, PPI, pre, post
Imazethapyr (Pursuit®)[37,280]		Soybeans, corn, legumes, and peanuts	130–260 early preplant, PPI, pre, post

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Imazamox	Mild irritant	Nonirritant	>5000	>4000	>6.3	Negative	Caution
Imazapyr	Irreversible	Nonirritant	>5000	>2000	>1.3	Negative	Danger
Imazaquin	Nonirritant	Slight irritant	>5000	>2000	>5.7	Negative	Caution
Imazethapyr	Slight irritant	Slight irritant	>5000	>2000	>2.6	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Imazamox[420]	Rat/2 years	1068	Mutagenicity	No evidence
	Dog/52 weeks	1165	Developmental	Not teratogenic
	Mouse/18 months	—	Reproductive	No evidence
	RfD	3.0	Oncogenicity	E (No evidence)
Imazapyr[403,421]	Rat/2 years	500	Mutagenicity	No evidence
	Dog/52 weeks	250	Developmental	Not teratogenic
	Mouse/18 months	1500	Reproductive	No evidence
	ADI	2.5	Oncogenicity	No evidence
Imazaquin[403,422]	Rat/2 years	500	Mutagenicity	No evidence
	Dog/52 weeks	25	Developmental	Not teratogenic
	Mouse/18 months	150	Reproductive	No evidence
	ADI	0.25	Oncogenicity	No evidence
Imazethapyr[403]	Rat/2 years	500	Mutagenicity	No evidence
	Dog/52 weeks	25	Developmental	Not teratogenic
	Mouse/18 months	750	Reproductive	No evidence
	ADI	0.25	Oncogenicity	No evidence

TABLE 16.57
Structure, Uses, and Hazard Profiles of Acetolactate Synthase (ALS)–Inhibiting Triazolopyrimidine and Pyrimidinylthiobenzoate Herbicides (HRAC Code B)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Cloransulam-methyl (FIRSTRATE®)[37,280]		Soybeans	35–44
Flumetsulam (Broadstrike®)[37,280]		Corn and soybeans	25–78
Pyrithiobac-sodium (Staple®)[37,280]		Cotton	35–105
Flucarbazone-sodium (SIERRA®)[37,280]		Postemergent control in wheat	21
Propoxycarbazone-sodium (Attribut®)[37,280]		Rye, triticale, and wheat	30–70

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Cloransulam-methyl	—	Nonirritant	>5000	>2000	>3.77	Negative	Caution
Flumetsulam	Slight irritant	Nonirritant	>5000	>2000	>5.9	Negative	Caution
Pyrithiobac-sodium	Irritant	Nonirritant	>3200	>2000	>6.9	—	Caution
Flucarbazone-sodium	Slight irritant	Nonirritant	>5000	>5000	>5.13	Negative	Caution
Propoxycarbazone-sodium	Nonirritant	Nonirritant	>5000	>5000	>5.03	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Cloransulam-methyl[37,423]	Rat/2 years	75	Mutagenicity	Not mutagenic
	Dog/52 weeks	10	Developmental	Not teratogenic
	Mouse/18 months	10	Reproductive	Not a reproductive toxin
	RfD	0.1	Oncogenicity	Not a likely human carcinogen
Flumetsulam[37,403]	Rat/2 years	35	Mutagenicity	Not mutagenic (Ames)
	Dog/52 weeks	100	Developmental	Not teratogenic
	Mouse/18 months	32	Reproductive	No evidence
	ADI	0.32	Oncogenicity	No evidence

TABLE 16.57 (continued)
Structure, Uses, and Hazard Profiles of Acetolactate Synthase (ALS)–Inhibiting Triazolopyrimidine and Pyrimidinylthiobenzoate Herbicides (HRAC Code B)

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Pyrithiobac-sodium[37,424]	Rat/2 years	58.7	Mutagenicity	Not mutagenic
	Dog/52 weeks	—	Developmental	Not teratogenic
	Mouse/18 months	217	Reproductive	No evidence
	ADI/RfD	0.6	Oncogenicity	C (Possible human carcinogen)
Flucarbazone-sodium[37,425]	Rat/2 years	125	Mutagenicity	Not mutagenic
	Dog/52 weeks	35.9	Developmental	Not teratogenic
	Mouse/2 years	275	Reproductive	No evidence
	cRfD	0.36	Oncogenicity	Not a likely human carcinogen
Propoxycarbazone-sodium[37,426]	Rat/2 years	43	Mutagenicity	Not mutagenic
	Dog/52 weeks	605	Developmental	Not teratogenic
	Mouse/18 months	—	Reproductive	Not a reproductive toxin
	cRfD	0.748	Oncogenicity	Not a likely human carcinogen

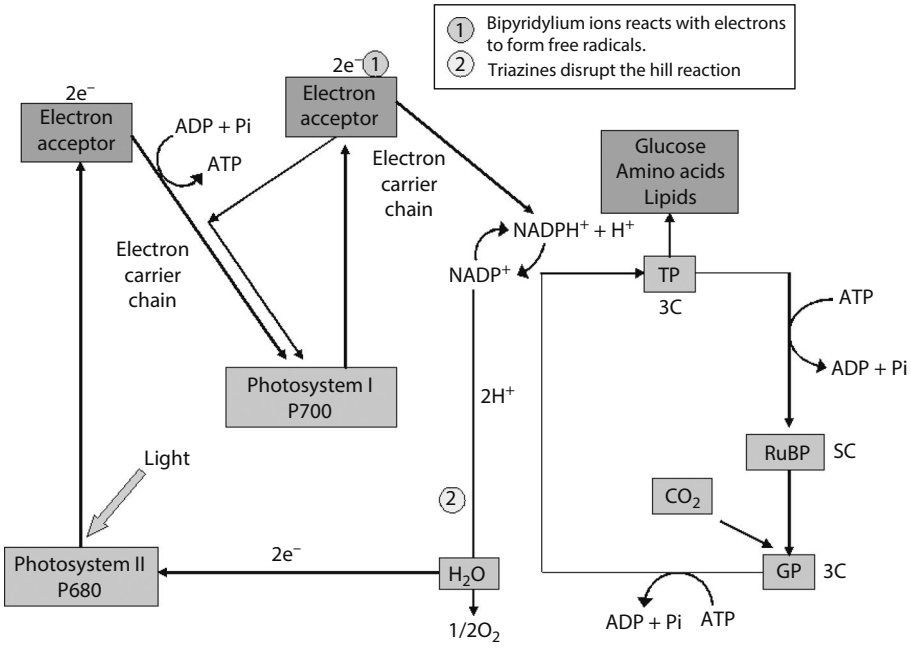


FIGURE 16.18 Photosynthesis: molecular targets.

pending; a slight elevation in the incidence of lymphoma was noted in the rat study on fluometuron.

Nitriles (HRAC Code C3)

The nitrile herbicides act against cellulose biosynthesis and are used as systemic herbicides.[37] The structures and uses of the nitriles dichlobenil and bromoxynil are presented, along with their hazard profiles, in Table 16.62. Dichlobenil and bromoxynil are classified as category C carcinogens, based on mouse liver tumors.

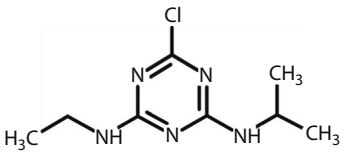
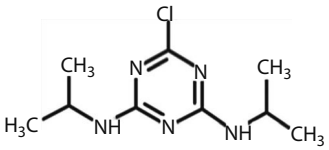
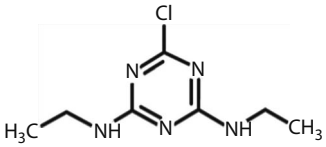
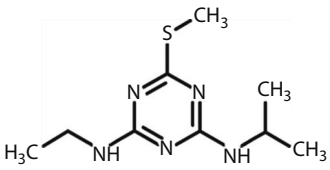
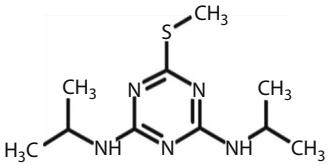
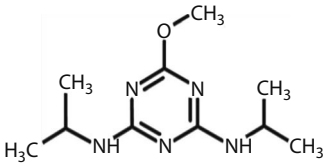
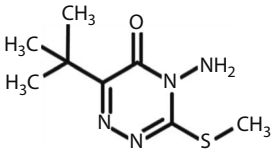
Benzothiadiazinones (HRAC Code C3)

This class of herbicides is represented by bentazon, which is also known as bentazone. Benzothiadiazinones interfere with photosynthetic electron transport via photosystem II receptors, and they are used as selective contact herbicides.[37] Bentazon is not toxic in either acute or repeated-dose studies.

Bipyridyliums (HRAC Code D)

Diquat and paraquat, which are bipyridylium photosynthesis inhibitors, are unlike the HRAC Code C1–C3 herbicides in

TABLE 16.58
Structures and Use Profiles of the Photosynthesis-Inhibiting Triazine and Triazinone Herbicides (HRAC Code C1)

Herbicide	Structure	Principal Crops/Use	Application Rate (kg a.i./ha)
Atrazine (Aatrex®)[37,280]		Pre- and postemergence control of annual broadleaved and annual grasses in corn, sorghum, sugarcane, and pineapple	1.5–2.5
Propazine (Milo-Pro®)[37,280]		Pre- and postemergence control of annual broadleaved and annual grasses in sorghum, carrots, chervil, and parsley	0.5–3
Simazine (Princep®)[37,280]		Pre- and postemergence control of annual broadleaved and annual grasses in pome fruit, stone fruit, citrus, vines, corn, sorghum, sugarcane, and pineapple	1.5–3
Ametryn (Evik®)[37,280]		Pre- and postemergence control of annual broadleaved and annual grasses in bananas, citrus fruit, corn, coffee, sugarcane, and pineapple	2–4
Prometryn (Caparol®)[37,280]		Preemergence in vegetables, cotton, sunflower, and peanuts, plus postemergence in cotton and vegetables	0.8–2.5 (preemergence) 0.8–1.5 (postemergence)
Prometon (Pramitol®)[37,280]		Control of most annual and many perennial broadleaved weeds, grasses, and brush weeds in noncrop areas	10–20
Metribuzin (Sencor®)[37,280]		Pre- and postemergence control of annual broadleaved and annual grasses in soybeans, potatoes, corn, cereals, sugarcane, alfalfa, and asparagus	0.07–1.45

that the bipyridyliums inhibit electron flow in photosystem I. The structures, uses, and hazard profiles for diquat and paraquat are provided in Table 16.63.

Diquat is less acutely toxic than paraquat, but they both are moderately toxic in long-term studies. Neither bipyridyliums are mutagenic, teratogenic, or carcinogenic, nor are they reproductive toxicants. However, both diquat and paraquat are capable of undergoing redox recycling, as they are reduced by electron donors, and they then undergo oxidization as they react with oxygen to form reactive oxygen species.[213,226] This redox potential is believed to account for tissue damage seen in the lung of animals treated with paraquat,[227] as well as cataractogenic effects in diquat-treated animals at low-to-moderate doses.[228] EPA concluded that while both diquat and paraquat are capable of generating oxygen free radicals, their effects are unlikely to be additive, because of differences in tissue distribution and hence target organ selectivity.[227]

Protoporphyrinogen Oxidase Inhibitors (HRAC Code E)

Protoporphyrinogen oxidase (PPO) inhibitors block the biosynthesis of chlorophyll by inhibiting PPO found in chloroplasts and mitochondria in plants. A similar action in animals

TABLE 16.59
Hazard Profiles of the Photosynthesis-Inhibiting Triazine and Triazinone Herbicides (HRAC Code C1)

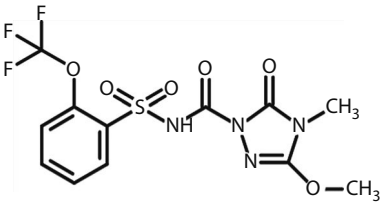
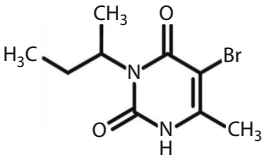
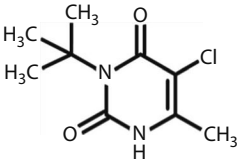
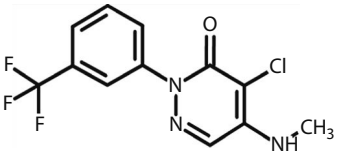
	Irritation		LD_{50} (mg/kg)				
Herbicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Atrazine	Nonirritant	Nonirritant	3090	>3100	>5.0	Positive	Caution
Propazine	Mild irritant	Nonirritant	>7000	>3100	>2.0	Negative	Caution
Simazine	Nonirritant	Mild irritant	>5000	>3100	>5.5	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Atrazine[221,223,403,427–430]	Rat/2 years	0.5	Mutagenicity	No evidence
	Dog/52 weeks	3.75	Developmental	Not teratogenic
	Mouse/18 months	1.2	Reproductive	No evidence
	ADI	0.005	Oncogenicity	Not likely carcinogenic in humans
Propazine[431]	Rat/2 years	5.8	Mutagenicity	No evidence
	Dog/52 weeks	1.3	Developmental	Not teratogenic
	Mouse/18 months	15	Reproductive	No evidence
	RfD	0.02	Oncogenicity	Category C with Q* (based mammary tumors in female Sprague-Dawley rats)
Simazine[223,225,403]	Rat/2 years	0.5	Mutagenicity	No evidence
	Dog/52 weeks	7.5	Developmental	Not teratogenic
	Mouse/18 months	5.7	Reproductive	No evidence
	ADI	0.005	Oncogenicity	Not likely carcinogenic in humans

	Irritation		LD_{50} (mg/kg)				
Herbicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Ametryn	Nonirritant	Nonirritant	1160	>2020	>5.1	Positive	Caution
Prometryn	Slight irritant	Nonirritant	4550	>2020	>5.1	Negative	Caution
Prometon	Irritant	Mild irritant	1518	>2020	>3.2	Negative	Warning
Metribuzin	Nonirritant	Nonirritant	1090	>20,000	>0.65	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Ametryn[403,432]	Rat/2 years	2.5	Mutagenicity	No evidence
	Dog/52 weeks	10	Developmental	Not teratogenic
	Mouse/18 months	1.5	Reproductive	No evidence
	RfD	0.025	Oncogenicity	E (No evidence)
Prometryn[403,433,434]	Rat/2 years	37	Mutagenicity	No evidence
	Dog/106-week oral	3.7	Developmental	Not teratogenic
	Mouse/102-week oral	1.0	Reproductive	No evidence
	RfD (based on the 2 years. dog study; UF = 100)	0.037	Oncogenicity	E (No evidence)
Prometon[403]	Rat/2 years	1.0	Mutagenicity	No evidence
	Dog/52 weeks	5.0	Developmental	Not teratogenic
	Mouse/18 months	70	Reproductive	No evidence
	RfD	0.01	Oncogenicity	No evidence
Metribuzin[403,435]	Rat/2 years	5.0	Mutagenicity	No evidence
	Dog/104-week oral	2.5	Developmental	Not teratogenic
	Mouse/18 months	120	Reproductive	No evidence
	RfD (based on 2-year dog study; UF= 100)	0.025	Oncogenicity	No evidence

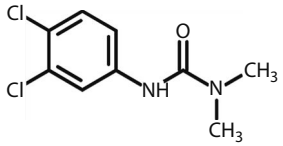
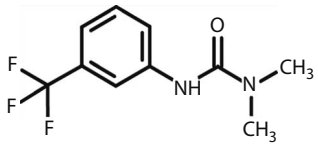
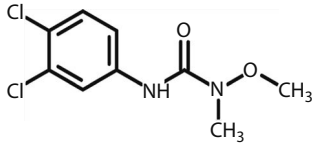
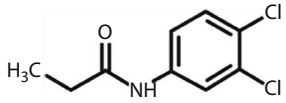
TABLE 16.60
Structures, Uses, and Hazard Profiles for the Photosynthesis-Inhibiting Triazolinone, Uracil, and Pyridazinone Herbicides (HRAC Code C1)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Flucarbazone (EVEREST®)[37,280]		Used postemergence for grass and broadleaved weeds in wheat	21
Bromacil (Hyvar®)[37,280]		Used to control grasses, broadleaf weeds, and brush in noncropland areas	1500–15,000
Terbacil (Sinbar®)[37,280]		Used to control grasses and broadleaf weeds in nut trees, mint, alfalfa, and fruit orchards	500–8000
Norflurazon (Predict®)[37,280]		Used to control broadleaf weeds and sedges in fruits, nuts, and berries; also used on right of ways	500–4000 9000 (right of ways)

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Flucarbazone	Slight irritant	Nonirritant	>5000	>5000	>5.13	Negative	Caution
Bromacil	Mild irritant	Mild irritant	5175	>5000	>4.8	Positive	Caution
Terbacil	Mild irritant	Nonirritant	1255	>5000	>4.4	Negative	Caution
Norflurazon	Nonirritant	Nonirritant	9000	>20,000	NA	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Flucarbazone-sodium[37,425]	Rat/2 years	125	Mutagenicity	Not mutagenic
	Dog/52 weeks	35.9	Developmental	Not teratogenic
	Mouse/2 years	275	Reproductive	No evidence
	cRfD	0.36	Oncogenicity	Not a likely human carcinogen
Bromacil[403,436]	Rat/2 years	2.5	Mutagenicity	Not mutagenic
	Dog/52 weeks	15.6	Developmental	Not teratogenic
	Mouse/18 months	—	Reproductive	Not a reproductive toxin
	RfD	0.1	Oncogenicity	C (liver tumors in male mice)
Terbacil[403]	Rat/2 years	2.5	Mutagenicity	Not mutagenic
	Dog/104 weeks	1.25	Developmental	Not teratogenic
	Mouse/18 months	7.1	Reproductive	Not a reproductive toxin
	ADI	0.013	Oncogenicity	E (No evidence)
Norflurazon[403,437,438]	Rat/2 years	19	Mutagenicity	Not mutagenic
	Dog/26 weeks	1.6	Developmental	Not teratogenic
	Mouse/18 months	41	Reproductive	Not a reproductive toxin
	RfD	0.02	Oncogenicity	C (Liver tumors in mice)

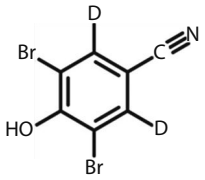
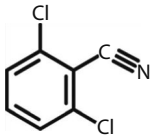
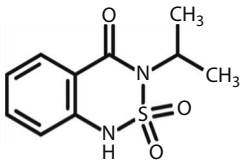
TABLE 16.61
Structures, Uses, and Hazard Profiles for the Photosynthesis-Inhibiting Urea and Amide Herbicides (HRAC Code C2)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Diuron (Diumate®)[37,280]		Used to control many annual weeds at lower rates and perennials at higher rates, including in nuts, berries, spices, and cereals	10,000–30,000
Fluometuron (Cotoran®)[37,280]		Used to control broadleaf weeds and grasses	1000–1500
Linuron (Lorox®)[37,280]		Used to control broadleaf weeds in vegetable and cereals	250–2240
Propanil (Stam®)[37,280]		Used to control broadleaved and grass weeds in rice, wheat, and citrus	2500–5000

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Diuron	Mild irritant	Nonirritant	3400	2000	>2.5	Negative	Caution
Fluometuron	Slight irritant	Nonirritant	6416	>10,000	>2.0	Negative	Caution
Linuron	Nonirritant	Nonirritant	1090	>20,000	>0.65	Negative	Caution
Propanil	Slight irritant	Nonirritant	>2500	>5000	>1.25	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Diuron[403,439,440]	Rat/2 years	<1.02	Mutagenicity	Not mutagenic
	Dog/104 weeks	0.625	Developmental	Not teratogenic
	Mouse/18 months	>50 (LDT)	Reproductive	Not a reproductive toxin
	RfD (UF = 300)	0.002	Oncogenicity	Known/likely (livers in mice; bladders in rats)
Fluometuron[403]	Rat/2 years	0.55	Mutagenicity	Not mutagenic
	Dog/52 weeks	10	Developmental	Not teratogenic
	Mouse/18 months	1.3	Reproductive	Not a reproductive toxin
	ADI[2]	0.0055	Oncogenicity	Classification pending
Linuron[403]	Rat/2 years	2.5	Mutagenicity	Not mutagenic
	Dog/104-week oral	0.77	Developmental	Not teratogenic
	Mouse/18 months	21	Reproductive	No reproductive toxin
	RfD	0.008	Oncogenicity	C (interstitial cell tumors in male rats)
Propanil[37,441]	Rat/2 years	400	Mutagenicity	Not mutagenic
	Dog/52 weeks	600	Developmental	No evidence
	Mouse/18 months	—	Reproductive	No evidence
	RfD	0.009	Oncogenicity	Not carcinogenic

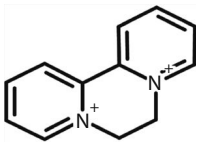
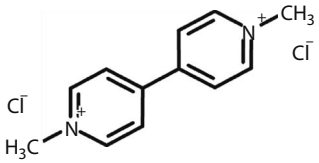
TABLE 16.62
Structure, Uses, and Hazard Profile for the Photosynthesis-Inhibiting Nitrile and Benzothiadiazone Herbicides (HRAC Code C3)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Bromoxynil (BUCTRIL®)[37,280]			
Dichlobenil (Acme®)[37,280]		Used to control annual, biennial broadleaf, and grasses in orchards, at industrial sites, under asphalt, and in noncrop areas	2700–8100
Bentazon (Basagran®)[37,280]		Used to control annual broadleaf weeds in soybeans, peas, peanuts, and cereals	1000–2240

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Bromoxynil	Corneal opacity, iritis, conjunctival irritation	Nonirritant	81	>2000	0.150	Negative	Warning
Dichlobenil	Nonirritant	Nonirritant	>1000	>2000	>0.25	Negative	Warning
Bentazon	Moderate irritant	Moderate irritant	1100	>2500	5.1	—	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Bromoxynil[442]	Rat/2 years	5.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.5	Developmental	Not teratogenic
	Mouse/18-month oral	2.6	Reproductive	Not a reproductive toxin
	RfD	0.015	Oncogenicity	C with Q_1* (liver tumors in mice)
Dichlobenil[403]	Rat/2 years	2.5	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.25	Developmental	Not teratogenic
	Hamster/18-month oral	10	Reproductive	Not a reproductive toxin
	RfD	0.013	Oncogenicity	C (liver tumors in female rats)
Bentazon[403,443]	Rat/2 years	17.5	Mutagenicity	Not mutagenic
	Dog/52 weeks	3.2	Developmental	Not teratogenic
	Mouse/18-month oral	50	Reproductive	Not a reproductive toxin
	RfD	0.03	Oncogenicity	E (No evidence)

TABLE 16.63
Structures, Uses, and Hazard Profiles for the Photosynthesis-Inhibiting Bipyridylium Herbicides (HRAC Code D)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Diquat (Weedtrine®)[37,280]		Used to control algae in ponds, lakes, and drainage ditches	400–1000
Paraquat (Cyclone®)[37,280]		Used to control existing vegetation at planting or for no-till cultivation	400–1000

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Diquat	Nonirritant	Slight irritant	>5000	>5000	>6	Negative	Caution
Paraquat	NA	Irritant	112	240	—	Negative	Warning

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Diquat[228,403,444]	Rat/2 years	0.6	Mutagenicity	Not mutagenic
	Dog/52 weeks	0.5	Developmental	Not teratogenic
	Mouse/18 months	3.5	Reproductive	Not a reproductive toxin
	RfD	0.005	Oncogenicity	E (No evidence)
Paraquat[227,403,445]	Rat/2 years	1.25	Mutagenicity	Not mutagenic
	Dog/52 weeks	0.45	Developmental	Not teratogenic
	Mouse/18 months	1.87	Reproductive	Not a reproductive toxin
	RfD	0.0045	Oncogenicity	E (No evidence)

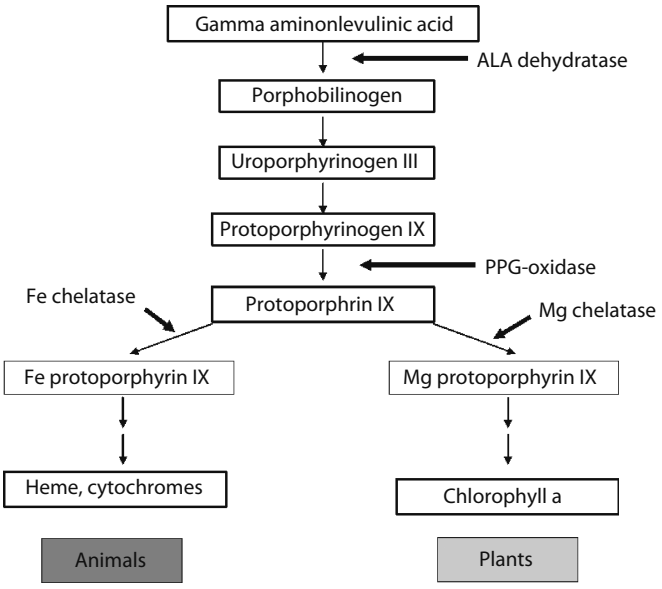


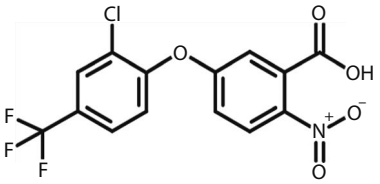
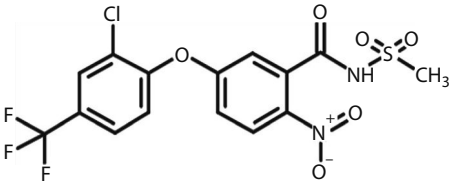
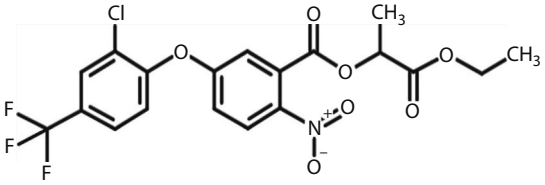
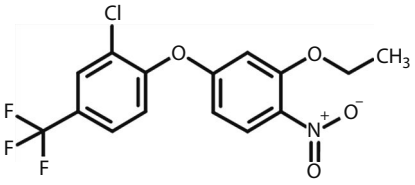
FIGURE 16.19 Protoporphyrin biosynthetic pathway.

interferes with the biosynthesis of heme and cytochrome P450 enzymes (Figure 16.19).

It is not uncommon to find evidence of anemia in rodents, especially rats, exposed to PPO-inhibiting herbicides. In addition to effects on heme synthesis, it is theorized that light-dependent (porphyria) and oxygen-dependent peroxidation of cell membrane lipids may lead to cell lysis and death, particularly in organs where protoporphyrin IX forms or bioconcentrates as a result of PPO inhibition.[229] Such a hypothesis is consistent with experimental observations that liver damage and liver tumor formation, particularly in mice, often result from high-dose exposures to PPO inhibitors.

It has also been postulated that liver damage and the subsequent tumor response seen in animals following high-dose exposures to PPO inhibitors may result from the peroxisome proliferative effects of these herbicides. Furthermore, it has been postulated that there may be a linkage between peroxisome proliferation and binding to PPARα receptors, as discussed in greater detail in the following text.[205,228]

TABLE 16.64
Structures, Uses, and Hazard Profiles for the Protoporphyrin-Inhibiting Diphenyl Ether Herbicides (HRAC Code E)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Acifluorfen (Scepter®)[37,280]		Used to control annual broadleaf weeds in peanuts, beans, and rice	200–600
Fomesafen (Flosil®)[37,280]		Used to control annual broadleaf weeds in soybeans	200–400
Lactofen (Cobra®)[37,280]		Used to control annual broadleaf weeds in cereals, potatoes, soy, and rice	70–220
Oxyfluorfen (Goal®)[37,280]		Used to control annual broadleaf weeds in conifers, vegetables, nuts, and vine crops	250–2240

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Acifluorfen	Nonirritant	Moderate irritant	1450	>2000	>6.9	Negative	Caution
Fomesafen	Moderate irritant	Mild irritant	1250	>1000	4.97	Negative	Caution
Lactofen	Severe irritant	Nonirritant	>5000	2000	—	—	Danger
Oxyfluorfen	Moderate irritant	Nonirritant	>5000	>5000	—	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Acifluorfen[403,446,447]	Rat/2 years	25	Mutagenicity	Not mutagenic
	Dog/52 weeks	NA	Developmental	Not teratogenic
	Mouse/18 months	38	Reproductive	Not a reproductive toxin
	RfD (rat reproduction)	0.013	Oncogenicity	B2 (liver/stomach tumors)
Fomesafen[363]	Rat/2 years	0.25	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.0	Developmental	Not teratogenic
	Mouse/18 months	1.0	Reproductive	Not a reproductive toxin
	RfD	0.0025	Oncogenicity	C-Q* (liver tumors in mice)
Lactofen[448,449]	Rat/2 years	25	Mutagenicity	Not mutagenic
	Dog/52 weeks	5.0	Developmental	Not teratogenic
	Mouse/18 months	1.5	Reproductive	Not a reproductive toxin
	RfD (mouse; UF = 1000)	0.002	Oncogenicity	B2 (liver/stomach tumors)
Oxyfluorfen[450,451]	Rat/2 years	2.0	Mutagenicity	Not mutagenic
	Dog/2-year oral	2.5	Developmental	Not teratogenic
	Mouse/18 months	0.3	Reproductive	Not a reproductive toxin
	RfD	0.003	Oncogenicity	C (liver tumors in mice)

Diphenyl Ethers (HRAC Code E)

The diphenyl ether PPO inhibitors are represented here by acifluorfen, fomesafen, lactofen, and oxyfluorfen. Their structures, uses, and hazard profiles are presented in Table 16.64.

Lactofen is a severe eye irritant, but otherwise the acute toxicity of the PPO inhibitors is not remarkable. Acifluorfen and lactofen are classified as B2 (probable human carcinogens) based upon an increased incidence of liver and stomach tumors. Oxyfluorfen and fomesafen are classified as category C (possible human carcinogens) based on an increased incidence of liver tumors.

A MOA underlying liver tumors commonly seen in this class of herbicide has been proposed.[205,230] The key events leading to tumor expression are redrawn in Figure 16.20 from the paper by Klaunig et al.[205] It has been proposed that diphenyl ethers serve as ligands to the PPARα receptor, thereby activating genes involved in peroxisome proliferation (key event 2a), the regulation of the cell cycle, the suppression of apoptosis (key event 2b), and lipid metabolism (key event 2c). Suppression of apoptosis, coupled with a stimulation of cell proliferation, allows cells with mutation to be selected for clonal expansion (key event 7), leading to preneoplastic foci and tumors. Peroxisome proliferation may cause oxidative stress (key event 5) and cell death, thereby further stimulating cell turnover.[205]

N-Phenylphthalimides, Thiadiazoles, and Triazolinones (HRAC Code E)

The structures, uses, and hazard profiles for the *N*-phenylphthalimide (flumiclorac-pentyl and flumioxazin), thiadiazole (fluthiacet-methyl), and triazolinone (carfentrazone-ethyl and sulfentrazone) PPO inhibitors are presented in Table 16.65. Flumiclorac-pentyl, which is applied at a rate of 30–90 g/ha, is not toxic in acute or repeat-dose studies. The thiadiazole, fluthiacet-methyl, which is also applied at a low rate of 4–15 g/ha, has a low reference dose, based upon results from the chronic mouse study. Fluthiacet-methyl caused liver tumors in mice and pancreatic tumors in rats. The reference dose for carfentrazone-ethyl (RfD = 0.03 mg/kg/day) is based upon the chronic rat study, where evidence of porphyrin deposits was seen in the liver at high doses.[231]

Oxadiazole and Pyrimidinedione Herbicides (HRAC Code E)

The structure, uses, and hazard profile for the oxadiazole PPO-inhibiting herbicide oxadiazon are presented in Table 16.65. Oxadiazon has an extremely low reference dose based on the hepatotoxicity and hemolytic anemia seen at high doses. Oxadiazon has been classified by EPA as a likely human carcinogen.

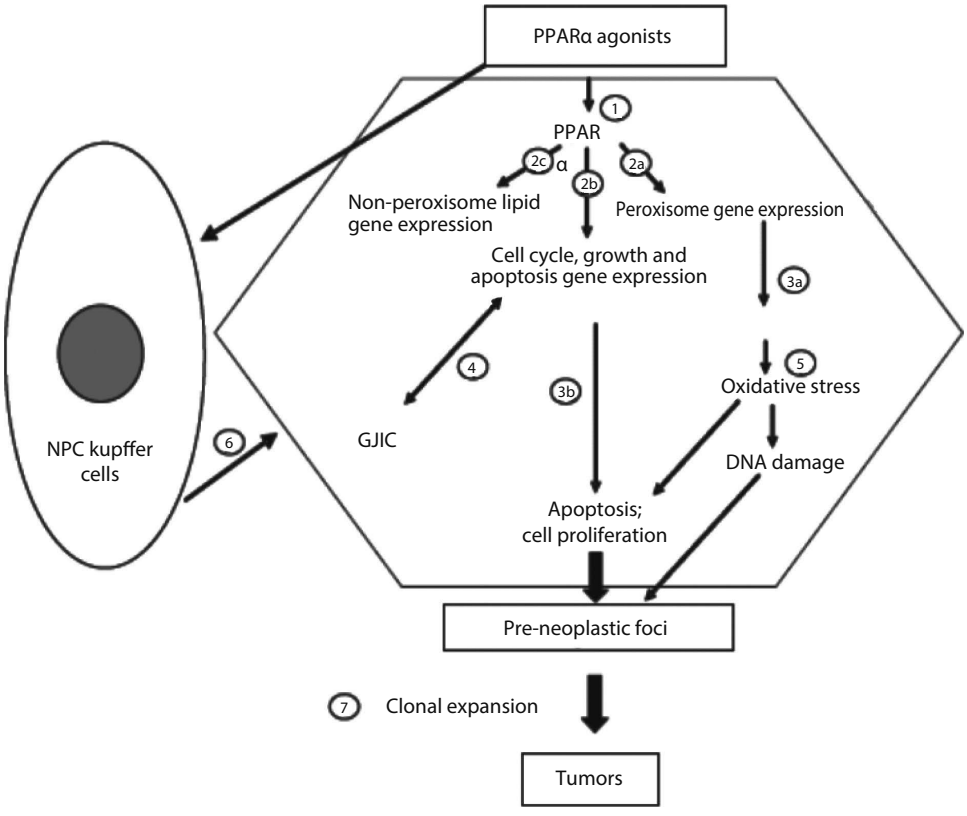
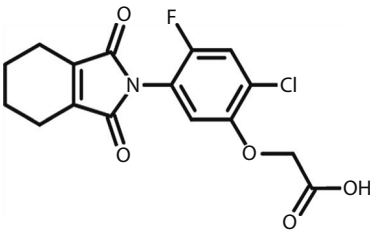
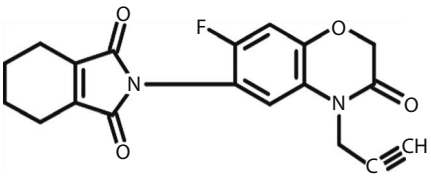
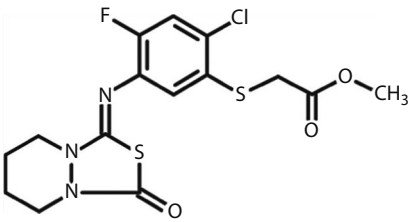
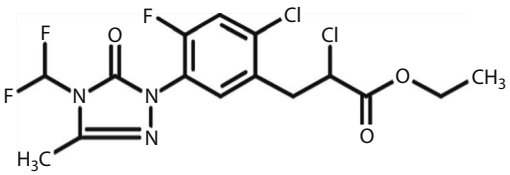
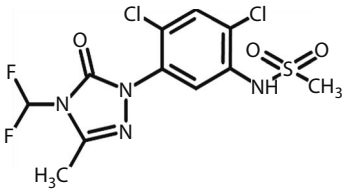
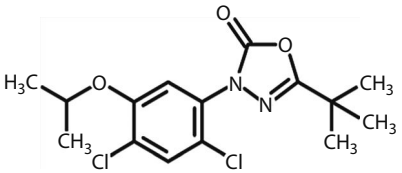


FIGURE 16.20 Proposed MOA for PPARα agonists.

TABLE 16.65
Structures, Uses, and Hazard Profiles for the Protoporphyrin-Inhibiting N-phenylphthalimide, Thiadiazole, Triazolinone, and Oxadiazole Herbicides (HRAC Code E)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Flumiclorac-pentyl (Resource®)[37,280]		Used to control broadleaf weeds in soybeans and corn	30–60
Flumioxazin (PLEDGE®)[37,280]			
Fluthiacet-methyl (Action®)[37,280]		Used to control annual broadleaf weeds in corn, soybeans, and cereals	4–15
Carfentrazone-ethyl (Affinity®, Aurora®)[37,280]		Used to control annual broadleaf weeds in cereals	9–35
Sulfentrazone (Spartan®)[37,280]			
Oxadiazon (Ronstar®)[37,280]		Used for control of bindweed and annual broadleaf weeds in flowers, fruit trees, bushes, sunflowers, and onions	1000–4000

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Flumiclorac-pentyl	Slight irritant	Nonirritant	>5000	>2000	>5.9	Negative	Caution
Flumioxazin	Nonirritant	Nonirritant	>5000	>2000	3.93	Negative	Caution
Fluthiacet-methyl	Slight irritant	Nonirritant	>5000	>2000	>5.0	NA	Caution
Carfentrazone-ethyl	Minimal irritant	Nonirritant	5143	>4000	>5.0	Negative	Caution
Sulfentrazone	Minimal irritant	Nonirritant	2855	>2000	>4.13	Negative	Caution
Oxadiazon	Slight irritant	Negligible irritant	>5000	>2000	>2.77	Negative	Caution

TABLE 16.65 (continued)
Structures, Uses, and Hazard Profiles for the Protoporphyrin-Inhibiting N-phenylphthalimide, Thiadiazole, Triazolinone, and Oxadiazole Herbicides (HRAC Code E)

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Flumiclorac-pentyl[403,452]	Rat/2 years	35	Mutagenicity	Not mutagenic
	Dog/52 weeks	100	Developmental	Not teratogenic
	Mouse/18 months	32	Reproductive	Not a reproductive toxin
	RfD	0.32	Oncogenicity	E (No evidence)
Flumioxazin[453]	Rat/2 years	2.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	100	Developmental	Not teratogenic
	Mouse/18 months	754.1	Reproductive	Not a reproductive toxin
	RfD	0.02	Oncogenicity	Not likely to be a human carcinogen
Fluthiacet-methyl[454,455]	Rat/2 years	2.1	Mutagenicity	Not mutagenic
	Dog/52 weeks	30	Developmental	Not teratogenic
	Mouse/18 months	0.1	Reproductive	Not a reproductive toxin
	RfD	0.001	Oncogenicity	Likely carcinogen (mouse liver tumors; rat pancreatic tumors)
Carfentrazone-ethyl[231]	Rat/2 years	3.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	50	Developmental	Not teratogenic
	Mouse/18 months	10	Reproductive	Not a reproductive toxin
	RfD	0.03	Oncogenicity	E (No evidence)
Sulfentrazone[456]	Rat/2 years	36.4	Mutagenicity	Not mutagenic
	Dog/52 weeks	24.9	Developmental	Not teratogenic
	Mouse/18 months	93.9	Reproductive	Not a reproductive toxin
	RfD	0.14	Oncogenicity	No evidence of carcinogenicity in rats or mice
Oxadiazon[457]	Rat/2 years	0.36	Mutagenicity	Not mutagenic
	Dog/52 weeks	—	Developmental	Not teratogenic
	Mouse/18 months	10	Reproductive	Not a reproductive toxin
	RfD	0.0036	Oncogenicity	Likely to be carcinogenic $Q_1^* = 7.11 \times 10^{-2}$ (mg/kg/day)$^{-1}$

Bleaching Herbicides (HRAC Code F1)

The bleaching herbicides disrupt the synthesis of carotenoid pigments, which protect chlorophyll pigments from photo-oxidation in strong light (see Figure 16.21). In the absence of carotenoids, chlorophyll is destroyed and turns white, thus the leaves of the plant have a bleached appearance. The pyridazinone, triketone, and isoxazole bleaching herbicides are considered here. The pyridazinones inhibit carotenoid biosynthesis at the phytoene desaturase step,[232] whereas the triketones, which were initially identified in the bottlebrush plant,[233,234] and the isoxazoles inhibit 4-hydroxyphenylpyruvate dioxygenase (HPDD).[233] The site of action for the triazoles and isoxazolidinones is unknown.[6] Of these MOA, only the HPDD inhibition is relevant to humans because blocking HPDD results in an increased incidence of tryrosinemia in animals.[235]

Pyridazinones (HRAC Code F1)

The structures, uses, and hazard profiles for norflurazon and fluridone are given in Table 16.66. Neither norflurazon nor fluridone is acutely toxic; the repeated-dose profile for fluridone is unremarkable. Norflurazon is classified as category C (possible human carcinogen) based on mouse liver tumors.

Triketones and Isoxazoles (HRAC Code F2)

The structures, uses, and hazard profiles for mesotrione and tembotrione (triketone), as well as for isoxaflutole (isoxazole), are presented in Table 16.67. Mesotrione is not mutagenic, carcinogenic, or neurotoxic. It has a low reference dose based on effects on tyrosine and its sequelae in rats. Mice and dogs are less sensitive and more similar to humans than are rats with respect to the inhibition of HPDD in vitro. Isoxaflutole is not acutely toxic, but it causes developmental and neurotoxic effects and liver tumors in mice and rats.

Triazoles and Isoxazolidinones (HRAC Code F3)

The chemical structures, uses, and hazard profiles for amitrole (triazole), clomazone (isoxazolidinone), and topramezone (isoxazolidinone) are presented in Table 16.68. These compounds are not acutely toxic. Clomazone has

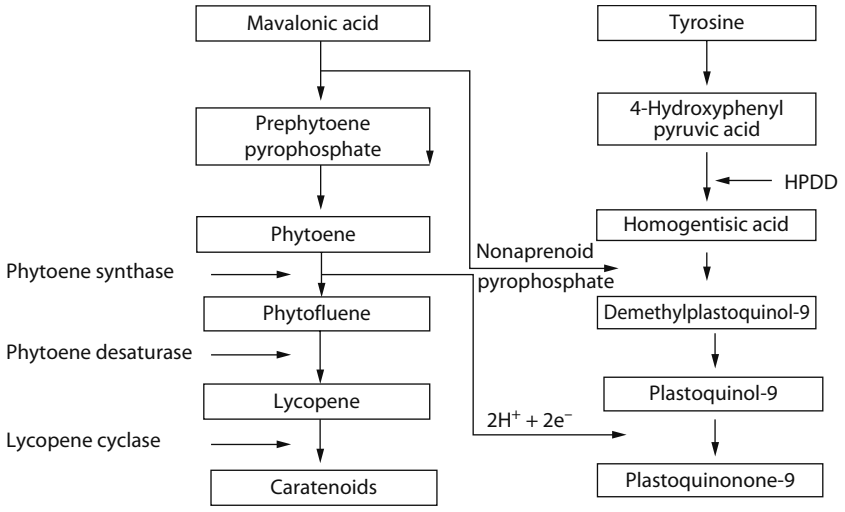
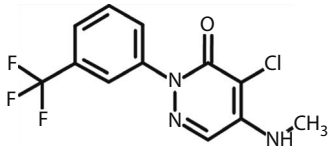
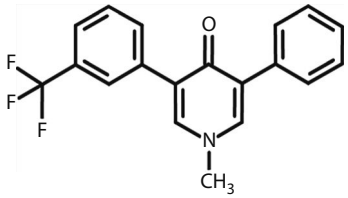


FIGURE 16.21 Carotenoid pigment and chlorophyll biosynthetic pathways.

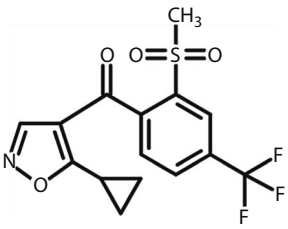
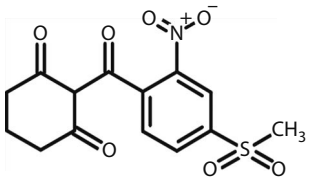
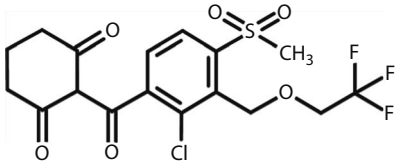
TABLE 16.66
Structures, Uses, and Hazard Profiles for the Photobleaching Pyridazinone Herbicides (HRAC Code F1)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Norflurazon (Evital®)[37,280]		Fruit trees, nut trees, vine crops, soybeans, peanuts, ornamentals, cotton, and IWC	500–3360
Fluridone (Sonar®)[37,280]		Aquatic herbicide	2240 (0.075–0.15 mg/L)

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Norflurazon	Nonirritant	Nonirritant	>9000	>20,000	>0.2	Negative	Caution
Fluridone	Slight	Nonirritant	>10,000	>5000	>4.12	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Norflurazon[403,438]	Rat/2 years	19	Mutagenicity	Not mutagenic
	Dog/52 weeks	1.5	Developmental	Not teratogenic
	Mouse/18 months	41	Reproductive	Not a reproductive toxin
	RfD	0.02	Oncogenicity	C (mouse liver tumors)
Fluridone[403]	Rat/2 years	8.0	Mutagenicity	Not mutagenic
	Dog/52 weeks	11.4	Developmental	Not teratogenic
	Mouse/18 months	11.6	Reproductive	Not a reproductive toxin
	RfD	0.08	Oncogenicity	E (No evidence)

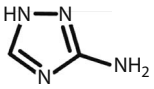
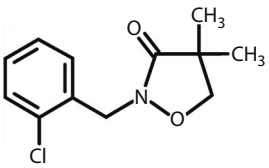
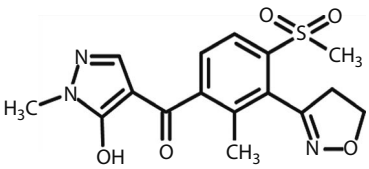
TABLE 16.67
Structures, Uses, and Hazard Profiles for the Photobleaching Triketone and Isoxazole Herbicides (HRAC Code F2)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Isoxaflutole (BALANCE®)[37,280]		Corn	75–140
Mesotrione (Callisto™)[37,280]		Controls broadleaf and some grass weeds in maize or corn	70–225
Tembotrione (Laudis®)[37,280]		Monocot and dicot weeds in corn	100

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Isoxaflutole	Mild irritant	Minimal	>5000	>2000	>5.3	Negative	Caution
Mesotrione	Mild irritant	Slight irritant	>5000	>5000	>5.19	Negative	Caution
Tembotrione	Moderate irritant	Nonirritant	>2000	>2000	>5.03	Negative (U.S.)	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Isoxaflutole[458–460]	Rat/2 years	2.0	Mutagenicity	No evidence
	Dog/52 weeks	45	Developmental	Developmental toxicity
	Mouse/18 months	3.2	Reproductive	No evidence
	RfD	0.002	Oncogenicity	Likely to be a carcinogen (liver tumors in both sexes of rats and mice)
Mesotrione[234,235]	Rat/2 years	0.16 (NOAEL)	Mutagenicity	Not mutagenic
	Dog/52 weeks	10	Developmental	Not teratogenic
	Mouse/18 months	56.2	Reproductive	Not a reproductive toxin
	RfD	0.007	Oncogenicity	Not carcinogenic
Tembotrione[461]	Rat/2 years	0.04	Mutagenicity	Not mutagenic
	Dog/52 weeks	2.5 (male)	Developmental	Not teratogenic
	Mouse/18 months	4.0 (male)	Reproductive	Not a reproductive toxin
	(LOAEL)	0.0004	Oncogenicity	Suggestive evidence of carcinogenic potential

TABLE 16.68
Structures, Uses, and Hazard Profiles for the Photobleaching Triazole and Isoxazolidinone Herbicides (HRAC Code F3)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Amitrole (Amizol®)[37,280]		Fruit trees, grapes, olives, ornamentals, cereals, IWC, and aquatic plants	1000–5000
Clomazone (Command®)[37,280]		Soybeans, peas, and peppers	560–1700
Topramezone (Armezon™)[37,280]		Postemergent corn weeds	90–180

	Irritation		LD_{50} (mg/kg)				
Herbicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Amitrole	Slight irritant	Slight irritant	>5000	>2000	—	—	Caution
Clomazone	Nonirritant	Minimal	2077	>2000	4.23 (female)	Negative	Caution
Topramezone	Slight irritant	Slight irritant	>2000	>2000	>5.0	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Amitrole[462]	Rat/2 years	0.5	Mutagenicity	No evidence
	Dog/52 weeks	NA	Developmental	Not teratogenic
	Mouse/18 months	1.4	Reproductive	No evidence
	RfD	Q* = 1.13 $(mg/kg/day)^{-1}$	Oncogenicity	B2 (thyroid tumor in both sexes of rats and mice and liver tumor in mice)
Clomazone[463]	Rat/2 years	4.3	Mutagenicity	No evidence
	Dog/52 weeks	12.5	Developmental	Not teratogenic
	Mouse/18 months	143	Reproductive	No evidence
	RfD	0.043	Oncogenicity	E (No evidence)
Topramezone[464,465]	Rat/2 years	0.4	Mutagenicity	Not mutagenic
	Dog/52 weeks	2.9	Developmental	Not teratogenic
	Mouse/18 months	19	Reproductive	Not a reproductive toxin
	RfD	0.004	Oncogenicity	Not likely to be carcinogenic

an unremarkable repeated-dose toxicity profile. Amitrole has been classified as category B2 (probable human carcinogen) based on thyroid tumors in rats and liver tumors in mice.[99] These tumor responses could be related to a *triazole-related action on the liver* (see the triazole fungicide section for a detailed discussion of this MOA and its relevance to man).

EPSP Synthase, Glutamine Synthase, and Dihydropteroate Synthase Inhibitors (HRAC Codes G, H, and I)

Glyphosate is a nonselective herbicide that inhibits 5-enolpyruvlshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway (Figure 16.22) of plant plastids.[236] Inhibition of EPSPS blocks the biosynthesis of the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp), as well as secondary products important for plant growth and development including lignans, alkaloids, flavonoids, and benzoic acids.[237] This pathway does not exist in mammals, and therefore this MOA is not relevant to man.

Glutamine synthetase is the initial enzyme in the pathway that assimilates inorganic nitrogen into organic compounds in plants. It is the pivotal enzyme in nitrogen metabolism in plants that, in addition to assimilating ammonia, recycles ammonia produced by other processes, including photorespiration and deamination reactions (Figure 16.23). Glutamine synthetase is found in analogous pathways in mammals, and it plays a similar role in recycling nitrogen. Glufosinate (DL-phosphinothricin) is a close structural analogue of glutamic

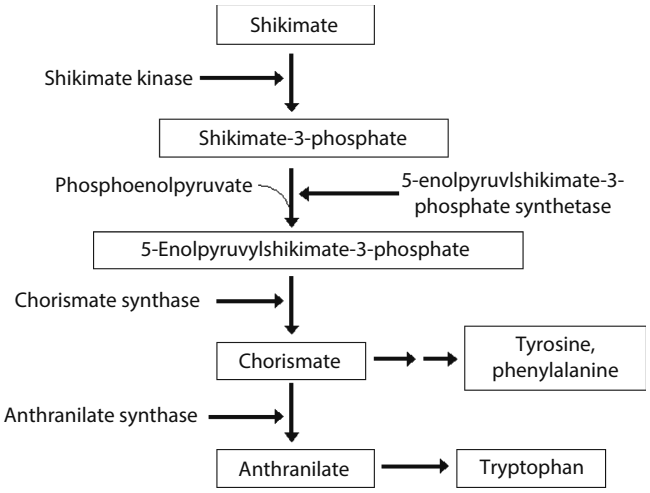


FIGURE 16.22 Aromatic amino acid biosynthetic pathway.

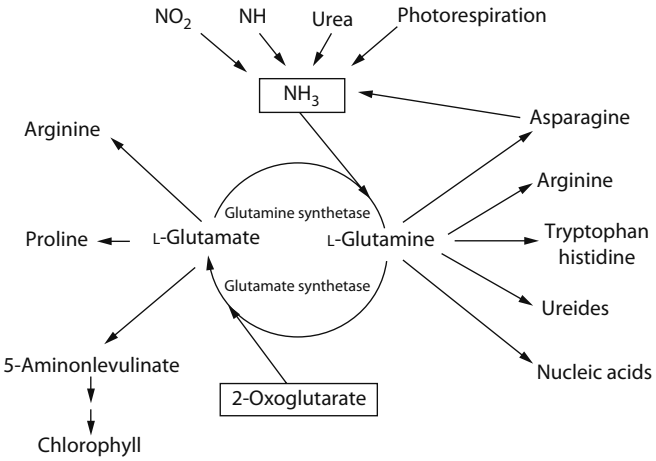


FIGURE 16.23 Nitrogen fixation and recycling in plants.

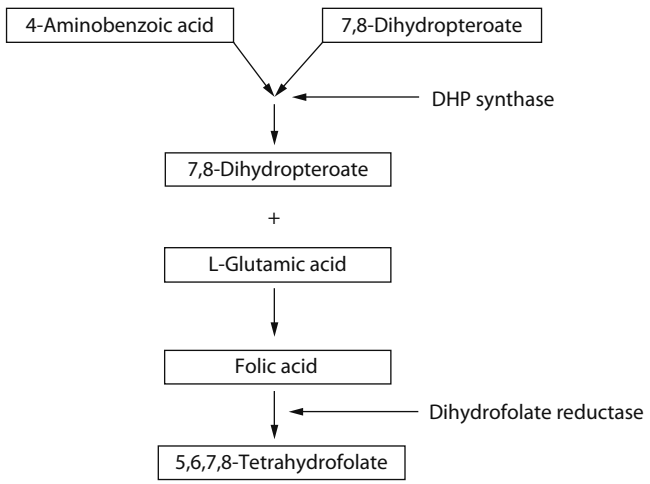


FIGURE 16.24 Folic acid synthesis.

acid and is considered to be the active pesticidal component in the natural tripeptide, phosphinothricin-alanyl-alanine (bialaphos), first discovered in *Streptomyces viridochromogenes*.[238] Glufosinate inhibits glutamine synthase, resulting in an accumulation of ammonium and the inhibition of photosynthesis.[237]

Folic acid, or its coenzyme form, serves as an intermediate carrier of hydroxymethyl, formyl, or methyl groups in enzyme-mediated reactions leading to the synthesis of amino acids, purines, and pyrimidines (Figure 16.24). Dihydropteroate (DHP) synthase catalyzes the first step of the folic acid biosynthetic pathway.[239]

Asulam inhibits DHP synthase, thereby blocking folic acid synthesis, which is needed for the formation of purine nucleotides required for cell division.[239] Asulam and other members of this class are structural analogues of 4-aminobenzoic acid and likely serve as substrates for DDP synthase, because the administration of 4-aminobenzoic acid reverses the phytotoxicity seen in plants and microbes treated with DHP synthase inhibitors.[239] The structures, uses, and hazard profiles for the representative EPSPS synthase, glutamine synthetase, and dihydropteroate synthase inhibitors are given in Table 16.69.

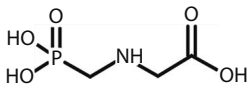
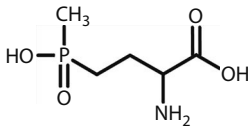
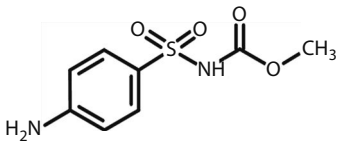
Glyphosate, glufosinate-ammonium, and asulam are not acutely toxic. Glyphosate and glufosinate-ammonium are not toxic in repeat-dose studies, and neither chemical is a developmental toxin, mutagen, or carcinogen. Asulam is not mutagenic, teratogenic, or a reproductive toxin, but a statistically significant increase in thyroid and adrenal gland tumors was observed in the male rat. A margin-of-exposure (MOE) approach was used to assess carcinogenic risk, likely because of asulam's structural similarity to products that occur naturally in plants.[240]

Dinitroaniline Microtubule Assembly Inhibitors (HRAC Code K1)

Several groups of herbicides, including the dinitroanilines (e.g., benfluralin, pendimethalin, and trifluralin), the phosphoroamidates (amiprophos-methyl and butamiphos), the pyridines (aminopyralid, dithiopyr, and thiazopyr), the benzamides (propyzamide and tebutam), and benzoic acid (DCPA), bind to tubulin monomers and prevent microtubule polymerization. This MOA is considered relevant to man (see the discussion on microtubule formation in the section on the benzimidazole and thiophanate fungicides).

The structures, uses, and hazard profiles of the three commercially important dinitroaniline microtubule assembly inhibitors are given in Table 16.70. Benfluralin, pendimethalin, and trifluralin are not acutely toxic. Benfluralin caused liver and thyroid tumors in rats at doses that exceeded the maximum tolerated dose and therefore was considered a suggestive human carcinogen; an MOE approach was used for cancer risk assessment.[241] Pendimethalin, which caused an increased incidence of thyroid tumors, was classified as a category C substance (possible human carcinogens) using an MOE approach

TABLE 16.69
Structures, Uses, and Hazard Profiles of EPSP Glutamine and DHP Synthase–Inhibiting Glycine and Phosphinic Acid Herbicides (HRAC Codes G, H, and I)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Glyphosate (Roundup®) EPSP synthase inhibitor[37,280]		Corn, soybeans, and IWC	1500–4300
Glufosinate-ammonium (Finale®) Glutamine synthase inhibitor[37,280]		Fruit trees, grapes, rubber, palm ornamentals, vegetables, and IWC	400–1500
Asulam (Asulox®)[37,280]		Sugarcane, alfalfa, banana, coffee, tea, cocoa, and pasture forestry	1000–10,000

	Irritation		LD_{50} (mg/kg)				
Herbicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Glyphosate	Slight	Nonirritant	5600	>5000	—	Negative	Caution
Glufosinate-ammonium	Nonirritant	Nonirritant	1620	4000	1.26	—	Caution
Asulam	Irritant	Slight irritant	>5000	>2000	>1.8	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Glyphosate[236,403,466]	Rat/2 years	400	Mutagenicity	Not mutagenic
	Dog/52 weeks	500	Developmental	Not teratogenic
	Mouse/18 months	4500	Reproductive	Not a reproductive toxin
	RfD	0.1	Oncogenicity	E (No evidence)
Glufosinate-ammonium[403,467]	Rat/2 years	2.1	Mutagenicity	Not mutagenic
	Dog/52 weeks	NA	Developmental	Not teratogenic
	Mouse/18 months	NA	Reproductive	Not a reproductive toxin
	RfD	0.02	Oncogenicity	Not carcinogenic
Asulam[240]	Rat/2 years	36	Mutagenicity	Not mutagenic
	Dog/52 weeks	60	Developmental	Not teratogenic
	Mouse/18 months	713	Reproductive	Not a reproductive toxin
	RfD or ADI	0.36	Oncogenicity	C (Thyroid and adrenal gland tumors in male rats)

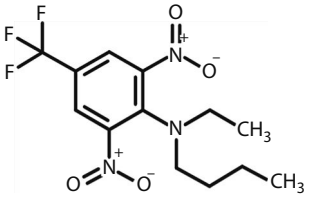
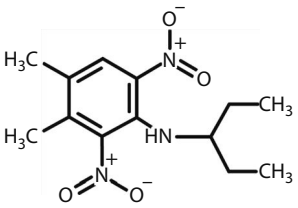
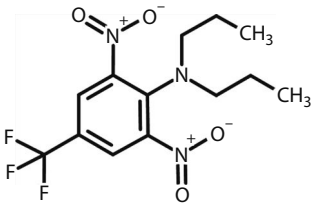
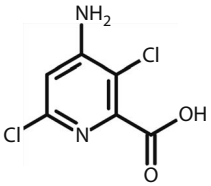
for cancer risk assessment.[242] Trifluralin caused thyroid, bladder, and kidney tumors, and its carcinogenic risk was regulated using Q_1*.[243,244] These tumorigenic effects are unlikely related to the herbicidal MOA of this class.

Chloroacetamide Inhibitors of Very-Long-Chain Fatty Acid Synthesis (HRAC Code K3)

Böger and Matthes reviewed the evidence suggesting that the chloroacetamides block the formation of very-long-chain saturated fatty acids (VLCFA) by inhibiting fatty acid elongase,[245] as shown in Figure 16.25. Vertebrates, including humans, have the biochemical mechanisms needed to synthesize long-chain fatty acids, including the enzyme, long-chain fatty acid acyl elongase.[246] Humans, however, lack the desaturase enzymes (not shown in Figure 16.25) that produce the health-promoting very-long-chain polyunsaturated fatty acids synthesized by plants and fish.[247] It is plausible that the chloroacetamides could perturb fatty acid synthesis in mammals, but there is no direct evidence of this in animal studies.

Aside from the herbicidal MOA of the chloroacetamides, alachlor, acetochlor, and butachlor have been identified as sharing a common mechanism of toxicity.[248] Alachlor, acetochlor, and butachlor all undergo dealkylation to form aniline and a reactive quinine imine (see Figure 16.26), the latter of which is thought to be the carcinogenic moiety ultimately responsible for nasal epithelial adenomas and carcinomas found in rats.[249] A sulfoxide metabolite of acetochlor was found in the plasma of rats treated with acetochlor, and this was bioconcentrated in the nasal epithelial tissue. Nasal epithelial tissue from humans apparently does not support the metabolic

TABLE 16.70
Structures, Uses, and Hazard Profiles for the Dinitroaniline Microtubule Assembly–Inhibiting and Pyridine Herbicides (HRAC Code K1)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Benfluralin (Balan®, Benefin®)[37,280]		Alfalfa, clover, lettuce, and tobacco	1260–1680
Pendimethalin (Prowl®)[37,280]		Corn, sorghum, rice, soybeans, cotton, potatoes, tobacco, sugarcane, beans, onions, and sunflower	560–3360
Trifluralin (Treflan®)[37,280]		Alfalfa, asparagus, beans, carrots, celery, cole crops, cucurbits, onions, okra, peas, peppers, potatoes, sunflower, tomatoes, wheat, barley, flax, soybeans, corn, sorghum, and ornamentals	500–1000
Aminopyralid[37,280]		Used on weeds in grassland as a foliar herbicide	—

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Benfluralin	Slight irritant	Slight irritant	>10,000	>5000	>2.3	Positive	Caution
Pendimethalin	Slight irritant	Nonirritant	1050	>5000	320 (nominal)	Negative	Caution
Trifluralin	Slight irritant	Nonirritant	>5000	>5000	>4.8	Positive	Caution
Aminopyralid	Nonirritant	Nonirritant	>5000	>5000	>5.79	Negative	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Benfluralin[241]	Rat/2 years	0.5	Mutagenicity	No evidence
	Dog/52 weeks	25	Developmental	Not teratogenic
	Mouse/18 months	6.5	Reproductive	No evidence
	RfD	0.005	Oncogenicity	Suggestive (liver and thyroid tumors at doses greater than the MTD)
Pendimethalin[242,403,468]	Rat/2 years	10.0	Mutagenicity	No evidence
	Dog/104 weeks	12.5	Developmental	Not teratogenic
	Mouse/18 months	75	Reproductive	No evidence
	RfD	0.13	Oncogenicity	C with RfD (thyroid follicular cell adenomas)
Trifluralin[243,244,403]	Rat/2 years	2.5	Mutagenicity	No evidence
	Dog/52 weeks	2.4	Developmental	Not teratogenic
	Mouse/18 months	7.5	Reproductive	No evidence
	RfD	0.024	Oncogenicity	C-Q* (bladder, kidney, thyroid tumors)
Aminopyralid[469]	Rat/2 years	50	Mutagenicity	Not mutagenic
	Dog/52 weeks	93	Developmental	Not teratogenic
	Mouse/18 months	1000	Reproductive	Not a reproductive toxin
	RfD	0.5	Oncogenicity	Not likely to be carcinogenic

conversion of the sulfoxide to the sulfoxide of the quinine imine, whereas the rat made this conversion.[250]

The structures, uses, and hazard profiles for alachlor, acetochlor, metolachlor, and dimethenamid are provided in Table 16.71. All four chloracetamide herbicides are potential skin sensitizers. Alachlor and acetochlor both exhibit mutagenic potential and significant oncogenic potential in both rats and mice. Dimethenamid also has exhibited weak genotoxicity and a tumor response in the female rat. Metolachlor showed a weak oncogenic response in the liver of the female rat. A common mechanism of action has been proposed by EPA for alachlor and acetochlor based upon the carcinogenic potential of the quinine imine reactive intermediate.[248]

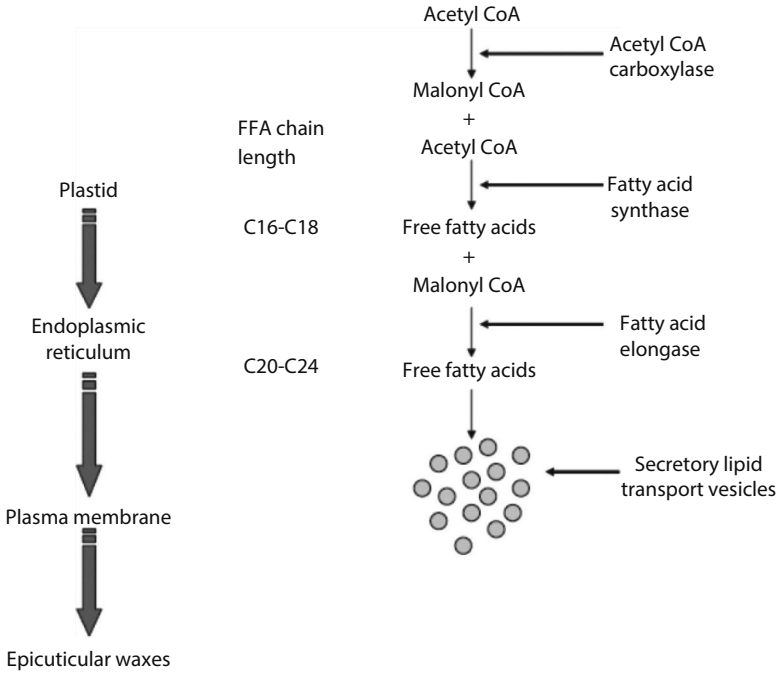


FIGURE 16.25 Long-chain fatty acid synthesis in plants.

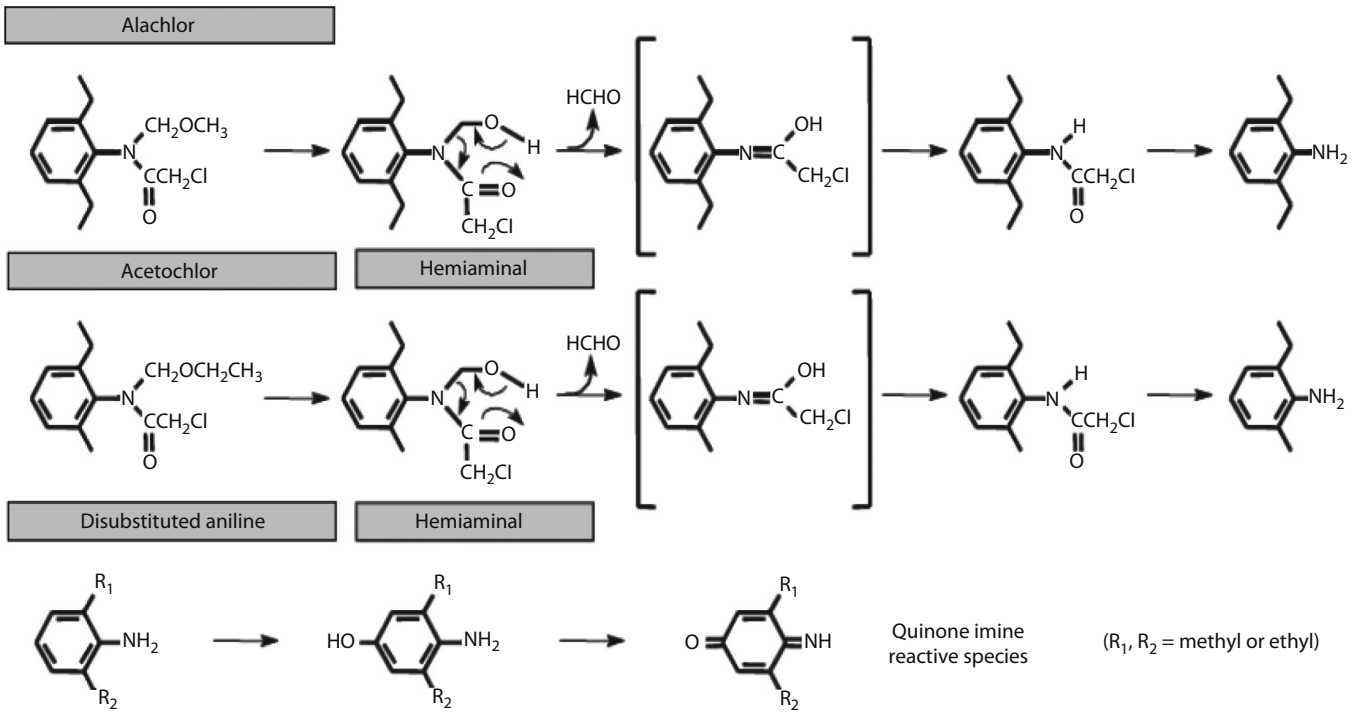
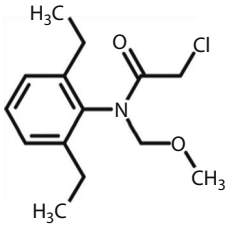
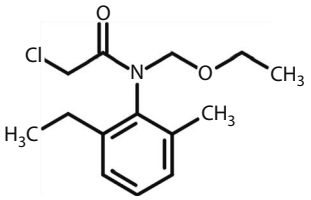
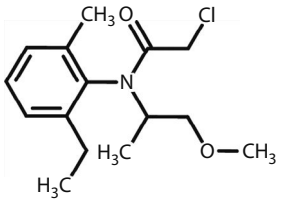
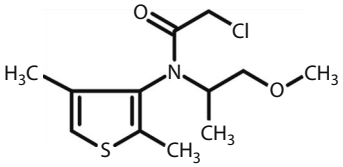


FIGURE 16.26 Chloroacetamide common mechanism grouping.

TABLE 16.71
Structures, Uses, and Hazard Profiles for the Chloroacetamide Inhibitors of Very-Long-Chain Fatty Acid Synthesis (HRAC Code K3)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Alachlor (Lasso®)[37,280]		Corn, beans, peanuts, sorghum, soybeans, sunflowers, and ornamentals	1500–4500
Acetochlor (Surpass®)[37,280]		Corn, soybeans, sorghum, and wheat	900–3360
Metolachlor (Dual®)[37,280]		Corn, soybeans, sorghum, cucurbits, onions, peas, pecans, peppers, potatoes, and sugar beets	1250–6200
Dimethenamid (Frontier®)[37,280]		Corn and soybeans	850–1440

	Irritation		LD_{50} (mg/kg)				
Herbicide	Eye	Skin	Oral	Dermal	LC_{50} (mg/L) Inhalation	Sensitization Potential	Signal Word
Alachlor	Nonirritant	Nonirritant	930	13,300	>1.04	Positive	Caution
Acetochlor	Slight irritant	Nonirritant	2148	4166	>3.0	Positive	Caution
Metolachlor	Nonirritant	Minimal irritant	>2780	>10,000	>1.75	Positive	Caution
Dimethenamid	Slight irritant	Nonirritant	1570	>2000	>5.0	Positive	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Alachlor[230,248,470]	Rat/2 years	2.5	Mutagenicity	Positive (UDS)
	Dog/52 weeks	1.0	Developmental	Not teratogenic
	Mouse/18 months	16.6	Reproductive	Not a reproductive toxin
	RfD	0.01	Oncogenicity	C/RfD (nasal in rats; lungs in mice)
Acetochlor[248,250,471,472]	Rat/2 years	8.0	Mutagenicity	Positive (CHO, UDS, mouse lymphoma)
	Dog/52 weeks	2.0	Developmental	Not teratogenic
	Mouse/18 months	13	Reproductive	Not a reproductive toxin
	RfD	0.02	Oncogenicity	B2 (liver, thyroid, and nasal tumors in rats; lung tumors in mice)
Metolachlor[248,472]	Rat/2 years	15	Mutagenicity	Not mutagenic
	Dog/52 weeks	10	Developmental	Not teratogenic
	Mouse/18 months	120	Reproductive	Not a reproductive toxin
	RfD	0.1	Oncogenicity	C/RfD (liver tumors in female rats)
Dimethenamid[473]	Rat/2 years	5.0	Mutagenicity	Weakly positive (CHO: UDS)
	Dog/52 weeks	9.6	Developmental	Not teratogenic
	Mouse/18 months	40	Reproductive	Not a reproductive toxin
	RfD	0.05	Oncogenicity	C/RfD (livers, ovaries in female rats)

Cellulose and Lipid Synthesis Inhibitors (HRAC Code L and N)

Isoxaben is a member of the benzamide class of herbicides that block the incorporation of glucose into cellulose.[251] This mechanism whereby isoxaben has this effect is not well understood, but it is unlikely relevant to man. Quinclorac also blocks the radiolabeled uptake of glucose into cellulose in certain monocot species.[252] However, it has also been suggested that quinclorac activates the auxin receptor, which has been tentatively identified,[253] leading to increased ACC (1-aminocyclopropane-1-carboxylic acid) synthase activity that results in not only the production of ethylene but also the increased amount of cyanide (Figure 16.27). Ethylene triggers biochemical changes leading to senescence in plants but cyanide, which is formed in grasses treated with quinclorac, is directly phytotoxic.[254] This pathway is not present in mammals, although TIR1 has homology with the human SKP2 (S-phase kinase–associated protein) and its corresponding gene.[255]

The herbicidal MOA for the thiocarbamate herbicide butylate is through the inhibition of fatty acid synthesis. Inhibition is achieved, not by blocking ACCase (Figure 16.16), but rather by inhibiting long-chain fatty acid synthesis (Figure 16.25). Some of the thiocarbamate herbicides (EPTC, pebulate, and cycloate) have been determined to belong to a common mechanism grouping with the carbamate insecticides, based upon their ability to inhibit acetylcholinesterase.[98] However, the most sensitive toxicological endpoint of this class of herbicide is sciatic nerve degeneration, and it is the NOELs for these endpoints that are used to assess acute and chronic risks.[256] The EPA did not establish a common mechanism grouping for the thiocarbamate herbicides based upon neuropathology because they concluded that the proposed mechanism underlying this response[257] was not adequately understood.[258]

The structures, uses, and hazard profiles of the cellulose synthesis inhibitors isoxaben and quinclorac, as well as the lipid synthesis inhibitors represented by butylate, are given in Table 16.72.

Isoxaben exhibits low acute toxicity and is only moderately toxic in repeat-dose studies (chronic RfD = 0.05 mg/kg/day), but it is a developmental toxin at maternally toxic doses. There was a positive micronucleus test, and there were adrenal gland and liver tumors.

The toxicity profile for butylate is unremarkable. It was not neurotoxic, although it was positive in the skin sensitization study.

Synthetic Auxin Mimics (Phenoxy, Benzoic, and Pyridine Acids) (HRAC Code O)

Indole 3-acetic acid, which is the plant hormone auxin, plays a critical role in regulating plant cell growth and differentiation by binding to its receptor(s).[259–261] The mechanism of auxin signaling has been the subject of intense research efforts,[262,263] and progress has been made in identifying the critical receptor proteins, the TIR1, F-box proteins (AFB1, AFB2, and AFB3 in *Arabidopsis*),[253,260,261] which are involved in the activation of these signaling pathways. Auxin binding mediates the association of a set of transcriptional repressor proteins (AUX/IAA proteins; 29 members in *Arabidopsis*) with another protein, the SCF complex. The SCF complex mediates polyubiquitination of the repressor proteins, which are then targeted for degradation by proteasome. With the repressor proteins removed, various sets of genes are induced, including those involved with cell elongation and cell division.[259] Grossman has suggested that the herbicidal action of the auxin mimics is through auxin-induced ACC synthase upregulation that results in ethylene-mediated senescence as well as cyanide-mediated phytotoxicity.[217,254] These pathways are not present in mammalian systems, although homologues of the F-box proteins have been identified in mammals.[255]

Table 16.73 provides the structures, uses, and hazard profiles for the auxin mimics, 2,4-D (phenoxy) and dicamba (benzoic), as well as for clopyralid and picloram (pyridine acids). The hazard profile for 2,4-D is unremarkable. Dicamba,

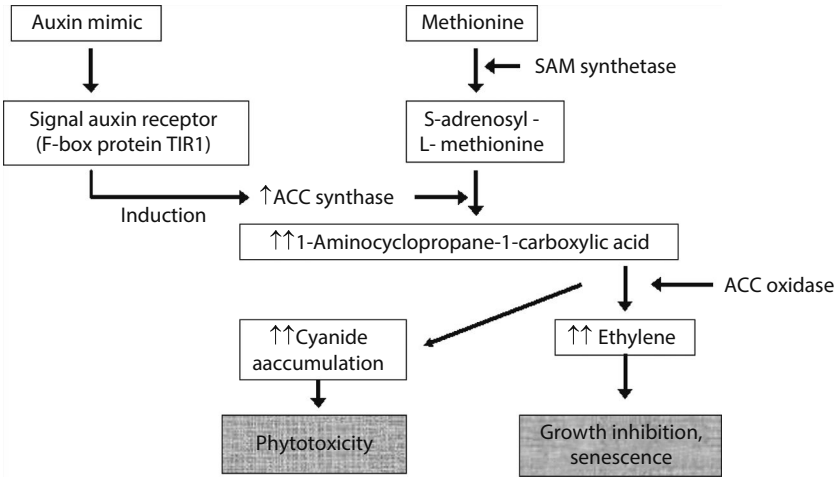
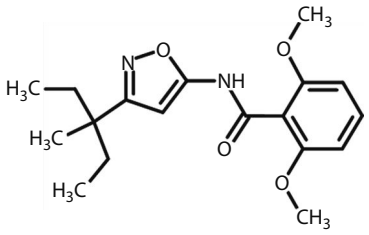
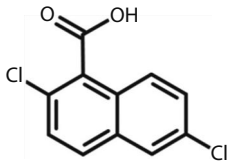
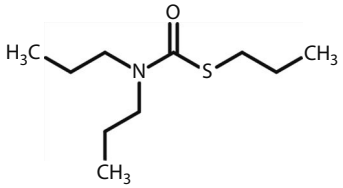


FIGURE 16.27 Auxin signal transduction.

TABLE 16.72
Structures, Uses, and Hazard Profiles for Benzamide, Quinoline Carboxylic Acid, and Thiocarbamate Inhibitors of Cellulose or Lipid Synthesis (HRAC Codes L and N)

Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
Isoxaben (Gallery®)[37,280]		Turf, ornamentals, nonbearing fruit trees, nut trees, and conifers	50–1000
Quinclorac (Facet®)[37,280]		Soybeans and rice	250–750
Butylate (Sutan®)[37,280]		Corn	3000–4000

	Irritation		LD_{50} (mg/kg)				
Herbicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Isoxaben	Moderate irritant	Sight irritant	>10,000	>2000	>2.68	Negative	Caution
Quinclorac	Nonirritant	Nonirritant	2680	>2000	>5.2	—	Caution
Butylate	Nonirritant	Mild irritant	4659	1659	4.64	Positive	Caution

Herbicide	Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Isoxaben[403]	Rat/2 years	5.0	Mutagenicity	Positive micronucleus test
	Dog/52 weeks	10.0	Developmental	Positive at maternal toxic doses
	Mouse/18 months	14.3	Reproductive	Not a reproductive toxin
	RfD or ADI	0.05	Oncogenicity	C (Adrenal and liver tumors)
Quinclorac[254]	Rat/2 years	675	Mutagenicity	Not mutagenic
	Dog/52 weeks	33	Developmental	Not teratogenic
	Mouse/18 months	42	Reproductive	Not a reproductive toxin
	RfD or ADI	0.38	Oncogenicity	Not carcinogenic
Butylate[403,475]	Rat/2 years	50	Mutagenicity	No evidence
	Dog/52 weeks	5.0	Developmental	Not teratogenic
	Mouse/18 months	20	Reproductive	Not a reproductive toxin
	RfD or ADI	0.05	Oncogenicity	E (No evidence)

clopyralid, and picloram are all eye irritants, and dicamba and picloram are potential skin sensitizers. Otherwise, the hazard profiles for the other chemicals are also unremarkable.

Semicarbazones (HRAC Code P)

The semicarbazones, represented here by diflufenzopyr-sodium, are often used in mixtures with dicamba to block auxin transport via carrier protein binding.[37] The structure, uses, and hazard profile of diflufenzopyr-sodium are listed in Table 16.74.

Herbicides with Unknown Mechanism of Action (HRAC Code Z)

The structure, uses, and hazard profile of monosodium methanearsonic acid (MSMA) are presented in Table 16.75.

MSMA, an organic arsenical, is a mild skin and eye irritant. MSMA caused decreased fertility in the rat reproduction study, and it is classified as a category B2 carcinogen (probable human carcinogen) based on bladder tumor incidence rates in rats.

TABLE 16.73
Structures, Uses, and Hazard Profiles for the Phenoxy, Benzoic, and Pyridine Acid Herbicides That Mimic Indole Acetic Acid (Auxin) (HRAC Code O)

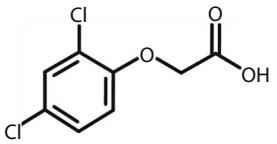
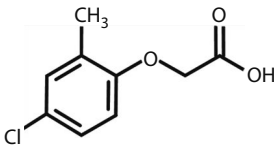
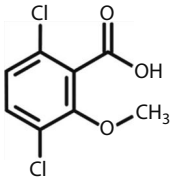
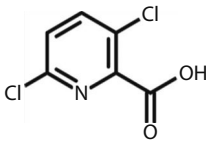
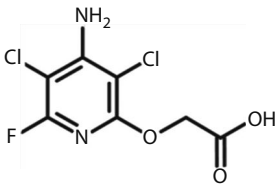
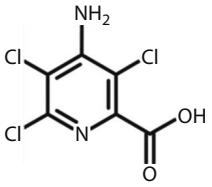
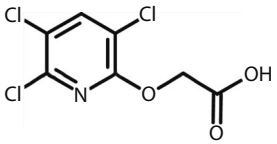
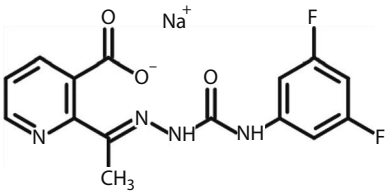
Herbicide	Structure	Principal Use/Crop	Application Rate (g a.i./ha)
2,4-D (Wedare®)[37,280]		Turf, cereals, sorghum, corn, soybeans, asparagus, and fruit trees	280–2240
MCPA (CHIPTOX®)[37,280]		Cereals, flax, rice, vegetables, grasslands, and under fruit trees	280–2250
Dicamba (Banvel®)[37,280]		Corn, turf, sorghum, cereals, pastures, and asparagus	100–400
Clopyralid (Reclaim®)[37,280]		Sugar beets, corn, grass seed, conifers, and pasture	70–560
Fluroxypyr (VISTA®)[37,280]		Small grains, apple orchards, plantations, conifer forests, and corn	180–400
Picloram (Tordon®)[37,280]		Industrial weed control, forestry, pasture, and range land	35–1120
Triclopyr (Brush-B-Gone®)		Plantation crops, pastures, conifer forests, and grasslands	720–8000

TABLE 16.73 (continued)
Structures, Uses, and Hazard Profiles for the Phenoxy, Benzoic, and Pyridine Acid Herbicides That Mimic Indole Acetic Acid (Auxin) (HRAC Code O)

	Irritation		LD_{50} (mg/kg)				
Herbicide	**Eye**	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
2,4-D	Severe irritant	Moderate irritant	639	>2000	1.8	Negative	Warning
MCPA	Corneal opacity	Nonirritant	1400	>2000	>6.3	Negative	Danger
Dicamba	Corrosive	Nonirritant	1851	>2000	>9.6	Positive	Danger
Clopyralid	Severe irritant	Slight irritant	4300	>2000	1.3	Negative	Warning
Fluroxypyr	Mild irritant	Nonirritant	>5000	>2000	>2.0	Negative	Caution
Picloram	Moderate irritant	Nonirritant	4012	>2000	>0.035	Positive	Danger
Triclopyr	Minimal irritant	Nonirritant	803	>2000	>4.8	Positive	Caution

Herbicide	**Species/Study**	**NOEL (mg/kg/day)**	**Toxicity Study**	**Hazard Indicator**
2,4-D[476,477]	Rat/2 years	5.0	Mutagenicity	No evidence
	Dog/52 weeks	1.0	Developmental	Not teratogenic
	Mouse/18 months	1.0	Reproductive	No evidence
	RfD or ADI	0.01	Oncogenicity	D (not classifiable)
MCPA[478]	Rat/2 years	4.4	Mutagenicity	Not mutagenic
	Dog/52 weeks	0.2	Developmental	Not teratogenic
	Mouse/18 months	15.4	Reproductive	Not a reproductive toxin
	RfD or ADI	0.0044	Oncogenicity	Not likely to be carcinogenic in humans
Dicamba[479,480]	Rat/2 years	125	Mutagenicity	Positive (*B. subtilis*; UDS)
	Dog/52 weeks	60	Developmental	Not teratogenic
	Mouse/18 months	108	Reproductive	No evidence
	RfD or ADI	0.6	Oncogenicity	D (not classifiable)
Clopyralid[481]	Rat/2 years	50	Mutagenicity	No evidence
	Dog/52 weeks	100	Developmental	Not teratogenic
	Mouse/18 months	500	Reproductive	No evidence
	RfD or ADI	0.5	Oncogenicity	E (No evidence)
Fluroxypyr[482]	Rat/2 years	100	Mutagenicity	Not mutagenic
	Dog/52 weeks	150	Developmental	Not teratogenic
	Mouse/18 months	300	Reproductive	Not a reproductive toxin
	RfD or ADI	0.5	Oncogenicity	No evidence for carcinogenicity in rats or mice
Picloram[483]	Rat/2 years	20	Mutagenicity	Not mutagenic
	Dog/52 weeks	35	Developmental	Not teratogenic
	Mouse/18 months	500	Reproductive	Not a reproductive toxin
	RfD or ADI	0.2	Oncogenicity	E (No evidence)
Triclopyr[484]	Rat/2 years	12	Mutagenicity	Not mutagenic
	Dog/52 weeks	5	Developmental	Not teratogenic
	Mouse/18 months	135	Reproductive	Not a reproductive toxin
	RfD or ADI	0.05	Oncogenicity	D (not classifiable)

TABLE 16.74
Structures, Uses, and Hazard Profiles for the Semicarbazone Herbicide Diflufenzopyr-sodium (Distinct®) (HRAC Code P)

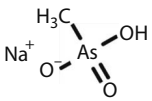
Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Inhibits transport of auxins via protein binding—used as a systemic postemergent herbicide in corn and noncrop pastures	9–185

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Mild irritant	Nonirritant	>5000	>5000	>2.93	Negative	Caution

Species/Study	NOEL (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	236	Mutagenicity	Not mutagenic
Dog/52 weeks	26	Developmental	Not teratogenic
Mouse/18 months	1004	Reproductive	Not a reproductive toxin
RfD	0.26	Oncogenicity	No evidence of carcinogenicity in rats or mice

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com; WSSA, *Herbicide Handbook of the Weed Science Society of America*, 9th edn., Weed Science Society of America, Champaign, IL, 2007; U.S. EPA, *Fed. Regist*, 1995; U.S. EPA, Diflufenzopyr-sodium: Conditional registration: Pesticide fact sheet, In: Office of Prevention P, and Toxic Substances, eds., U.S. Environmental Protection Agency, Washington, DC, 1999.

TABLE 16.75
Structure, Uses, and Hazard Profile for the Organoarsenical Herbicide Monosodium Methanearsonate (BUENO®) (HRAC Code Z)

Structure	Principal Use/Crop	Application Rate (g a.i./ha)
	Controls broadleaf weeds in noncrop areas, cotton, and turf	2220–2770

Irritation		LD_{50} (mg/kg)				
Eye	**Skin**	**Oral**	**Dermal**	**LC_{50} (mg/L) Inhalation**	**Sensitization Potential**	**Signal Word**
Mild irritant	Mild irritant	1059	>2000	>6.0	—	Caution

Species/Study	NOEL[1] (mg/kg/day)	Toxicity Study	Hazard Indicator
Rat/2 years	3.2	Mutagenicity	NA
Dog/52 weeks	2.0	Developmental	Not teratogenic
Mouse/18 months	9.3	Reproductive	Decreased fertility
RfD	0.01	Oncogenicity	B2 (bladder fibrosarcomas)

Sources: BCPC, The Pesticide Manual: A World Compendium, Tomlin, C., ed., British Crop Protection Council, Alton Hampshire, U.K., 2011; Royal Society of Chemistry, Chemical structure, ChemSpider, Cambridge, U.K., 2012 [September 12, 2012], Available from: http://www.chemspider.com; WSSA, *Herbicide Handbook of the Weed Science Society of America*, 9th edn., Weed Science Society of America, Champaign, IL, 2007; U.S. EPA, *Fed. Regist*, 1995.

CONCLUSIONS AND QUESTIONS

According to a Byzantine proverb, "He who has bread may have many problems, but he who lacks it has only one." In a world where the current population is burgeoning, one might say that collectively we face more than 6.6 billion individual problems, ultimately leading to only one question: How will we sustain and possibly increase food production in the face of declining arable land and the future demand to convert a part of biomass production into energy production? A corollary question is: How will we produce food locally where it is needed or redistribute it from regions of high productivity to other needy parts of the world? All this must be done in the face of strident attacks, at least in the developed countries, against the basic sciences and the economy of food production that up to now have permitted the diversion of human resources away from growing food to other economic, political, and cultural activities.

So, how will we overcome these apparently insurmountable challenges? Perhaps we should look to the past 60 years, the period of time that might be considered the period of modern agriculture, for clues as to what has worked and what has failed. The most noticeable advances have included the following:

- The development of strains of plants that are resistant to disease or result in a greater yield using conventional breeding techniques or, more recently, molecular marker–assisted breeding methods
- The introduction of mechanized farming methods that resulted in a reduction in the reliance on human or animal labor
- The utilization of cropping practices and fertilizers to enhance productivity
- The use of agricultural chemicals to enhance yield by reducing loss to disease and the destruction by pests
- The development of highly effective pesticides that control pests with a near-pharmacologic level of potency (i.e., grams active ingredient per hectare)

One must also acknowledge, however, that there have been some failures to achieve the main goal of pesticide science—namely, to achieve the selectivity of effect and thereby to prevent unwanted effects on animals or the environment. Thus, pesticide development is about one thing and one thing only: selectivity. The mission of pesticide scientists is to find chemicals that control pests at some reasonable and economically achievable dose, ultimately by interfering with a fundamental life process of the pest, without having any effect on beneficial plants, insects, or mammals, including humans.

In reviewing the more than 50 different MOA discussed in this chapter, one cannot help but be amazed at the ingenuity of the researchers who have spent their lives in this hunt for better and more effective pesticides. These men and women have achieved success mainly using relatively crude biological screening tools combined with ingenious chemical synthesis strategies, which, with luck and a lot of persistence, have led to the discovery of pesticide candidates that have then undergone extensive optimization to finally become useful agricultural tools. In hindsight, once the molecular mechanisms have been described, it is incredible that the specificity of effect was achieved using what could be best described as an intelligent random search process. Even today, with high-throughput screening methods and the use of combinatorial chemistry, the crop protection industry has not turned to rational design, largely because the science still is not there to build a molecule from the ground up based on what is currently known about molecular targets. Using traditional methods, then, it may still be possible to increase selectivity, lower use rates, and identify chemicals with new MOA. The incorporation of genes that create tolerance in beneficial plants and the expression of pesticides by the species that are to be protected are examples from the recent past. In the future, it is likely that plants and animals will continue to be genetically modified or bred using more sophisticated selection methods to achieve improved traits that create greater health benefits or facilitate the preprocessing of food and fiber. Targeted delivery of pesticides, whether derived from natural sources or that are the result of chemical optimization of what has been designed by nature, may be another way to move toward solutions that have lower environmental impact. Whatever the outcome, the next 50 years should prove to be critical, given current population predictions and the emerging environmental and energy crises.

QUESTIONS

16.1 Farmers must contend with some 80,000 plant diseases, 30,000 species of weeds, 1000 species of nematodes, and more than 10,000 species of insects. Today, national and international agricultural organizations estimate that as much as 45% of the world's crops continue to be lost to these types of pests. In the United States alone, about $20 billion worth of crops (one-tenth of production) is lost each year. What do you think would be the status of our national food production capacity without the use of pesticides?

16.2 Who ensures that pesticides can be used without unacceptable hazard to the consumer to protect food crops and maximize yields?

16.3 How stringent are the testing requirements for the registration of a pesticide when compared to those for products used in the household, the yard, industrial situations, or even in the pharmaceutical industry?

16.4 Has the introduction of pesticides into your food supply had a positive or negative impact on your life?

16.5 What crop protection chemical's human life-saving potential has been rivaled only by the introduction of penicillin?

16.6 Are organically grown foods really less hazardous or more nutritious than those grown with the aid of pesticides, and if so, why?

KEYWORDS

Fungicides, Insecticides, Herbicides, Pesticides, Human, Health

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17 Metals

Janis E. Hulla

CONTENTS

The views expressed in this chapter are those of the author and do not necessarily represent the views of the U.S. Army Corps of Engineers, Army, or Department of Defense.

INTRODUCTION

Scope

The U.S. laws that are related to exposure to toxic substances, and the federal agencies responsible for implementing them, are discussed in Chapter 2 of this textbook and appear in Table 2-1 of the fifth edition.[1] The list includes agencies responsible for managing exposures related to drugs, consumer products, and environmental contaminants. Included are laws that protect plants and animals in addition to humans. The scope of this chapter includes fundamental concepts that are relevant to each of these three receptor groups. The chapter's focus is the human health effects of essential and nonessential metals. The radiation biology and toxicity of radioactive metals are discussed in Chapter 18 of this text.

A history of the U.S. government's management of risks associated with exposure to chemicals is summarized in Chapter 2 of this book. Included is a discussion of the 1983 National Research Council publication, *Risk Assessment in the Federal Government: Managing the Process*.[2] This publication defined four components of the federal risk assessment process still used today: (1) hazard identification, (2) dose–response assessment, (3) exposure assessment, and (4) risk characterization. Components of these four processes that are specific to essential and nonessential metals are discussed in this chapter.

In the scientific vernacular, a metal is any element characterized by ductility, luster, being electropositive with a tendency to lose electrons, and having the property of conducting heat and electricity. Scientists may refer to the periodic table of elements where metals are distinguished from alkali metals, alkaline earth metals, metalloid metals,

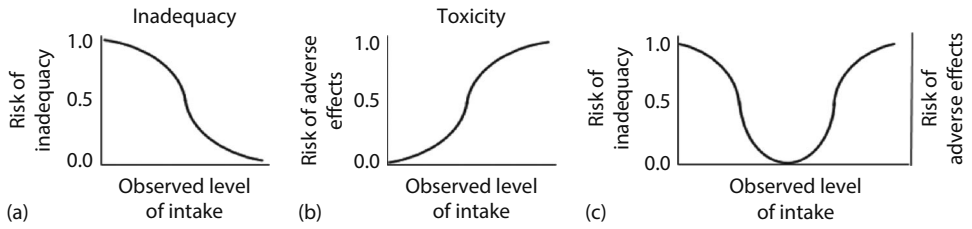

FIGURE 17.1 Hypothetical U-shaped dose–response curve for essential trace element. (a) Adverse effects at low doses due to dietary deficiency. (b) Adverse effects at high dose due to toxicity. (c) U-shaped curve for a hypothetical trace metal essential nutrient. (Adapted from Olin, S.S., *J. Nutr.*, 128, 364S, 1998.)

lanthanide metals, and actinide metals based on their atomic structure. In this chapter, *metals* is used as a general term referring to all 286 elements within these categories. Of the 286 metals, only the dietary essential metals and the metals for which there is evidence of toxicity are described in this chapter. The toxicological characteristics of metal-containing nanomaterials are outside this chapter's scope.

Essential and Nonessential Metals

The scope of this chapter includes (1) dietary essential metals and (2) toxic metals. Quantifying dose is fundamental to defining the toxicity and/or essentiality of metals. This concept is perhaps best illustrated by the U-shaped dose–response curves of metals that are both trace essential nutrients and toxic at high dose. Toxicity at high dose, as illustrated by Figure 17.1b, is generally understood. Perhaps less well recognized is that, for trace essential nutrients, toxicity can result from dietary insufficiency as illustrated in Figure 17.1a. Metals that are both essential at low dose and toxic at high dose demonstrate U-shaped dose–response curves as illustrated in Figure 17.1c.

Physical and Chemical Determinants of Metal Essentiality and Toxicity

The chemical determinants of metal toxicity include the strength with which a metal complexes with a ligand and the significance of the biological function of that molecular ligand. Metals are Lewis acids forming cations, and they are electron-accepting species. Lewis bases form anions, are electron donors, and are ligands for metals. *Soft* metals and ligands are characterized as having electrons that are mobile within the electron cloud. The electron clouds of soft species are easily deformed and polarized. Soft species preferentially form covalent bonds. Conversely, *hard* metals and ligands are species with an electron cloud that is relatively rigid and relatively less likely to be polarized. Hard metal species preferentially form ionic bonds during complex formation. The hard, borderline, and soft acid metals that are most significant biologically are listed in Table 17.1. Bonds between (1) hard acid metals and soft base ligands or (2) soft acid metals and hard base ligands are relatively weak and, therefore, relatively rare.

Hard acid metals are oxygen-seeking. They tend to bond to hard base ligands that contain, for example, –OH, –COO–, and $–OPO_3H^-$, greater than C=O. Soft acid metals preferentially bond to soft base ligands containing nitrogen, for example, $–NH_2$ and greater than NH, and sulfur, for example, –SH and –S–S–.[3] The borderline metals demonstrate relatively equal affinity for oxygen, nitrogen, and sulfur. The soft acid metals are more toxic than hard acid metal because (1) the processes of biological absorption, distribution, metabolism, and excretion put them in contact with biomolecule ligands that function to maintain homeostasis and (2) the thermodynamics favor reactions that result in dysfunction, for example, the displacement of endogenous essential metals in metalloenzymes.

TABLE 17.1
Classification of Metal Ions as Hard, Borderline, or Soft Acids

Hard acids	Al^{3+}, Ba^{2+}, Be^{2+}, K^+, Na^+, Mg^{2+}, Sr^{2+}, Ca^{2+}, Li^+
Borderline between hard and soft acids	Sn^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Mn^{2+}
Soft acids	Ag^+, Cu^+, Hg^{2+}, Au^+, CH_3Hg^+, Tl^{3+}, Tl^+, Bi^{3+}

Source: Framework for Metals Risk Assessment, Office of the Science Advisor, Risk Assessment Forum, EPA 120/R-07/001, March 2007.

The metal coordination cavities of metalloproteins match physical characteristics, such as ionic radius and geometry, of their endogenous essential metals. Replacement of coordinated essential metals by nonessential metals results in different stability or conformation such that the normal function is disrupted. This is an important principle in metals toxicology.

Biological Determinants of Metal Toxicity

In addition to the ionic states mentioned earlier, metals occur as elements and as organometallic compounds and adhere to air particulates. Each of these chemical forms is a unique exposure, kinetic and dynamic determinant. Another important component of pharmaco- and toxicokinetics of metals is the potential to bind to transport proteins and to storage proteins. For example, the metal-binding protein, metallothionein, sequesters both essential and toxic metals. Some

TABLE 17.2
Examples of Metal-Binding Storage and Transport Proteins

Metal-Binding Proteins	Metals	Function
Metallothionein	Silver, mercury, copper, bismuth, cadmium, lead, and zinc	Transportation
Transferrin	Iron, aluminum, and manganese	Transportation
Ferritin	Iron, cadmium, zinc, aluminum, and beryllium	Storage

common examples of metal-binding proteins are shown in Table 17.2. The location of these binding proteins defines the organ sensitivity for some metals. Further, the disruption of the normal function of metal binding is a common component of toxicological modes of action.

Chemical State and Toxicokinetics

Exposure to metals most commonly occurs via ingestion and inhalation. Exposure to metals bound to particulates occurs via inhalation. Exposure to elemental mercury can also occur via inhalation because it is volatile. Exposure to elemental metals and organometals can occur via the dermal route since they are uncharged and lipophilic. Ingestion is the common route of exposure for the water-soluble metal ions. Ingestion is also a common route of exposure for metals that bioaccumulate in the food chain. Some organometallic compounds of arsenic, lead, mercury, and tin are listed in Table 17.3. These compounds also have the potential to bioaccumulate.

Duration of exposure impacts both qualitative and quantitative toxicology. The amount of impact changes in exposure duration depends on the toxicokinetics of the metal. For example, daily doses of cadmium will accumulate in the kidneys and toxicity will develop. Conversely, essential elements are excreted so efficiently that daily intakes below the upper limits are tolerated over a lifetime. In this context, physiologically based pharmacokinetic (PBPK) modeling is a very useful tool for evaluating the toxicity of metals. Models integrate information on the effects of exposure routes, chemical forms, age at exposure, duration of exposure, and interindividual variation on absorption, distribution, excretion, and target organ sensitivity. Also useful are the data held in the National Library of Medicine's Comparative Toxicogenomics Database. This database links human diseases and data generated by high-throughput chemical screening experiments.[4] The database is designed to elucidate molecular mechanisms by which environmental chemicals affect humans. The increasing availability of information on modes of action increases the reliability of PBPK modeling.

TABLE 17.3
Examples of Organometallic Compounds

Metal	Organometallic Compound
Arsenic	Methylarsonic acid, dimethylarsenic acid, trimethylarsine, trimethylarsine oxide
Lead	Tetraethyllead, ethyltrimethyllead, dimethyldiethyllead
Mercury	Methylmercury, dimethylmercury
Tin	Tributyltin, tributyltin oxide

Chemical State and Toxicodynamics

The essentiality of some metals is related to the essential physiological role of the metal in, for example, enzymatic reactions, energy metabolism, and neurotransmission or as a structural component such as bone. The toxicity of a nonessential metal may be related to a dynamic substitution for an essential metal in these functional roles. Metal toxicity may also be related to its covalent or noncovalent reaction with enzymes, membranes, or DNA. Toxicity may also be induced by stimulating the production of active oxygen species.[5] Redox active metals like iron, chromium, manganese, nickel, copper, and cobalt can participate in redox cycling, producing reactive radicals such as the superoxide anion radical and nitric oxide. Exposure to metals with subsequent formation of reactive oxygen species can overwhelm the body's antioxidant protection and subsequently induces DNA damage, lipid peroxidation, and/or protein modification. Such chemical changes underlie numerous diseases, including cancer, cardiovascular disease, diabetes, atherosclerosis, neurological disorders, and chronic inflammation.

The toxicodynamics of lead, cadmium, and arsenic are thought not to involve redox cycling. Their toxic effects are primarily mediated through bonding to sulfhydryl groups of proteins and depletion of antioxidants such as glutathione. Zinc, also a nonredox cycling metal, is an essential component of numerous proteins involved in the defense against oxidative stress and is thought to play a neuroprotective role. In these contexts, zinc deficiency is potentially a special case among the essential metals.[6]

The variety of potential physiological effects makes it difficult to determine which action is responsible for toxicity and to identify the most sensitive target organ. In some cases, organs are most sensitive for a biochemical reason. For example, thallium interferes with energy metabolism, and the target organs are those with the highest energy requirement. In other cases, the most sensitive organ is simply the organ in which the accumulation is greatest. For example, cadmium and uranium accumulate in the kidney.

In summary, the fundamental determinants of metal toxicity are the strength with which a metal complexes with a ligand, the biological significance of the function of that molecular ligand, and the potential of a metal to participate in the generation of reactive oxygen species.

Treatment of Toxic Effects of Metals

Chelators are usually the preferred medical treatment of metal toxicity. Unlike many other drugs, chelators do not target specific effectors in a toxicity mode of action. Rather, metal chelation therapy relies on metal ions' reactivity to

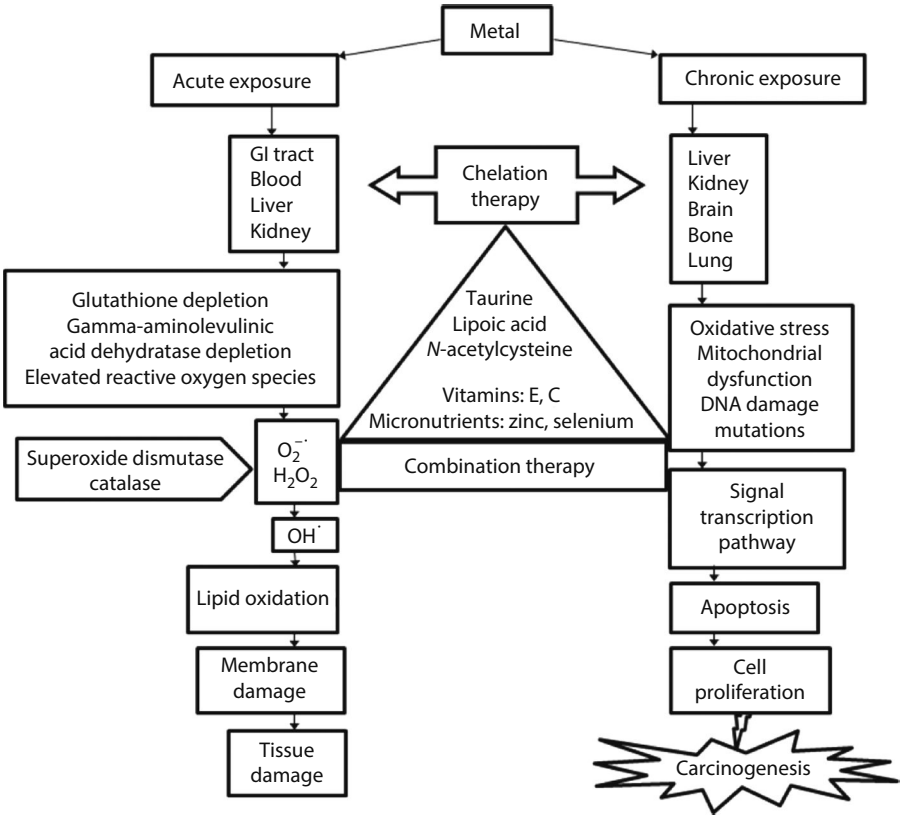

FIGURE 17.2 Strategies for the treatment of acute and chronic metal exposures. (From Flora, S.J.S. and Pachauri, V., *Int. J. Environ. Res. Public Health*, 7(7), 2745, 2010, an open source publication.)

form complex structures that are efficiently excreted. For example, 2,3-dimercaprol has long been the preferred therapy for lead or arsenic poisoning.

Serious side effects have been associated with therapy using some of the metal chelators. This has led to the development of less toxic analogues. Hydrophilic chelators like meso-2,3-dimercaptosuccinic acid effectively promote renal metal excretion. Yet, the trade-off for efficient excretion is that the hydrophilicity limits access to intracellular metals. Newer strategies attempt to address these limitations by combining structurally different chelating agents or by combining an antioxidant with a chelating agent (see Figure 17.2). Prevention of excessive exposure is still the best way to reduce the potential for metal toxicity.[7]

U.S. GOVERNMENT'S RISK MANAGEMENT PARADIGM FOR METALS

The four components of the U.S. federal risk assessment, (1) hazard identification, (2) exposure assessment, (3) dose–response, and (4) risk characterization, are applied to both essential and nonessential metals. The National Academy of Sciences' Food and Nutrition Board of the Institute of Medicine (IOM) evaluates each component during the development of dietary reference intake (DRI) values.[8] The U.S. Environmental Protection Agency's (EPA) 2007 document titled *Framework for Metals Risk Assessment* describes how metal-specific principles should be considered in each of the components of the health risk assessment process.[9]

Safety Evaluation and Hazard Identification

Since metals may be intended and unintended components of commercial products such as food, food supplements, drugs, cosmetics, pesticides, as well as environmental contaminants, they are subject to a multiplicity of U.S. statutes designed to advise on and/or to regulate exposure. The initial step of the U.S. government's risk assessment process is hazard identification. *Hazard identification* is used when the substance being evaluated is an environmental contaminant. *Safety evaluation* is more commonly used when the substance being evaluated is a drug or nutrient. The Food and Drug Administration (FDA) safety evaluation process[10] shares many of the components of other agencies' hazard identification framework.[11] Toxicity testing is frequently conducted as part of the hazard identification and safety evaluation processes. Guidelines for toxicity testing have been established by the U.S. National Toxicology Program (NTP)[12] and the International Conference on Harmonisation.[13]

Notable among the U.S. laws that dictate toxicity testing of metals to which humans are intentionally or unintentionally exposed are the Food, Drug, and Cosmetic Act (FDCA)

and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). The federal agencies responsible for their implementation are the FDA and EPA, respectively. The FDCA dictates that safety evaluations be conducted before pharmaceuticals and food additives are approved. The FIFRA dictates that safety evaluations be conducted before pesticides are registered. In contrast, the Toxic Substances Control Act (TSCA), which controls new and existing industrial chemicals that are not regulated by other statutes, does not require manufacturers to conduct toxicity testing. However, under TSCA, the EPA may dictate that toxicity testing be conducted in response to environmental concerns. The EPA also is responsible for setting a tolerance for pesticide residues under the FDAC and has the authority to implement safety assessments in response to environmental concerns under other federal legislations, including the Safe Drinking Water Act (SDWA), Clean Water Act (CWA), Clean Air Act (CAA), Resource Conservation and Recovery Act (RCRA), Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), and the National Pollutant Discharge Elimination System (NPDES). The Occupational Safety and Health Administration (OSHA) and the National Institute of Occupational Safety and Health (NIOSH) have statutory authority to do hazard identifications under the Occupational Safety and Health Act. The NTP is charged with coordinating federal toxicity testing and developing testing methods.

Metals Exposure Assessment and Intake Assessments

Intake assessment is the phrase used when the exposure being assessed is to a drug or nutrient. *Exposure assessment* is used when the exposure being assessed is to an environmental contaminant. The goal of both types of assessments is to characterize the anticipated amount of the substance, duration of exposure, exposure routes, and, for environmental contaminants, the source.

Human activities such as mining and smelting, fossil fuel burning, and fertilizer-intensive agriculture have increased human exposure to metals far above those of the preindustrial environment. Some of the earliest information that linked adverse health effects to environmental exposure was the toxicity observed in workers who inhaled metals as fumes or dusts. Workers commonly displayed adverse lung effects that included mild self-limiting metal fume fever due to acute exposure. As a consequence of chronic exposures, these workers displayed benign pneumoconiosis or severe chronic obstructive lung disease.

Development of biomarkers as a tool to quantify environmental exposure to metals is a major advancement in the science of exposure assessment. Biomonitoring for blood and/or urine exposure biomarkers enables investigators to link exposure to health effects in the general population. Biomarkers provide more precise quantification of tissue exposure than is possible by traditional means, particularly for the general population that may be exposed by several routes, all of which may vary in time and location.

Several metals are considered to be carcinogenic by the inhalation route, but not by the oral route. This determination is based on the observations of (1) an increased rate of lung, but not other forms of cancer among workers and experimental animals exposed by inhalation and (2) no increase in cancer rate among experimental animals exposed orally. However, it cannot be ruled out that this may be due to a difference in potency that is related to the route of exposure. It could be argued that most mechanisms by which a metal is carcinogenic to lung tissue could operate in other tissues too. In these contexts, it is noteworthy that certain metals such as arsenic are known to be both lung carcinogens and systemic carcinogens.

Exposure to metals via the dermal route is less common than oral or inhalation exposures. A few metals are readily absorbed through the skin. A case of transdermal exposure to dimethylmercury that resulted in the death of a laboratory researcher was reported in 1999.[14] The parenteral route of exposure is also less common. However, cancers can be induced in experimental animals by implantation of solids. *Solid-state* carcinogenesis in humans is relevant in the context of implanted metal prosthetic devices.[15] Therefore, studies using parenteral routes of exposure are useful if the goal is to evaluate human parenteral exposure from medical procedures.

The age at which an individual is exposed is relevant to the exposure assessment. Infants and young children may be particularly sensitive to toxic effects of metals. They often absorb a greater fraction of ingested metals than older children or adults because some developing systems, particularly the nervous system, are more sensitive to toxic effects than mature systems. Additionally, the high rate of DNA replication during development is thought to make fetuses, infants, and children more sensitive than adults to genotoxicants. Further, the elderly may be more sensitive than healthy adults to the toxic effects of metals due to diminution of homeostatic and adaptive mechanisms. For these reasons, an uncertainty factor for individual variability may be applied when advisory, screening or regulatory levels of exposure are being calculated.

Metals Dose–Response Assessment

In 2009, the U.S. EPA revised its risk assessment process and, in response to recommendation from the National Academy of Sciences, made further improvement in 2011.[16] An EPA Integrated Risk Information System (IRIS) risk assessment is now a seven-step process that includes assembling a set of relevant data and surveying the data to determine the organ exhibiting an adverse effect at the lowest dose. The assessment of noncarcinogens identifies the no-observed-adverse-effect level (NOAEL) or the lowest-observed-adverse-effect level (LOAEL). Uncertainty factors are applied in the face of scientific uncertainties such as those encountered in extrapolating animal data to humans. The dose–response assessment of noncarcinogens yields a reference level below which no effects are expected to occur. A different process is used to quantify the cancer risk. It uses a weight-of-evidence approach to determine whether the element has the

potential to cause cancers in humans.[17] The cancer assessment assumes that any exposure carries some cancer risk and builds in safety margins by using the most sensitive sex/species/organ carcinogenic response and by using the upper 95th confidence limit of the dose–response slope. In 2005, the EPA revised its cancer classification scheme, and now it uses standard descriptors of conclusions rather than letter designations. However, many EPA IRIS profiles retain the EPA's 1986 letter classifications of cancer groups.

Risk Characterization of Essential Metals and Nonessential Metals

The characterization of risks related to exposure to either essential or nonessential metals integrates information about the hazards, exposures, and dose–response. The risk characterization provides an estimate of the likelihood that any of the identified adverse effects will occur. An integral component of risk characterization is definition of the assumptions and descriptions of the qualitative and quantitative uncertainties. Depending on the goals of the risk assessment, the foundation for establishing an advisory, screening, or regulatory level may be provided in the risk characterization.

U.S. GOVERNMENT'S RISK MANAGEMENT OF EXPOSURE TO METALS

The EPA, FDA, USDA (U.S. Department of Agriculture), NIH (National Institutes of Health), OSHA, and CDC (Centers for Disease Control and Prevention)/Agency for Toxic Substances and Disease Registry (ATSDR) are among the U.S. federal government agencies involved in managing metal-related risks. These agencies conduct research and evaluate research data to derive concentrations of essential and nonessential metals. These concentrations are used as advisory, screening, or regulatory levels. Only some of the concentrations derived by the EPA, ATSDR, NIOSH, and OSHA are cited in this chapter.[18–20] Other nations and international governmental and nongovernmental organizations also develop advisory, screening, and regulatory levels for metals. For example, the American Conference of Governmental Industrial Hygienists (ACGIH) publishes an annual list of chemicals for which the organization has derived, or is in the process of deriving, threshold limit values (TLVs). The reader is encouraged to become familiar with the various types and sources of advisory, screening, and regulatory levels. Some can be found in the ATSDR's toxicology profiles of the individual metals. The International Toxicity Estimates for Risk (ITER) database is also an excellent source for advisory, screening, and regulatory levels.[21]

Advisory Levels

Dietary Reference Intakes

In 2006, the Food and Nutrition Board of the IOM, in partnership with Health Canada, developed the latest DRIs.[8] The DRIs are advisory concentrations and include (1) an estimated average requirement (EAR), (2) a recommended dietary allowance (RDA), (3) an adequate intake (AI) level, where an RDA cannot be estimated, and (4) tolerable upper intake levels (ULs) above which risk of toxicity would increase. DRIs are revised and published periodically by the board through a process of convening expert committees to estimate the mean dietary requirement for the general population. DRIs are developed for specific female and male life stages, pregnant women, and lactating women. The dietary essential metals for which the board derived at least one of the DRIs include chromium, copper, iron, magnesium, manganese, molybdenum, potassium, sodium, and zinc. The board also derived ULs for three nonessential metals, boron, nickel, and vanadium, because they may be present in the diet. Arsenic and silicon may also be in the diet, but it was determined that there was insufficient data on which to derive upper limits for these elements. DRIs are not enforced as regulations. Rather, federal agencies use them to advise their constituents. The USDA uses DRIs to plan and assess the diets of healthy people and to advise the public.[22] The FDA uses DRIs as tools for advising industry on how to respond to product premarket requirements and labeling. The DRIs' relationships between levels of intake and risks of adverse effect are depicted graphically in Figure 17.3.

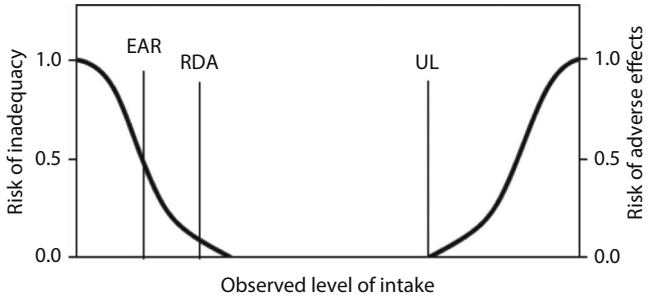

FIGURE 17.3 Relationships between levels of intake and risks of adverse effect: EAR—estimated average requirement; RDA—recommended dietary allowance; and UL—tolerable upper intake level. (Reprinted from *Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*, National Academies Press, Washington, DC, 2006. With permission.)

Screening Levels

Minimal Risk Levels

The CDC, through the ATSDR, develops minimal risk levels (MRLs).[23] The MRL is defined as an estimate of the daily human exposure to a hazardous substance that is likely to be without appreciable risk of adverse, noncancer, health effects over a specified duration of exposure. MRLs serve as a screening tool to help public health professionals decide where to look more closely to evaluate risk of adverse health effects from exposure. The CDC's legislative authority to define MRLs is derived from the CERCLA. The derivation of a metal's MRL is published as a component in the agency's toxicological profile of that metal as an environmental contaminant.

Toxicity Values

EPA's "Framework for Metals Risk Assessment" was released in 2007. It outlines key principles about metals and describes how they should be considered in conducting human health and ecological risk assessments.[9] The EPA derives oral reference doses (RfDs) and inhalation reference concentrations (RfCs) based on nonneoplastic endpoints. These reference values are derived using a process similar to the ATSDR's process for developing MRLs. ATSDR's MRLs and EPA's reference values estimate an exposure to the human population, including susceptible subgroups, that is likely to be without an appreciable risk of adverse health effects over a lifetime. For cancer endpoints, the EPA derives a slope factor. The slope factor is defined as an upper bound, approximating a 95% confidence limit, on the increased cancer risk from a lifetime exposure to a substance.[24] Derivation of the three types of toxicity values is the responsibility of the EPA's IRIS, and the process is commonly referred to as the IRIS risk assessment process.[16] Toxicity values, and related documentation, are deposited in the IRIS database.[19]

Regional Screening Levels

The EPA uses toxicity values and default exposure assumptions to calculate regional screening levels (RSLs). RSLs are used on environmental cleanup sites to screen concentrations of contaminants as a first step in determining if cleanup is required. If a site has metal concentrations in the media, that is, air, soil, or water, that are higher than the corresponding RSLs, a quantitative site-specific risk assessment may be conducted. In this process, site-specific exposure parameters are developed and used with the IRIS toxicity values according to "Risk Assessment Guidelines for Superfund" to determine the need for, and extent of, remediation.[25]

Recommended Exposure Limits

NIOSH develops recommended exposure limits (RELs) for hazardous substances, including metals, in workplace air. A REL is a time-weighted average (TWA) concentration for up to a 10 h work shift during a 40 h work week. NIOSH also develops other advisory levels, for example, short-term exposure limits (STELs). The STEL is a 15 min TWA and an exposure that should not be exceeded at anytime during the work week. OSHA considers NIOSH advisory limits in developing the enforceable permissible exposure limit (PEL) regulation (see the following).

Threshold Limit Values

The ACGIH is a nongovernmental organization that publishes an annual list of chemicals for which the organization has derived, or is in the process of deriving, advisory levels such as the TLVs.[26] TLVs are TWA workplace air concentrations for a normal 8 h workday and a 40 h workweek. Like the NIOSH RELs, TLVs are advisory and not enforceable regulations.

Regulatory Levels

Permissible Exposure Limits

The OSHA has jurisdictions that include promulgation and enforcement of PELs. PELs are regulatory limits on the amount or concentration of a substance in the air. OSHA PELs are based on an 8 h TWA exposure. Approximately 500 PELs have been established.

Maximum Contaminant Levels

The National Primary Drinking Water Regulation gives the EPA the authority to derive maximum contaminant levels (MCLs) for hazardous substances including metals. MCLs are enforceable standards that apply to public water systems. MCLs protect drinking water quality by limiting the levels of specific contaminants that can adversely affect public health and are known or anticipated to occur in water.

ESSENTIAL METALS

Calcium

Sources of Calcium Exposure

Calcium is naturally occurring in calcite, gypsum, fluorspar, and dolomite rock formations. Calcium compounds may enter the atmosphere in the form of dust or other fine particles. Occupational exposure to calcium occurs via inhalation of particulate matter in settings where commercial products are made from these materials. The general population may be exposed to calcium via inhalation of ambient air, ingestion of food, and drinking water that contain calcium compounds or ions.[27]

Calcium Essentiality

Calcium is essential both for the physical structure of bone and for normal physiological functions such as nerve conduction, muscle contraction, blood clotting, regulation of vascular smooth muscle tone, the calcium (II) component of current across membranes, enzyme activation, and acetylcholine synthesis. Calcium (II) is also a second messenger.[28,29] The bodies of healthy men and women contain about 1300 and 1200 g of calcium, respectively. About 99% of calcium is located in bone and teeth with the remaining 1% in extracellular fluids, intracellular structures, and cell membranes. In adults, blood serum calcium ranges from 8.5 to 10.4 mg/dL with tight physiological controls. Decreased body calcium leads to loss of bone mineralization, reduction of bone strength, increased susceptibility to fractures, and may increase blood pressure, particularly among pregnant women. Calcium deficiency is also associated with convulsions and tetany.[30]

Adverse Health Effects of Calcium

Calcium is not a very toxic metal, but adverse effects may occur at intakes greater than the tolerable upper limit (UL). Intestinal absorption of calcium decreases as intake

increases. However, very large intakes of calcium can increase the calcium body burden as well as interfere with the absorption of magnesium,[31] zinc, and iron.[32] Very large chronic intakes are associated with hypercalcemia and/or hypercalciuria. Mild hypercalcemia leads to constipation, anorexia, nausea, and vomiting. With increased hypercalcemia, symptoms include confusion, delirium, stupor, and coma. Symptoms of chronic calcium excess include soft tissue calcification, kidney stones, and renal failure. Calcium supplementation is associated with a modest increased risk of cardiovascular events.[33,34]

Toxicokinetics of Calcium

Twenty to thirty percent of ingested calcium is absorbed in the small intestine depending on the presence of vitamin D metabolites, pH in lumen, and on dietary factors, such as calcium binding to fiber or phytates. Calcium absorption is increased when a calcium deficiency is present. Intracellular calcium overload has been proposed to result in a breakdown of high-energy phosphates.[30]

Toxicodynamics of Calcium

Calcium plays an important role in muscle contraction. Calcium release from the sarcoplasmic reticulum is initiated by an action potential. The intracellular calcium activates contraction by binding to troponin and preventing its inhibition of the actin–myosin interaction. As calcium is pumped back into the sarcoplasmic reticulum, troponin inhibition is restored and the muscle relaxes. Cytotoxic effects induced by toxic concentration of calcium also include blebbing of the plasma membrane, activation of calcium-dependent phospholipases, stimulation of calcium-dependent neutral proteases, and calcium-activated DNA fragmentation.[35]

Advisory, Screening, and Regulatory Levels of Calcium

In 2006, the Food and Nutrition Board released their new DRI for calcium.[8] An RDA was not derived for calcium. There was an insufficiency of data to derive the requisite EAR (Figure 17.3) from which an RDA is calculated. In these cases, an AI is usually developed (Table 17.4). The AI for calcium is 1300 mg/day for ages 9–18, 1000 mg/day for ages 19–50, and 1200 mg/day for ages greater than 51.[8] The intakes are designed to maximize calcium retention, promote bone strength, and prevent osteoporosis. The UL is 2.5 g/day. The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Chromium

Sources of Chromium Exposure

The chromium content of food varies widely and is thought to be influenced by geochemical factors.[36] Cereals are usually a significant source of chromium (III) in the diet, but content varies widely. Whole grains typically contain more chromium (III) than refined grains. Meats, poultry, and fish contribute one to two micrograms per serving. Some beers and wines also contain chromium.

Di- and trivalent chromium compounds occur in natural ores. Hexavalent chromium (VI, or chromate) rarely occurs naturally but is produced from anthropogenic sources. The production and use of chromium compounds in metal alloys, pigments, metal finishing, leather tanning, and wood preservation may result in their release to the environment. Chromium compounds are also released into the atmosphere attached to particulates via the combustion of natural gas, oil, and coal. Chromic acid mists are air contaminants in metallurgical operations. Dermal exposure to chromium occurs during the processing of cement.

Chromium Essentiality

Chromium (III) plays a role in the metabolism of glucose and lipids.[8] Dietary deficiency is associated with increased risk of diabetes and cardiovascular diseases.[37] However, there are insufficient data to determine with confidence the value of dietary chromium supplementation as a treatment for diabetes.[38] The typical daily food intake of chromium ranges from 25 to 224 mg with an average of 76 mg. Chromium picolinate is used as a dietary supplement.[27]

Toxicokinetics of Chromium

Only about 1% of oral intake of chromium (III) is absorbed in the gastrointestinal tract. Absorption of chromium (VI) is higher, 3%–6%.[39] Chromium that is absorbed gets excreted in the urine, and chromium not absorbed is excreted in the feces.[8] Chromium (VI) readily crosses cell membranes via ion transport systems. Once inside the cells, chromium (VI) gets reduced to chromium (III) by thiols. In the blood, chromium (III) is mainly bound to transferrin while chromium (VI) is taken up by erythrocytes. Chromium is stored in the liver, spleen, soft tissues, and bone. Chromium may be transferred to infants via breast milk.[27]

Adverse Health Effects of Chromium

Patients who did not receive chromium supplementation in their total parenteral nutrition displayed unexplained weight loss, peripheral neuropathy, impaired glucose transport, increased insulin requirement, elevated plasma free fatty acids, and low respiratory quotient.[8]

Chromium (III) has low toxicity due to poor membrane permeability. In contrast, chromium (VI) readily crosses cell membranes and is toxic due to its strong oxidation characteristics. Symptoms of acute toxicity include vomiting, gastrointestinal bleeding, and cardiovascular shock. Liver necrosis, kidney tubular necrosis, and damage to the blood-forming tissues can occur. Long-term occupational exposure to chromium is associated with either low molecular weight proteinuria or with elevated levels of the proteins normally found in the urine.[40] The endpoint on which the EPA's chromium acid mist RfC was calculated is nasal septal atrophy.[30,41]

TABLE 17.4
Examples of Advisory DRIs, Chronic Toxicity Values, and Cancer Classifications for Essential Metals

U.S. Dietary Reference Intake and Toxicity Values for Essential Metals

	Dietary Reference Intake[a]		Chronic Oral Toxicity		Chronic Inhalation Toxicity		Carcinogenicity	
Essential Metal	RDA or AI[b] (mg/d)	UL (mg/d)	RfD[c] (mg/kg/day)	MRL[d] (mg/kg/day)	RfC[c] (mg/m^3)	MRL[d] (mg/m^3)	IUR[c] per ($\mu g/m^3$)	Cancer Classification[e]
Potassium	4700	—	—	—	—	—	—	—
Calcium	1000	2500	—	—	—	—	—	—
Sodium	1500	2300	—	—	—	—	—	—
Magnesium	**420 males** **320 females**	350[f]	—	—	—	—	—	—
Iron	**8 males** **18 females**	45	—	—	—	—	—	—
Zinc	**11 males** **8 females**	40	0.3	0.3	—	—	—	—
Manganese	2.3 males 1.8 females	11	0.14	—[g]	5×10^{-5}	4×10^{-5}		D
Copper[h]	**0.9**	10	—	—	—	—	—	D
Molybdenum	**0.045**	2	0.005		—	—	—	—
Chromium (III)	0.035 males		1.5[i]	—	—	—	—	D
Chromium (VI)[g]	0.025 females	—	0.003	0.001	8×10^{-6}[j] 1×10^{-4}[k]	5×10^{-6}	1.2×10^{-2}	Inhalation: A Ingestion: D
Cobalt[h,l]	—	—	—	—	—	0.0001	—	Group 2B-IARC

Dashes indicate the absence of a value or that no information is available.

[a] The RDA values are specific for males and/or nonpregnant, nonlactating females, 31–50 years of age. The UL values are specific for males and/or nonpregnant, nonlactating females, 19–70 years of age.[8]

[b] Adequate intakes are shown in plain text, RDAs in bold. The value shown is for both males and females except where a gender-specific value is shown.

[c] *Source:* The RfDs, RfCs, and IUR are taken from the EPA's Integrated Risk Information System.[19]

[d] *Source:* The chronic MRLs are from the Agency for Toxic Substances and Disease Registry (ATSDR).[298]

[e] In 2005, the EPA revised its classification scheme such that now it uses standard descriptors of conclusions rather than letter designations. However, many EPA IRIS profiles retain the EPA's 1986 letter classifications of cancer groups: Group A (known human carcinogen) and Group D (not classifiable as to human carcinogenicity). The IARC Group 2B is classified as possibly carcinogenic to humans.

[f] The UL for magnesium represents intake from a pharmacological agent only and does not include intake from food and water.

[g] ATSDR has not derived a chronic MRL. The agency did derive an interim guidance value of 0.16 mg/kg/day that it recommended for use in ATSDR public health assessments.

[h] Copper, chromium (VI), and cobalt are undergoing EPA reassessment or review.[361]

[i] The RfD was derived for insoluble chromium (III) salts.

[j] The RfC was derived for chromic acid mists and soluble chromium (VI) aerosols.

[k] The RfC was derived for chromium (VI) particulates.

[l] Cobalt is an essential metal as it is a component of vitamin B_{12}. However, DRIs are not established for cobalt specifically.

Toxicodynamics of Chromium

There is evidence that chromium (III) potentiates the action of insulin. Although the mechanism of action is still being investigated, there is evidence that chromium (III) increases insulin binding to cells, increases insulin receptor number, and activates insulin receptor kinase.[42]

Hexavalent chromium is the most important valence from a toxicological perspective because it is readily absorbed by all tissues. Chromate is structurally similar to phosphate and sulfate and enters cells via the general anion channel protein.[40] Dermal exposure to potassium dichromate and other chromium compounds can lead to the development of a sensitization reaction. The resulting hypersensitivity results from chromium binding to proteins and becoming antigenic.

The carcinogenicity of chromium in the respiratory system was established in the late nineteenth century with the nasal tumors first described among Scottish chrome pigment workers.[43] Chromium-induced carcinogenesis has been reviewed in the recent literature.[40,44] The mechanism of action is believed to be from a direct modification of DNA.[45,46] After hexavalent chromate enters a cell, it is rapidly reduced to chromium (III). During the reduction process, unstable and reactive

intermediates, including chromium (IV), chromium (V), hydroxide, thiyl radicals ($RS^{\bullet}$) and organic radicals ($R^{\bullet}$), and active oxygen radicals are formed. It is believed that these moieties are responsible for chromium carcinogenicity.[44] Because chromium (VI) is readily absorbed by all tissues, one could postulate that chromium-induced cancers should be noted in other organs. Although the evidence is not as strong, exposure to hexavalent chromium is associated with an increased incidence of many types of cancers.[30,40]

Advisory, Screening, and Regulatory Levels of Chromium

In 2006, the Food and Nutrition Board developed gender-specific AI levels for chromium that are not valence-specific (see Table 17.4). In an earlier publication, board members found that there was not sufficient evidence to raise concern regarding the safety or toxicity of chromium picolinate for intake up to 1.6 mg of chromium picolinate/day, or 200 µg of chromium (III)/day, for 3–6 months.[47] This conclusion is consistent with the findings published in the 2000 ATSDR toxicological profile for chromium, and the Food and Nutrition Board did not derive an upper limit for chromium. The U.S. EPA places chromium (III) in Group D (not classifiable as to human carcinogenicity), and as of May 2012, an RfC was not developed. In contrast, the EPA determined that chromium (VI) is a Group A human carcinogen via inhalation. In 1998, the agency published an inhalation unit risk (IUR) value of 1.2×10^{-2}. The U.S. EPA's toxicity values and the ATSDR's MRLs for chromium are shown in Table 17.4. To protect workers, OSHA establishes regulatory levels, PELs, for air in occupational environments. In May 2012, the PELs for chromium (VI) and chromium (III) were 5×10^{-6} and 5×10^{-4} µg/m^3, respectively. Chromium is also regulated as a hazardous air pollutant under section 112 of the CAA. It is noteworthy that a bill to amend the SDWA to require a health advisory and drinking water standard for chromium (VI) was proposed in the U.S. House of Representatives, in March 2012. If implemented, an MCL for chromium (VI) would be the first valence-specific federal drinking water regulation. The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Cobalt

Sources of Cobalt Exposure

Cobalt is a hard silvery metal widely distributed in rocks and soils and always occurs with nickel and usually with arsenic.[48] It is primarily used in the production of superalloys, as a drier in paints, in magnets, and the production of prosthetic devices. Occupational exposure occurs in the hard metal industry, among cobalt blue dye plate painters and coal miners. This exposure is reflected in elevated levels of cobalt in tissues and body fluids. Exposure to cobalt also occurs via the use and implantation of medical devices. Patients with cobalt-containing metal-on-metal total hip replacements were found to have blood cobalt concentrations up to 50 times higher than controls.[49]

Cobalt Essentiality

Cobalt is an essential component of vitamin B_{12}, also called hydroxycobalamin, which is involved in intermediary metabolism, nucleic acid synthesis, and single-carbon metabolism. It is required to prevent macrocytic megaloblastic anemia, atrophic gastritis, achlorhydria, neurological degeneration, and dementia.[28] The only recognized requirement for cobalt is a component of vitamin B_{12}.

Adverse Health Effects of Cobalt Intoxication

Cobalt can cause allergic dermatitis and cross-reaction with nickel is frequent.[50] Inhalation exposure to cobalt alloyed to tungsten carbide is associated with hard metal disease, which is characterized by interstitial fibrosis and restrictive respiratory impairment.[51] Cobalt by itself has caused occupational asthma in diamond polishers, and the effect has been attributed to an immunologic mechanism with cobalt acting as a hapten.[52]

The carcinogenicity of cobalt is uncertain. Animal studies are positive only for subcutaneous, intramuscular, or intratracheal administration, but not for inhalation. The excess rates of lung cancer observed in men occupationally exposed to cobalt dust could be explained by simultaneous exposure to nickel, arsenic, and/or tobacco.[53] In a 2006 review of cobalt and its compounds, the International Agency for Research on Cancer (IARC) concluded that cobalt metal without tungsten carbide, cobalt sulfate, and other soluble cobalt (II) salts are possibly carcinogenic to humans (Group 2B).[54]

Toxicokinetics of Cobalt

For the general population, ingestion is the primary route of exposure to cobalt.[48] The absorption, distribution, and excretion of cobalt is not well studied. However, a biokinetic model, developed to estimate levels of cobalt in whole blood and urine resulting from ingestion of supplements, returned predicted levels consistent with those measured in humans following ingestion of known doses.[55]

Toxicodynamics of Cobalt

Cobalt-caused occupational asthma is attributed to an immunologic mechanism with cobalt acting as a hapten.[52] Inhalation exposure to cobalt is also associated with hard metal disease. The toxic mechanism of hard metal particles is thought to involve both cobalt sensitivity and the generation of oxygen radicals by the carbide particles.[51,56] More recent data indicate hard metal dust is genotoxic both in *in vitro* and *in vivo* systems.[57] Based on these findings, and evidence that the production of active oxygen species and inhibition of DNA repair are the likely modes of action, IARC classifies cobalt as a Group 2B possible human carcinogen.[21,27]

Advisory, Screening, and Regulatory Levels of Cobalt

Although cobalt is an essential metal, the Food and Nutrition Board has not developed DRI values for cobalt. Rather, the

requirement for cobalt is implicit in the recommendation for vitamin B_{12} intake. The U.S. EPA has not derived toxicity values for cobalt. However, the ATSDR established an MRL for chronic inhalation toxicity, 0.1 μg/m^3 (Table 17.4). OSHA's regulatory PEL for cobalt metal dust and fume is 0.1 mg/m^3 whereas the NIOSH advisory REL is 0.05 mg/m^3. The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Copper

Sources of Copper Exposure

Copper occurs naturally as free metal and occurs in compounds in (I) or (II) valence states. Copper enters the environment by discharge from steel and pulp mills and erosion of natural deposits. The ingestion of copper in the diet is the primary source for copper intake. Occupational exposure is primarily due to inhalation of particulates containing copper present in smelting, welding, or other metal works.[27]

Copper Essentiality

Copper is incorporated into several enzymes involved in (1) hemoglobin formation, (2) carbohydrate metabolism, (3) catecholamine biosynthesis, and (4) cross-linking of collagen, elastin, and hair keratin.[58] These enzymes include cytochrome C oxidase, dopamine β-hydroxylase, ascorbic acid oxidase, and superoxide dismutase. Cytochrome C oxidase is important in energy production because it catalyzes a key reaction. Superoxide dismutase is present in the liver, lung, blood, and brain where it plays a protective role in reducing superoxide radicals to hydrogen peroxide. Copper deficiency can be a result of malnutrition or excess intake of zinc. Clinical manifestations of copper deficiency include macrocytic anemia, neutropenia, and bone abnormalities.[27]

Adverse Health Effects of Copper

Copper toxicity from ingestion is characterized by vomiting, diarrhea, nausea, abdominal pain, hemolytic anemia, hepatic and renal necrosis, and death. Industrial exposure may occur to copper fumes, resulting in metal fume fever with dyspnea, chills, headache, and nausea.[59] Dermal irritation and contact allergic dermatitis have been associated with copper jewelry, intrauterine contraceptive devices, and occupational exposure to electroplating and copper-containing agricultural products.[60]

Toxicokinetics of Copper

Copper is readily absorbed following oral ingestion, but homeostatic mechanisms limit further intake once requirements are met. Newly absorbed copper is transported to body tissues primarily by albumin, transcuprein, and ceruloplasmin. Copper can be dermally absorbed from copper-containing topical products. Copper is deposited mainly in hepatocytes with lesser amounts in the kidney.[61] Copper is either active or in transit with little or no excess copper being normally stored.[62] Biliary excretion is the major route with small amounts excreted in the urine. Considering these homeostatic mechanisms following oral intake, absorption through the inhalation or dermal routes may allow toxic levels to pass unimpeded into the blood.[63]

Toxicodynamics of Copper

The role of copper in normal immune response is beginning to be elucidated. There is evidence that the mode of action for impaired DNA synthesis, observed in T lymphocyte from copper-deficient lab animals, involves limitation of interleukin 2 activity.[64] There are two well-characterized genetic disorders that provide information on the role of copper transport proteins in disease mechanisms. Wilson's disease is due to an autosomal recessive miscoding of the gene *ATP7B*.[65] Defects in this gene are associated with accumulation of copper in the liver and brain. In addition, there is decreased concentration of plasma ceruloplasmin, impaired biliary copper excretion, and hypercupremia, resulting in hepatic and renal lesions and hemolytic anemia.[58] Menkes' disease is a systemic lethal disorder characterized by neurodegenerative symptoms and connective tissue manifestations. It is an X-linked recessive inherited disease that is attributable to defects in the *ATP7A* gene, which codes for a copper transport protein.[66] In Menkes' disease, copper transport from the intestine to the portal vein and transport into the brain is blocked. Copper accumulates in red blood cells while there is systemic copper deficiency.

Advisory, Screening, and Regulatory Levels of Copper

The RDA and upper limit for copper intake, for a selected segment of the adult population, are provided in Table 17.4. For adult men and nonpregnant women aged 19–70 years, the RDA is 900 μg/day and the UL is 10 mg/day. The EPA's MCL for drinking water is 1.3 mg/L.[67] The NIOSH REL and OSHA PEL for copper fume are each 0.1 mg/m^3 and for copper dusts and mists, 1 mg/m^3.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Iron

Sources of Iron Exposure

Iron is a silver white solid metal found in the natural environment mainly in combination with other elements such as oxides, carbonates, sulfides, and silicates. It is the second most abundant metal and the fourth most abundant element in the earth's crust. The use of iron in agriculture, metallurgy, drugs, or other consumer products results in its release to the environment. The mining and processing of iron ores also may result in the release of iron compounds to the environment. Dietary iron is available as either heme or nonheme iron. Heme iron is found in meats and is relatively

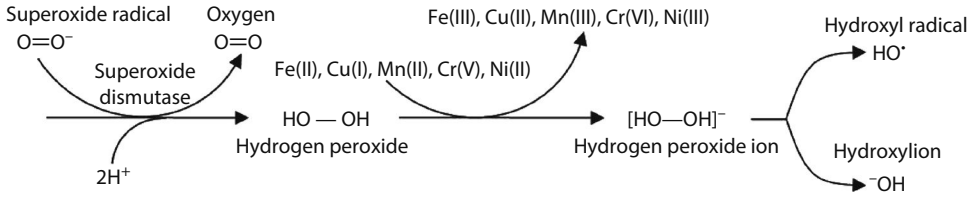

FIGURE 17.4 Conversion of superoxide anion radical to hydrogen peroxide can occur spontaneously, or it can be catalyzed by superoxide dismutase. Cleavage of hydrogen peroxide to hydroxyl radical and hydroxyl ion is catalyzed by the metal ions shown and is called the Fenton reaction.

well-absorbed compared with nonheme iron, which is also found in meats, grains, and vegetables.[30]

Iron Essentiality

Iron exists in two stable oxidation states, oxidized ferric (III) and reduced ferrous (II), which accounts for its essentiality as a trace element and its crucial role in the oxygen and electron transport reactions of living cells. Early symptoms of iron deficiency are nonspecific and include fatigue and weakness. This progresses to iron deficiency anemia characterized by microcytic hypochromic anemia. These symptoms resolve after administration of iron.[30]

Adverse Health Effects of Iron

Acute iron poisoning is the most common fatal poisoning in children reported to poison control centers in the United States.[68] Acute iron poisoning via the oral route is characterized by four distinct clinical stages, but individual patients do not always demonstrate each stage.[69] Stage I occurs 0.5–2 h after ingestion and is characterized by the onset of acute gastrointestinal symptoms including vomiting and diarrhea, but central nervous system (CNS) symptoms such as lethargy and coma may be present in severe cases. Stage II occurs 6–24 h after ingestion, and the victim may be asymptomatic or appear to have improved. Stage III occurs 12–48 h after ingestion and is characterized by gastrointestinal perforation, coma, convulsions, cardiovascular collapse, hepatic and renal failure, and metabolic acidosis. Stage IV occurs three to four weeks after ingestion with the appearance of gastrointestinal scarring.

Chronic oral iron intoxication leads to hemosiderosis, a condition in which there is a generalized increase in the iron content in the body tissues, particularly the liver and spleen. Oxidative damage associated with elevated brain iron has been suggested as a risk factor for early age onset of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.[70] Increased iron stores have been reported to contribute to the development of noninsulin-dependent diabetes.[71] Also, hepatotoxicity is typically seen in patients with chronic iron overload with progression from portal fibrosis to cirrhosis.[72]

Toxicokinetics of Iron

Intestinal absorption of iron depends on iron status. Ten percent of the total (heme plus nonheme) is absorbed when the iron status is normal, but up to 20% can be absorbed in deficiency states. Iron is lost through the shedding of cells, sweat, nails, hair, blood loss, menstruation, and in the urine. Humans are unable to eliminate iron as efficiently as it is absorbed. Thus, the body regulates iron stores by limiting absorption.[73] Dietary calcium phosphate, bran, phytic acid, and plant polyphenols inhibit absorption of nonheme iron. Divalent iron is taken up by intestinal mucosa and converted to the trivalent form. The trivalent form is bound to transferrin.[59] Iron is transported to the liver or spleen bound to transferrin and stored as ferritin, which has a large iron storage capacity and prevents iron from participating in the Fenton reaction (Figure 17.4). Of the typical 4 g of body iron stores found in adults, 66% is bound as hemoglobin, 10% in myoglobin, a minute amount in iron-containing enzymes, and the rest as intracellular storage proteins, ferritin and hemosiderin.

Toxicodynamics of Iron

Ferrous iron catalyzes lipid peroxidation. This can cause disruption of mitochondrial membranes and the Krebs cycle. The subsequent accumulation of lactic acid from anaerobic metabolism may result in severe metabolic acidosis. Iron also shunts electrons from the electron transport system by acting as an electron sink. The result is also metabolic acidosis. Free iron is an oxygen-reactive substance, is highly toxic to cells, and enhances the formation of free radicals and peroxidation of membrane lipids.[74] Free radical stress and lipid peroxidation have both been suggested as factors in the etiology of diabetes.[75]

Advisory, Screening, and Regulatory Levels of Iron

The RDA and UL for iron for men and nonpregnant women aged 19–70 are shown in Table 17.4.[8] The U.S. EPA has not derived toxicity values and the ATSDR has not established chronic MRLs for iron. The NIOSH REL and OSHA PEL for iron oxide dust and fume are 5 and 10 mg/m^3, respectively. NIOSH also established an REL for soluble iron salts, 1 mg/m^3. The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Magnesium

Sources of Magnesium Exposure

Magnesium is widely distributed in the environment in the form of rocks and minerals such as olivine, montmorillonite, and dolomite. Magnesium salts are also a component of sea salt and are released to the air as sea spray. Commercial

uses of magnesium salts include lightweight metal alloys, fertilizer, construction materials, pharmaceuticals, cosmetics, and chemical intermediates. Magnesium is released to the environment by erosion and in industrial waste streams. Occupational exposure may occur through inhalation and dermal contact with magnesium compound at workplaces where magnesium-containing products are produced or used. Environmental monitoring data indicate that the general public is exposed to magnesium via inhalation of ambient air, ingestion of food and drinking water, and by dermal contact.[27]

Magnesium Essentiality

Magnesium is essential to a large number of biochemical and physiological processes, including neuromuscular conduction in skeletal and cardiac muscles.[59] It is also an important structural component of bone.[8] Magnesium deficiency may cause metabolic changes that contribute to heart attacks and strokes and may be a risk factor for postmenopausal osteoporosis. Magnesium deficiency also results in reduced levels of potassium and calcium, as well as symptoms of nausea, muscle weakness, irritability, and mental confusion.[76] Magnesium deficiency is associated with alcoholism. Alcoholics are more likely to experience dietary insufficiency than members of the general population. They excrete more magnesium in their urine and, with cirrhosis of the liver, there may be less albumin available to bind magnesium.[77]

Adverse Health Effects of Magnesium

Oral exposure to magnesium is not toxic except in individuals with impaired renal function. These individuals may experience nausea, vomiting, and hypotension, followed by central nervous system (CNS) depression with a drop in blood pressure and respiratory paralysis.[8] Inhalation exposure to magnesium oxide can cause metal fume fever.[59]

Toxicokinetics of Magnesium

Magnesium is absorbed mainly in the small intestine. Calcium and magnesium are competitive with respect to their absorption sites, and excess calcium may partially inhibit the absorption of magnesium. Plasma concentrations of magnesium are regulated within a narrow range (0.65–1.0 mM), primarily by adjustments in the reabsorption of filterable magnesium in the loop of Henle, but also by the passive buffering by bone magnesium.[8] In blood plasma, about 65% is in the ionic form while the remainder is bound to protein. The former is what appears in the glomerular filtrate. Excretion also occurs in the feces, sweat, and milk.

Toxicodynamics of Magnesium

Magnesium is a cofactor of many enzymes involved in intermediary metabolism. In the glycolytic cycle that converts glucose to pyruvate, there are seven enzymes that require magnesium (II). Magnesium is also involved in the citric acid cycle and in the beta-oxidation of fatty acids.[78] The neurological effects of magnesium toxicity include synaptic blockage and impaired reuptake of neurotransmitters. Magnesium-mediated neuromuscular toxicity involves decreases in acetylcholine release from the prejunctional motor neuron and decreases motor endplate sensitivity of acetylcholine.[27,30]

Advisory, Screening, and Regulatory Levels of Magnesium

The RDA and UL for magnesium are shown in Table 17.4. The EPA has not derived toxicity values for magnesium nor has the ATSDR derived chronic MRLs. NIOSH has not established an REL for magnesium oxide fume. The OSHA PEL is 15 mg/m^3. RELs and PELs are developed for total and respirable magnesium carbonite.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Manganese

Sources of Manganese Exposure

Manganese is a silver-gray-colored soft metal, which occurs in ores mainly as oxides.[59] Manganese and its compounds are used in numerous products and applications, including iron and steel alloys, dry cell batteries, paints, inks, fertilizers, and fungicides.[79] Manganese cyclopentadienyl tricarbonyl (MMT) is a gasoline octane enhancer in use since 1970. The major combustion products of MMT are manganese particulates of manganese phosphate with some sulfates and a small amount of oxides. Manganese occurs in human diets that include meats, poultry, nuts, grains, green leafy vegetables, and tea.[27]

Manganese Essentiality

Manganese is an essential trace metal that is a component of several mitochondrial enzymes, pyruvate carboxylase, and superoxide dismutase. It activates a wide variety of enzymes, including decarboxylases, transferases, and hydrolases. Although manganese deficiency has not been observed in the human population, suboptimal manganese intake may be a concern because in animals, manganese deficiency can cause impaired growth, skeletal abnormalities, and altered metabolism of carbohydrates and lipids.[80]

Adverse Health Effects of Manganese

The primary toxic effect of occupational inhalation exposure is neurological damage.[81] However, inhalation exposure to manganese can also affect the lung directly, causing metal fume fever, pneumonitis, chronic obstructive lung disease, and pneumonia.[80] The neurological effects of inhalation of manganese dusts, termed manganism, typically begin with weakness and lethargy and may progress to disturbances in speech and gait, a masklike face, tremor, and possibly hallucinations and psychosis. Symptoms may resemble Parkinson's disease, but there is only minimal response to L-dopa therapy.[81]

Toxicokinetics of Manganese

Occupational inhalation exposure is the primary route for manganese toxicity. Manganese applied to the nasal cavity in rats is taken up in the olfactory receptor cells and transported

along the primary neurons to the olfactory bulb, with subsequent migration into most parts of the brain. This route circumvents the blood–brain barrier.[82,83] Only between 3% and 10% of dietary manganese is absorbed in normal adults, and total body stores are controlled by a complex homeostatic mechanism regulating absorption and excretion. Calcium, iron deficiencies, age, and other factors may increase manganese absorption.[80]

Toxicodynamics of Manganese

The pathobiochemical aspects of manganism involve the striatum and globus pallidus. Cell damage may be due to the autooxidation of dopamine with the formation of free radicals.[84]

Advisory, Screening, and Regulatory Levels of Manganese

The AI, UL, RfD, RfC, and chronic MRLs for manganese are shown in Table 17.4. The EPA places manganese in Group D, not classifiable as to human carcinogenicity. This classification is based on inadequate evidence in humans and animals.[19] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Molybdenum

Sources of Molybdenum Exposure

The primary molybdenum-containing ore is molybdenite (MoS_2), with minor ores being powellite ($CaMoO_4$) and wulfenite ($PbMoO_4$). Metallic molybdenum is used in a number of important applications, such as in high temperature and tool steel alloys, and in missile and aircraft parts. Molybdenum disulfide is used as a dry lubricant or as a component in lubricants. Dental technicians exposed to dust of vitallium alloy, which contains chromium, cobalt, and molybdenum, can develop pneumoconiosis that is clearly different from hard metal lung disease associated with cobalt exposure. Occupational exposure to molybdenum compounds may also occur through inhalation and dermal contact at workplaces where molybdenum compounds are used. As molybdenum occurs naturally in the environment, the general public can be exposed to small amounts of molybdenum via inhalation and ingestion of food and drinking water.[27] Dietary sources of molybdenum include legumes, grains, and nuts. The content of molybdenum in these foods is highly dependent on the soil content in which they were grown. Animal products, fruits, and vegetables are generally low in molybdenum.[8]

Molybdenum Essentiality

Molybdenum is a cofactor of several enzymes, including aldehyde oxidase, xanthine oxidase, and sulfite oxidase. In this context, molybdenum functions in the catabolism of purines and pyrimidines and sulfur amino acids. The essentiality of molybdenum was established based on a genetic defect that prevents sulfite oxidase synthesis and leads to severe neurological damage and early death. A molybdenum deficiency syndrome has not been observed in humans or animals. The UL for molybdenum is based on impaired reproduction and growth in animals.[8]

Adverse Health Effects of Molybdenum

The oral toxicity of molybdenum is low presumably because it is rapidly excreted in the urine at relatively high levels of intake.[8] The acute oral toxicity of high concentrations of some molybdenum compounds is related to their solubility. In animal studies, acutely toxic oral doses of molybdenum resulted in severe gastrointestinal irritation with diarrhea, coma, and death from cardiac failure. Animal subchronic and chronic oral exposures can result in growth retardation, anemia, hypothyroidism, bone deformities, sterility, liver and kidney abnormalities, and death.[27]

In studies conducted in regions of high molybdenum soil concentrations, human residents were found to have elevated concentrations of uric acid in the blood and urine, increased blood xanthine oxidase activity, and gout-like symptoms. Studies of workers chronically exposed to molybdenum showed increased incidences of elevated levels of molybdenum in blood plasma and urine and high levels of ceruloplasmin and uric acid in blood serum. Occupational exposure to molybdenum may also result in increased serum bilirubin levels and decreased blood IgA/IgG ratios due to a rise in alpha-immunoglobulins. Pulmonary effects of chronic exposure are reported in one study of 3 of 19 workers. The findings were indicative of pneumoconiosis.[27]

Toxicokinetics of Molybdenum

The oral absorption of molybdenum is thought to be passive rather than actively mediated because absorption is very efficient over a wide range of intake levels. It is thought that molybdenum is transported in the plasma by alpha-2-macroglobulin and that up to 97% of molybdenum in erythrocytes is protein-bound. Urinary excretion is the primary pathway of elimination and the amount eliminated is directly related to the dietary intake.[8]

Toxicodynamics of Molybdenum

Little is known about the mechanism of action of molybdenum toxicity. However, in animals, excessive intake of molybdenum causes a physiological copper deficiency. It is suggested that the mechanism may involve formation of a copper–tetrathiomolybdate complex in the reductive environment of the gastrointestinal tract. It is believed that this might reduce the biological utility of copper.[85]

Advisory, Screening, and Regulatory Levels for Molybdenum

The RDA, UL, and RfD for molybdenum are shown in Table 17.4. The ATSDR has not established chronic MRLs and the EPA has not established an RfC or IUR level. Neither the EPA nor IARC provides information on the cancer categorization of molybdenum. The OSHA PEL for molybdenum metal is 15 mg/m^3. The PEL for soluble compounds of molybdenum is 5 mg/m^3. NIOSH has not established an REL for either form of molybdenum. The advisory, screening, and

regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Potassium

Sources of Potassium Exposure

Elemental potassium is a highly reactive soft metal with a silver-colored appearance. Elemental potassium is not found in nature. However, potassium compounds are common in the earth's crust. Dietary sources of potassium as bicarbonate precursors include leafy greens, root vegetables, and vine produce such as tomatoes, cucumbers, and pumpkins. Meat, milk, and grains contain more.[27]

Potassium Essentiality

Potassium is the principal cation of intracellular fluid, accumulating to a concentration about 30 times higher than in plasma. Potassium in plasma is involved in nerve transmission, muscle contraction, and blood pressure homeostasis. The gastrointestinal absorption of potassium is nearly complete. Plasma concentrations are kept within a narrow range by regulation of urinary excretion, and by depletion of body stores in cases of low potassium intake. Dietary potassium deficiency is rare. Moderate potassium deficiency is characterized by increased blood pressure, increased risk of kidney stones, increased salt sensitivity, and possibly increased risk of stroke. Frank hypokalemia results in cardiac arrhythmias, muscle weakness, and glucose intolerance.[8]

Adverse Health Effects of Potassium

Dermal and ocular thermal burns and necrosis due to the formation of potassium hydroxide are the primary effects following exposure to elemental potassium. Dietary potassium is not toxic in healthy individuals if sufficient water is ingested and renal function is adequate to maintain homeostasis. Cardiac and related effects are the most important risks of supplemental potassium chloride overdose. The symptoms may range from nausea, vomiting, and diarrhea with abdominal discomfort to weakness, muscle cramps, ascending paralysis, dysarthria, hypotension, and arrhythmias.[27]

Toxicokinetics of Potassium

Healthy individuals absorb about 85% of their dietary potassium. The steady-state correlation between intake and urinary excretion is high with about 77%–90% of injected potassium being excreted in the urine. The rest is excreted in the feces and sweat.[27]

Toxicodynamics of Potassium

The high intracellular concentrations of potassium are maintained by the sodium–potassium ATPase pump. Changes in plasma insulin can affect extracellular and plasma potassium concentrations because insulin stimulates the pump. Potassium appears to moderate the effect of increased sodium intake on elevating blood pressure, probably by affecting renal sodium excretion.[86]

Advisory, Screening, and Regulatory Levels for Potassium

The AI for potassium is 4.7 g/day for both adult men and women (Table 17.4). The EPA has not derived toxicity values for potassium and the ATSDR has not derived chronic MRLs. Neither the EPA nor IARC provides information on the cancer categorization of potassium. OSHA has not established a PEL for potassium or potassium compounds other than potassium cyanide. The NIOSH ceiling REL for potassium hydroxide (synonymous to caustic potash and lye) is 2 mg/m^3.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Sodium

Sources of Sodium Exposure

Sodium is a highly reactive soft metal with a silver appearance that is not found in the elemental form in nature. Sodium compounds are present in seawater and are ubiquitous in nature in the form of halides, silicates, and carbonates. Sodium is typically consumed as sodium chloride, which accounts for about 90% of total dietary intake.[27]

Sodium Essentiality

Sodium is the principal cation of extracellular fluid and the primary regulator of extracellular fluid volume. Sodium also regulates osmolarity, acid–base balance, and membrane potential and participates in active transport across cell membranes. Sodium deficiency is very uncommon but may occur after heavy and prolonged sweating, chronic diarrhea, or renal disease and constitutes a medical emergency.

Adverse Health Effects of Sodium

Dietary sodium is not toxic if sufficient water is ingested and renal function is adequate to maintain homeostasis. Lifelong excess intake of sodium may predispose sensitive individuals to hypertension, and individuals diagnosed with high blood pressure are commonly advised to limit sodium intake to 1–2 g/day or less.[86] Dermal and ocular thermal burns and necrosis due to the formation of sodium hydroxide are the primary effects following exposure to elemental sodium.

Toxicokinetics of Sodium

Sodium is typically consumed as sodium chloride and is 98% absorbed in the small intestine. Renal excretion of sodium maintains homeostasis, over a wide range of intakes and losses, via aldosterone control of tubular excretion.[30]

Toxicodynamics of Sodium

Sodium regulates extracellular fluid and plasma volumes and functions to regulate the electrical potential and active transport across the cell's plasma membrane. The rennin-angiotensin-aldosterone axis regulates blood volume and

pressure by promoting sodium retention and reabsorption in the kidneys. These effects are counterbalanced by atrial natriuretic peptide, which functions to reduce blood volume and pressure via increased glomerular filtration with increased sodium excretion.[30]

Advisory, Screening, and Regulatory Levels for Sodium

The dietary AI and UL for sodium are shown in Table 17.4. The UL is based on sodium's effect on blood pressure. The Food and Nutrition Board acknowledges that a lower UL may be appropriate for sensitive individuals such as older individual, African Americans, and individual with hypertension. The EPA has not derived toxicity values for sodium and the ATSDR has not derived chronic MRLs. Neither the EPA nor IARC provides information on the cancer categorization of potassium. OSHA has not established a PEL for sodium bisulfite; the NIOSH REL is 5 mg/m^3. The OSHA PEL and NIOSH REL for sodium hydroxide (caustic soda) are both 2 mg/m^3.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Zinc

Sources of Zinc Exposure

Zinc is found in almost all minerals in the earth's crust. Zinc is a bluish-white, soft metal extracted from ore and is used in alloys, for galvanizing iron to prevent corrosion and oxidation, and in cosmetics, pharmaceuticals, and dry cell batteries.[87] At temperatures approaching its boiling point, zinc volatilizes and oxidizes to the white fume of zinc oxide. Dietary sources of zinc include red meat, some seafood, whole grains, and fortified breakfast cereals.[27]

Zinc Essentiality

Zinc is an essential trace element and is a required component of many enzymes. Zinc is stored in bone and muscle but is not readily released from these stores during deficiency. The enzymes in which zinc is a catalytic component include RNA polymerase, alcohol dehydrogenase, and alkaline phosphatase. In other enzymes, such as copper-zinc superoxide dismutase, zinc is a structural component. Zinc is also a structural component in some proteins involved in gene regulation. These proteins include the retinoic acid receptor and vitamin D receptor. Zinc may also influence apoptosis and protein kinase C activity.[8] Zinc is involved in many metabolic functions. Symptoms of mild deficiency are diverse and inconsistent. Severe zinc deficiency causes hypogonadism and dwarfism, which are alleviated with zinc supplementation. While the signs and symptoms of zinc deficiency include loss of appetite, growth retardation, and slow wound healing, no single enzyme function has been identified as associated with these signs of zinc deficiency. Low zinc status is observed in 30%–50% of people with alcoholism.[8]

Adverse Health Effects of Zinc

Inhalation exposure to zinc oxide fume can cause metal fume fever. Zinc chloride fume is a corrosive material, which has caused chemical pneumonitis, alveolar and bronchial obliteration, and death. Zinc has low human toxicity by the oral route, but high levels can cause gastrointestinal distress.[88] Long-term oral intakes of zinc at levels of 18.5–25 mg/day can interfere with copper absorption, and intakes 10–30 times the RDA can impair immune responses and decrease serum high-density lipoprotein.[8]

Toxicokinetics of Zinc

Zinc compounds are absorbed orally and excreted primarily in the feces. Plasma zinc concentrations remain relatively stable unless low or high intakes occur over prolonged periods. Gastrointestinal absorption of zinc is higher when body stores are lower and is also higher from more refined diets. Zinc is absorbed via both carrier-mediated and passive diffusion. The carrier-mediated absorption mechanism may be saturable. Intestinal metallothionein can inhibit zinc absorption by competing with the carrier-mediated transport.[27] Absorbed zinc is bound to albumin and transferred to the intestine via the portal system. Most of the body's zinc is stored in muscle and bone with about 0.1% of total zinc present in the plasma. Excretion is primarily in the feces with less than 10% excreted in the urine.[27]

Toxicodynamics of Zinc

Zinc has catalytic, structural, and regulatory biological functions. For example, zinc is an essential component of numerous proteins involved in the defense against oxidative stress. It has been shown that depletion of zinc may enhance DNA damage via impairments of DNA repair mechanisms.[6]

Advisory, Screening, and Regulatory Levels for Zinc

The RDA, UL, RfD, and oral chronic MRL for zinc are shown in Table 17.4. The critical effect on which the UL is based is zinc's effect on copper metabolism, that is, reduced copper status. The RfC, IUR, and chronic inhalation MRL have not been derived for zinc. Neither the EPA nor IARC has developed a cancer classification for zinc. The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

HIGHLY TOXIC METALS

The cancer classifications and some of the available quantitative toxicity advisory, screening, and regulatory values for the highly toxic metals, lead, cadmium, mercury, and arsenic, are provided in Table 17.5. These values are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

TABLE 17.5
Examples of Advisory Chronic Toxicity Values and Cancer Classifications for Highly Toxic Metals

U.S. Toxicity Values for Highly Toxic Metals

	Chronic Oral Toxicity		Chronic Inhalation Toxicity		Carcinogenicity		
Metal	MRL (mg/kg/day)	RfD (mg/kg/day)	MRL (mg/m^3)	RfC (mg/m^3)	Oral Slope Factor (mg/kg/day)	Inhalation Unit Risk ($\mu g/m^3$)	Cancer Classifications[a]
Tetraethyllead	—	0.0000001	—	—	—	—	—
Lead	—	—	—	—	—	—	B2-EPA Group 2A-IARC
Methylmercury	0.0003	0.0001	—	—	—	—	C-EPA Group 2B
Mercury	—	—	0.0002	0.0003	—	—	D-EPA Group 3-IARC
Arsine	—	—	—	0.00005	—	—	—
Dimethyl-arsenic	0.02	—	—	—	—	—	D-EPA
Arsenic	0.0003	0.0003	—	—	1.5	0.0043	A-EPA Group 1-IARC
Cadmium	0.0001	0.0005 for drinking water 0.001 for dietary exposure	0.00001	—	—	0.0018	B1-EPA Group 1-IARC

[a] Many IRIS profiles retain the EPA's 1986 letter classifications of cancer groups. These include the following: Group A (carcinogenic to humans), Group B1 (probable human carcinogen [limited human data]), Group B2 (probably carcinogenic to humans [inadequate human data]), Group C (possibly carcinogenic to humans), Group D (not classifiable as to human carcinogenicity), and Group E (evidence of noncarcinogenicity for humans). In 2005, the EPA revised its classification scheme such that now it uses standard descriptors of conclusions rather than letter designations. IARC classifications are as follows: Group 1 (carcinogenic to humans), Group 2A (probably carcinogenic to humans), Group 2B (possibly carcinogenic to humans), Group 3 (not classifiable as to its carcinogenicity to humans), and Group 4 (probably not carcinogenic to humans).

Arsenic

Sources of Arsenic Exposure

Arsenic is a component of copper, cobalt, and nickel ores; and arsenic trioxide, As_2O_3, is a toxicologically significant form that is released into the environment by smelting. Other anthropogenic sources include wood preservatives and some agricultural chemicals. Arsenic is also used to make gallium arsenide for light-emitting diodes, lasers, and solar devices and as a doping agent.[27] Arsine, AsH_3, is a gaseous form of arsenic that is formed whenever arsenic is in the presence of hydrogen. For example, it can be generated in metal tanks storing acids that contain arsenic impurities.

By modeling data from the National Health and Nutrition Examination Survey, investigators defined a mean total arsenic exposure from food. That value, 0.38 μg/kg/day, is approximately 14 times higher than the mean arsenic exposures from the drinking water. The mean inorganic arsenic exposure from food is 0.05 μg/kg/day (1.96 μg/day), which is approximately two times higher than the mean inorganic arsenic exposures from the drinking water. The modeled exposure and dose estimates matched well with the duplicate diet data and measured arsenic biomarkers. The major food contributors to inorganic exposure were vegetables (24%); fruit juices and fruits (18%); rice (17%); beer and wine (12%); and flour, corn, and wheat (11%). Approximately 10% of total arsenic exposure from foods is the more toxic inorganic arsenic.[89–91]

Adverse Health Effects of Arsenic

Acute ingestion of several hundred milligrams or more of a soluble arsenic compound, such as sodium arsenite or arsenic trioxide, is characterized by systemic findings that appear in stages over the course of hours to weeks. The initial stage typically begins 30 minutes to several hours following ingestion. Gastrointestinal hemorrhaging occurs, leading to profound losses of fluid and electrolytes. It is accompanied by vasodilation, hypotension, and metabolic acidosis. In severe acute intoxication, death may occur within hours from shock or ventricular arrhythmias. If the individual survives the initial phase, a second phase of cardiovascular symptoms, for example, congestive heart failure, noncardiogenic pulmonary edema, and ventricular arrhythmia, may appear within 1–7 days. The third phase of acute arsenic intoxication is characterized by pancytopenia and sensorimotor peripheral neuropathy. This phase generally appears 1–4 weeks after the initial high dose ingestion.[92]

The therapeutic use of inorganic arsenite in the treatment of cancer has yielded information on the clinical features of low-level arsenic intoxication. Recent therapeutic protocols for the treatment of refractory or relapsed acute promyelocytic leukemia and numerous other malignancies have administered between 10 and 20 mg of intravenous As_2O_3 daily for 2-month courses. Cardiotoxic effects have included frequent prolongation of the QTc interval on the electrocardiogram.

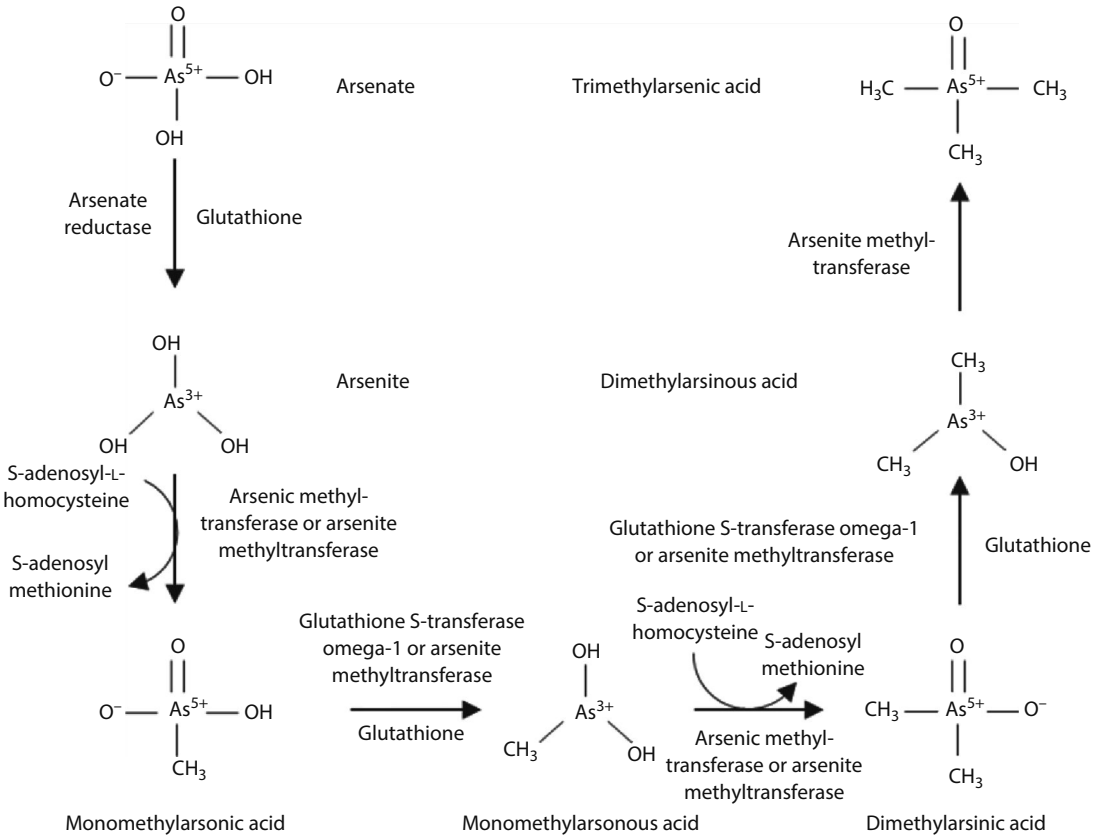

FIGURE 17.5 The interconversion of arsenate and arsenite and their conversion to monomethylarsonic acid, dimethylarsenic acid, monomethylarsonous acid, dimethylarsinous acid, and trimethylarsenic acid take place primarily in the liver.

Malignant ventricular arrhythmias, including fatal torsades des pointes, have been reported.[93] Other notable toxicity in this setting has included peripheral neuropathy, gastrointestinal disturbance, and hepatotoxicity.[94]

The most common early signs of chronic arsenic poisoning are muscle weakness and hyperpigmentation of the skin. Epidemiological studies have linked chronic arsenic ingestion, particularly from naturally occurring arsenic in drinking water, to a strikingly broad spectrum of serious chronic illness. These include peripheral vascular occlusion and a type of gangrene termed blackfoot disease. Chronic exposure to inorganic arsenic induces cancer in human lungs, urinary bladder, skin, kidney, and liver. The majority of deaths are from lung and bladder cancer.[95] Epidemiological investigations conducted in Taiwan, Chile, and Argentina made the link between arsenic ingestion and risk for lung cancer and bladder cancer. Epidemiological studies of smelter workers, pesticide-manufacturing workers, and case reports of lung cancer in arsenical pesticide applicators showed strong associations between exposure and an increased incidence of lung cancer.

Toxicokinetics of Arsenic

Major routes of absorption of inorganic arsenic in the general population are inhalation and ingestion. Inhaled inorganic arsenic deposited in the lungs is eventually absorbed. Most ingested soluble inorganic arsenic is absorbed and excretion occurs primarily in the urine.[30] Insoluble forms pass through the gastrointestinal tract with negligible absorption.[96] Two processes are involved in the metabolism of arsenate and arsenite: (1) the interconversion of arsenate and arsenite and (2) the conversion of these moieties to monomethylarsonic acid and dimethylarsenic acid. The methylation process, illustrated in Figure 17.5, was once thought to be a detoxification mechanism since the methylated forms of arsenic were believed to be less toxic and methylation results in lower tissue retention.[97] However, the identification of highly toxic trivalent methylarsenicals has challenged this hypothesis.[98]. Long-term accumulation of inorganic arsenic does not generally occur in physiologically active compartments in the body. However, arsenic concentrates in the skin, hair, and nails because arsenic reacts with thiol groups, and Mees' lines, horizontal white lines on the fingernails, appear in exposed individuals as the exposed nail grows.[99]

Toxicodynamics of Arsenic

Although arsenic can exist in several valence states, the (III) and (V) states are the most prevalent, with arsenite (III) being more toxic than arsenate (V). Arsenic is believed to exert its toxic effects through at least two mechanisms, depending on its valence state. Through the process of arsenolysis, arsenate inhibits adenosine triphosphate (ATP) synthesis by

uncoupling mitochondrial oxidative phosphorylation. It is thought that arsenate is substituted for inorganic phosphate in the formation of ATP and that the unstable arsenate ester is then rapidly hydrolyzed. Arsenite reacts with thiol groups on the active sites of many enzymes and tissue proteins, such as keratin in skin, nails, and hair.[100] One mechanism of carcinogenicity is postulated to involve the multistep metabolism of pentavalent arsenic to dimethylarsenic acid during which free radicals are produced.[59,100] There is also evidence that reduced glutathione can chemically reduce pentavalent arsenicals to trivalent arsenicals, which then disrupt tubulin polymerization directly. In this context, arsenic may indirectly induce stable chromosome aberrations that can lead to cancer.[101]

Arsine, AsH_3, is a highly toxic gaseous hydride of trivalent arsenic, and its toxic effects are distinct from the other arsenic compounds. Arsine binds hemoglobin, resulting in rapid hemolysis and hematuria. The third effect of the classic arsine triad is abdominal pain.[59]

Screening and Regulatory Levels of Arsenic

The EPA classifies arsenic as a Group A known human carcinogen. IARC places arsenic in Group 1, carcinogenic to humans. The EPA's RfD, oral slope factor and unit risk value for arsenic, and the RfC for arsine are shown in Table 17.5.[19] The ATSDR's MRL for chronic oral toxicity is also shown in Table 17.5. OSHA's PELs for inorganic arsenic, organic arsenic, and arsine are 0.010, 0.5, and 0.2 mg/m^3, respectively.[20] An estimated lethal dose of arsenic (III) for a 70 kg human is 70–180 mg.[27] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Cadmium

Sources of Cadmium Exposure

Cadmium is a soft silver-white transition metal. Cadmium compounds are naturally occurring and often found in association with zinc ore. In the environment, cadmium occurs as cadmium (II) and usually as particulate matter when released to air.[59] Cadmium is used primarily in the production of batteries but also for pigments in plastics, ceramics, and glasses, as stabilizers for polyvinyl chloride, as coatings on steel and some nonferrous metals, and as a component of specialized alloys.[102]

Adverse Health Effects of Cadmium

Cadmium affects nearly every organ system if the dose is high enough. Acute effects depend on the route of exposure. Symptoms of acute inhalation exposure to cadmium develop 4–10 h postexposure and initially simulate metal fume fever (fever, nausea, vomiting, headache, cough, dyspnea, nasopharyngeal irritation). These adverse effects can progress to chemical pneumonitis and a potentially fatal pulmonary edema.[103,104] A fatal dose can be inhaled by exposed individuals who are unaware of the presence of cadmium.[105] Fatal doses have been estimated at 50 mg/m^3 for 1 h and 9 mg/m^3 for 5 h.[106] Recovery following acute high-level exposure or chronic exposure at lower levels may be accompanied by pulmonary fibrosis.

Oral exposure to cadmium is rarely fatal because the associated gastrointestinal irritation leads to vomiting, eliminating most of the dose before absorption. Chronic inhalation or ingestion of cadmium results in kidney damage, characterized by tubular and/or glomerular dysfunction with proteinuria and low concentrating capacity.[107]

Excess inhalation or ingestion of cadmium can also lead to abnormalities of calcium metabolism, and susceptible individuals may develop a painful bone disease that was first discovered in a cadmium-contaminated area in Japan and termed itai-itai (ouch-ouch) disease.[108] The disease is characterized by osteomalacia and osteoporosis with an increased tendency to spontaneous fracture. It is associated with bone pain and renal tubular dysfunction. Cadmium increases bone resorption and inhibit bone formation in both *in vivo* and *in vitro* systems.[109] It is hypothesized that cadmium causes prolonged urinary calcium loss, leading to the skeletal demineralization and increased risk of fractures.[110]

Cadmium exposed populations are not reported to have elevated death rates associated with cardiovascular disease. Although intravenous cadmium administration produces a hypertensive response in rats, no difference in blood pressure was found between high- and low-exposure workers after adjusting for age, weight, and cigarette smoking.[111]

Cadmium-induced testicular damage leads to infertility in experimental animals and may damage the reproductive ability of cadmium-exposed workers.[112]. Maternal and fetal toxicity of cadmium is well documented in rodents.[102,113] Elevated levels of cadmium in neonates are associated with a decreased birth weight. Further research is required to determine if developmental effects of cadmium are of concern at environmental levels.

The NTP and IARC have linked cadmium to lung cancer in humans. Cadmium's link to other human cancers is less clear. Prostate tumors have been reported in male rats after oral cadmium exposure, and several studies suggest a role for cadmium in human prostate cancer.[114–117] Additionally, occupational exposure to cadmium is associated with an increased risk of renal cell carcinoma.[118]

Toxicokinetics of Cadmium

Dermal absorption of cadmium compounds is negligible. The primary routes of exposure for humans are inhalation and ingestion. Cadmium absorption following inhalation exposure is dependent on particle size and solubility. Between 10% and 50% of inhaled cadmium will be absorbed. The absorption is greater for small particles and fumes than for large particle dust. Poorly soluble inhaled particles are deposited on the ciliated tracheobronchial mucosa, transported to the pharynx, and swallowed into the gastrointestinal tract.[27] When exposure occurs by smoking cigarettes, cadmium is in the form of submicron aerosols and absorption of the inhaled

amount is between 25% and 50%. Tobacco plants are known to concentrate cadmium independent of the soil content and it is estimated one pack/day smokers can absorb 1–3 μg cadmium/day. Cadmium oxide is reported to be highly bioavailable, and smokers are known to have higher levels of cadmium in their blood (4–5 times higher) and kidneys (2–3 times higher) than nonsmokers.[110]

In humans, about 5% of ingested cadmium is absorbed. However, cadmium and iron are both absorbed through a common pathway involving the divalent metal transporter-1 (DMT-1).[119] Iron deficiency results in increased expression of intestinal DMT-1 and is therefore associated with increased cadmium absorption.[110,120] In individuals with iron deficiency, the gastrointestinal absorption rate may be as high as 20% but has been reported to be as high as 90%.[107,121]

Toxicodynamics of Cadmium

Absorbed cadmium is first transported to the liver where it induces metallothionein synthesis and is sequestered as cadmium-metallothionein. Small amounts of liver cadmium-metallothionein are released into the plasma following normal cell turnover, filtered with the primary urine, reabsorbed into the proximal tubular cells where lysosomes degrade the metallothionein portion releasing cadmium. Once released, cadmium again induces renal metallothionein synthesis. Renal damage results when the kidneys can no longer produce sufficient metallothionein to sequester the cadmium ion and prevent its interaction with critical macromolecules. Free cadmium inactivates metalloenzymes, activates calmodulin, and/or damages cell membranes through activation of oxygen.[122] Increased urinary excretion of β_2-microglobulin, a low molecular weight protein normally reabsorbed in the proximal tubule, is an early indicator of renal damage.

The mechanism(s) of cadmium carcinogenesis are not clearly understood. Cadmium is a poor mutagen but may act as an epigenetic or indirectly genotoxic carcinogen. Potential mechanisms include inhibition or faulty DNA repair, aberrant gene activation, and suppressed apoptosis.[121]

Although the mechanism of cadmium-induced testicular toxicity is poorly understood, cadmium treatment is known to increase vascular permeability in rat testis, and one theory is that the damage is the result of testicular blood vessel toxicity.[123] Other evidence suggests cadmium-induced testicular toxicity is associated with oxidative damage through the production of reactive oxygen species.[124] In support of this theory, it has been reported that zinc-deficient rats are more susceptible to cadmium-induced testicular damage.[125] Other researchers report ascorbic acid and/or alpha-tocopherol supplementation protect rats from cadmium-induced testicular damage.[126,127]

Screening and Regulatory Levels of Cadmium

The EPA has derived an RfD for cadmium based on the highest level of human renal cadmium not associated with significant proteinuria. Separate values were derived for food and water exposure, assuming 2.5% absorption of cadmium from food and 5% from water with a 0.01% per day excretion. A kidney concentration of 200 μg/g wet human renal cortex is considered the no-observable-effect-level.[19] The resulting RfD values are 0.001 mg/kg/day (food) and 0.0005 mg/kg/day (water) (Table 17.5). An RfC for chronic cadmium inhalation exposure was not developed.

The ATSDR has calculated chronic MRLs for cadmium based on human studies with measured exposures. The chronic oral MRL is 0.0001 mg/kg/day and the chronic inhalation MRL is 0.00001 mg/m^3 (Table 17.5). The average nonsmoking American absorbs approximately 1–3 μg of cadmium per day from the diet, which is only two to four times lower than the oral MRL.[102]

The EPA places cadmium in Group B1, a probable human carcinogen based on evidence in humans and animals.[19] The EPA's IUR is 1.8×10^{-3} per μg/m^3. The World Health Organization's IARC designated cadmium and cadmium compounds as carcinogenic to humans (Group 1) based on evidence of lung cancer (Table 17.5).[112,128] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Lead

Sources of Lead Exposure

Lead is the most widely used nonferrous metal. The usual valence state of lead in inorganic lead compounds is (II). Lead and lead compounds have been used in many industrial applications, including batteries, ammunition, paints and varnishes, gasoline, radiation shields, medical equipment, solder, glass, and ceramic glazes. Children are primarily exposed by ingestion. Lead poisoning in children caused by ingestion of lead paint was first noted in Australia and became recognized as a public health problem in the United States in the 1920s. Children have also been exposed by household renovation, fishing sinkers, imported lead-contaminated candy, and the use of imported ceramic dinnerware.[129–132] Adults are primarily exposed occupationally by inhalation. Excessive blood lead levels, greater than 40 μg/dL, have been reported in automobile radiator repair mechanics, and *take-home* lead is a potential source of elevated blood lead level in their children.[133] Excessive blood lead levels are also reported in other lead-related industries such as ceramics and furniture-stripping.[134]

A less common source of exposure is the lead in crystal that leaches into alcoholic beverages. Lead contents as high as 21.5 mg/L are reported in beverages stored in crystal decanters.[135] The lead content of various calcium supplements has been tested, and significant levels of lead was detected in over one-quarter of the 70 different brands examined.[136] Additionally, dissolution of retained lead gunshot has resulted in lead poisoning.[137]

The major sources of exposure have been reduced by the actions of federal agencies.[138] The EPA's phaseout of lead in gasoline started in 1973 and was completed in 1995. The EPA also banned lead in plumbing, fixtures, fittings, and solder.

In 1978, the U.S. Consumer Product Safety Commission banned the use of paint containing more than 0.06% lead by weight for interior/exterior residential surfaces, toys, and furniture. In 1995, FDA eliminated lead-solder in food cans. Subsequently, results of two National Health and Nutrition Examination Surveys, NHANES II (1976–1980) and NHANES III (phase I, 1988–1991; phase II, 1991–1994), indicate a substantial decline in blood lead level.[139–142] In 1994, it was estimated that 890,000 (4.4%) U.S. preschool children had a blood lead level of 10 μg/dL or higher.[140] Further reductions in blood lead level will require primary prevention efforts to reduce exposure to lead remaining in housing and soil.[143]

Adverse Health Effects of Lead

Early symptoms of chronic poisoning in children are often nonspecific, including headaches, anorexia, vomiting, and constipation. The adverse effects progress to anemia with basophilic stippling of red cells, Burton's line (a bluish grey line along the gums), chronic nephritis, peripheral neuropathy (manifested as wrist and/or foot drop), and radiographs of long bones revealing lead deposits. Frank encephalopathy occurs when blood lead concentrations are greater than 80 μg/dL and is characterized by ataxia, coma, convulsions, cerebral edema, and death. The long-term neurological consequences of childhood lead poisoning became well recognized in 1943 when Byers and Lord followed up 20 cases and found poor academic performance in all but one. In 1975, de la Burde and Choate reported school failure due to learning and behavioral problems in asymptomatic lead-exposed children. Asymptomatic children in first and second grade with elevated dentine lead levels scored lower on standardized tests, especially in areas measuring verbal performance and auditory processing. They were also more likely to exhibit disruptive behavior relative to controls.[144] Reexamined 11 years later as adolescents, those with greater lead exposure were more at risk for dropping out of school, reading disability, absenteeism, poor hand–eye coordination, and low scholastic class standing.[145,146]

In adults, early symptoms are often nonspecific and include fatigue, depression, sleep disturbance, anorexia, intermittent abdominal pain, nausea, constipation, diarrhea, and myalgia. Blood lead level is the single best diagnostic test for lead exposure. Animal experimentation and many epidemiologic studies suggest low increases in blood lead levels may elevate blood pressure, but the results are not definitive.[147,148] No consistent relationship between blood pressure and blood lead level was found after the examination of the NHANES III dataset.[149] However, in more recent studies, blood lead levels were significantly correlated with higher blood pressure among black men and women, but not white or Mexican-American participants.[150] Blood lead levels were also correlated with higher blood pressure among individuals working in the battery industry.[151] Other researchers suggest long-term lead accumulation, measured as bone lead, as opposed to blood lead level, which reflects recent exposure, may be associated with developing hypertension.

There is evidence of neurotoxicity, nephrotoxicity, and reproductive effects in adults. Reversible slowing of nerve conduction velocity has been observed at blood lead levels as low as 30 μg/dL and adverse effects on reaction time, mood, and visual–motor coordination at 30–50 μg/dL. Overt neurotoxicity, wrist drop, is reported at levels in excess of 80 μg/dL. Chronic irreversible nephropathy requires high and sustained exposure, but low-level lead exposure, blood lead level less than 10 μg/dL, is associated with renal impairment. Morphological alterations and decreases in sperm count, density, and motility have all been reported in heavily exposed males at blood lead level greater than 40 μg/dL.[152] Paternal occupational lead exposure has been reported to increase the risk of low birth weight and prematurity and to decrease cognitive scores in their children. Lead readily crosses the placenta to the fetus, and maternal blood lead levels in excess of 15 μg/dL are associated with low birth weights and preterm delivery, and blood lead in excess of 30 μg/dL, with spontaneous abortions.[153] Anemia is not seen until blood lead levels are in excess of 50 μg/dL.

Toxicokinetics of Lead

Inhalation and ingestion are the main routes of exposure for inorganic lead. In cases of occupational exposure, 35%–40% of inhaled lead dust or fume is deposited in the lungs with extensive (95%) blood absorption.[154,155] Children absorb 50% of an ingested dose through the gastrointestinal tract. In contrast, adults absorb 10% of an ingested dose, but gastrointestinal absorption will vary with solubility, nutritional status, fasting, and inversely with particle size. Elimination is mainly in the urine, with lesser amounts in the feces, sweat, hair, and nails.

Toxicodynamics of Lead

Lead's ability to substitute for divalent cations and bind sulfhydryl groups underlies systemic toxic effects that are manifested in, for example, the immune, cardiovascular, urinary, and nervous systems. The vulnerability of astrocytes to the toxic effects of lead is suggested as a key etiologic factor in lead-induced neurotoxicity. Astrocytes sequester lead up to a 55 times greater concentration than the surrounding extracellular fluid, and lead reduces astrocytes' glutamine synthetase activity in a dose-dependent manner. This compromises the ability to maintain extracellular levels of glutamate, a function crucial to homeostatsis.[156] There is also evidence to support the hypothesis that lead substitution for calcium in several intracellular regulatory pathways underlies some aspects of lead neurotoxicity. In this context, it is noteworthy that lead activates calcium calmodulin-dependent phosphodiesterase and calcium calmodulin inhibitor-sensitive potassium channels, and, at picomolar concentrations, lead activates calcium calmodulin-independent protein kinase C.[27]

Lead inhibits a large number of enzymes by binding to sulfhydryl groups. Lead enzyme inhibition is a component in some of the mechanisms proposed for lead-induced carcinogenesis. Possible mechanisms include inhibition of

regenerative repair, inhibition of DNA synthesis or repair, generation of reactive oxygen species with oxidative damage to DNA, substitution of lead for zinc in transcriptional regulators, interaction with DNA-binding proteins, and aberrant gene expression.[98]

Screening and Regulatory Levels of Lead

Since 1970, the CDC has repeatedly reduced the action level for blood lead. As new information has emerged about the neurological, reproductive, and possible hypertensive toxicity of lead, and as parameters that are more sensitive are developed, the blood lead levels of concern for lead exposure have been progressively lowered by the CDC from 70 to 10 µg/dL (Figure 17.6).

Although the CDC set 10 µg/dL as the blood lead level of concern in 1991, this is not interpreted to mean there are no adverse effects below this level. Cognitive deficits are associated with blood lead levels below 5 µg/dL.[157] There is evidence to support that there is no threshold for the toxic effects of lead.[158–160] In this context, it is important to note that in January 2012, a committee of experts recommended that the CDC establish a reference value of 5 µg/dL based on the 97.5 percentile distribution of blood lead levels among children aged 1–5.[161] The committee also recommended that the CDC replace the term *blood lead level of concern* because it has the potential to be misinterpreted as a toxicity threshold.

Because the adverse health effects associated with lead occur at blood lead levels so low as to be without a threshold, the EPA considers it inappropriate to derive toxicity values for lead.[19] However, the EPA did derive an RfD for tetraethyllead, 0.0001 µg/kg/day (Table 17.5). The ATSDR has not derived a chronic oral MRL or a chronic inhalation MRL for lead (Table 17.5). Although the EPA classifies lead as a Group B2 probable human carcinogen, the agency considers it inappropriate to quantify the carcinogenic risk from oral exposure because toxicokinetic differences between humans and animals cannot be taken into account using the standard procedures. IARC considers inorganic lead compounds as *probably carcinogenic to humans* (Group 2A) and organic lead as *not classifiable as to their carcinogenicity to humans* (Group 3).[162] These screening values and cancer classifications are shown in Table 17.5. Epidemiological data suggest a role for lead in human carcinogenicity.[163] Lead has been suggested to play a *facilitative* role in carcinogenicity by inhibiting DNA repair and/or otherwise enhancing the DNA damage of other genotoxic compounds.[164]

The NIOSH REL for metallic lead, lead oxides, and lead salts (including lead soaps but excluding lead arsenate) is 0.050 mg/m^3. NIOSH further recommends that air concentrations be maintained so that worker blood lead levels remains below 0.060 mg/dL. The OSHA PEL for lead is also 0.050 mg/m^3. However, OSHA considers *lead* to mean all inorganic lead compounds (lead oxides and lead salts) and the class of lead compounds called soaps. All other lead compounds are excluded from the OSHA definition. The NIOSH REL is an advisory level while the OSHA PEL is a regulatory level.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Mercury

Sources of Mercury Exposure

Mercury is a silver-white fluid trace metal found in igneous and sedimentary rocks and as mercury sulfide in cinnabar ore. Mercury occurs naturally as elemental mercury and as inorganic and organic mercury compounds. Release of elemental mercury vapor from the earth's crust with direct discharges to the atmosphere is the major natural source of mercury in the environment. Other important sources include smelting, gold refining, cement production, and burning of fossil fuel. Industrial applications of mercury include its use as a cathode in the electrolysis of sodium chloride. Mercury has also been used in pesticides. Agricultural products treated with mercurial fungicides are a major dietary source of mercury compounds. Atmospheric fallout from burning fossil fuels deposits inorganic mercury compounds in soil and water where it may be methylated by the microflora to form methylmercury. Edible fish can concentrate methylmercury. Fish harvested from mercury-polluted water

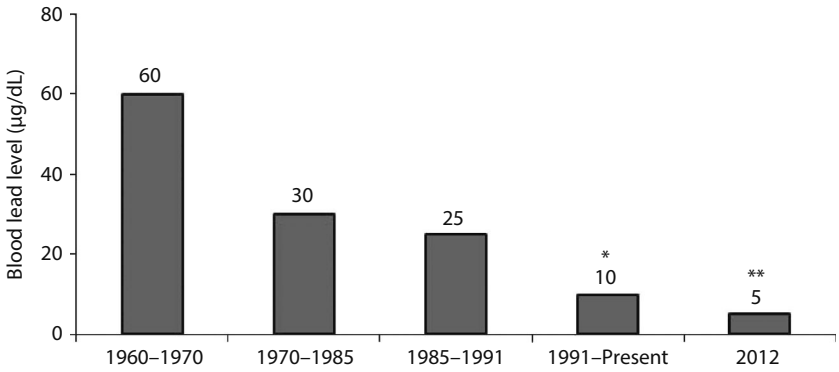

FIGURE 17.6 The graphic illustrates the progressive lowering of CDC-recommended action level for blood lead in children. *The current CDC-recommended blood lead action level is 10 µg/dL. **In 2012, the CDC proposed to reduce the action level to 5 µg/dL.

is a major source of methylmercury in the diet. Due to the potential for environmental release, the use of mercury in some commercial products, for example, paint and batteries, is now restricted.[27]

The use of dental amalgam fillings has generated concern, both because exposure of dental workers may exceed occupational standards and because a variety of illnesses in the general public, including developmental, neuronal, and renal illnesses, have been attributed to dental mercury exposure.[165,166] Conditions other than contact dermatitis have not been firmly linked to dental amalgam.[167–169] Whether amalgam should continue to be used for new dental fillings remains controversial[170] even in the face of reviews that supported its safety.[171,172] Additional research is needed before the potential for dental amalgams to cause harm, and the benefit of substituting more costly or less durable materials, can be fully evaluated. The evaluation must include the potential risk of effects on the fetus.[173] Mercury is also present in the pharmaceutical preservative thimerosal. The scientific evidence accumulated to date does not support a causal association between autism and exposure to thimerosal as a component of vaccines.[174]

Adverse Health Effects of Mercury

The CNS is the critical organ for mercury vapor exposure. Acute inhalation exposure to mercury vapor irritates the respiratory tract and may be followed by chest pains, dyspnea, coughing, hemoptysis, and interstitial pneumonitis leading to death. Low-level exposure gives rise to psychotic reactions characterized by delirium, hallucinations, and suicidal tendency. Chronic exposure to the vapors produces CNS toxicity, including muscle weakness and tremors, nervousness, memory loss, and anorexia. Occupational exposure to metallic mercury has long been associated with the development of proteinuria. Acute elemental mercury ingestion is usually of no significance due to poor absorption from the gastrointestinal tract. Inorganic mercury compounds generally demonstrate local irritant or corrosive activity. The kidney is the critical organ following the ingestion of inorganic divalent mercury salts. Ingestion of mercuric compounds, for example, mercuric chloride, can cause ulcerative gastroenteritis and tubular necrosis leading to death. Chronic effects of inorganic mercury compounds produce CNS toxicity similar to that noted for elemental mercury.[175]

Methylmercury is neurotoxic and the effects are both dose- and time-dependent. Ataxia is an early symptom followed by slurred speech, weakness, vision and hearing loss, tremors, coma, and death. Well-documented poisonings from contaminated fish and grains occurred in Japan and Iraq.[176,177] Additionally, methylmercury is a well-known neuroteratogen. The fetus is more sensitive to the toxic effects of methylmercury than adults. In the fetus, it affects normal neuronal development, leading to altered brain architecture and decreased brain size. Contact dermatitis may occur to both inorganic and organic mercurials with cross-sensitivity to each being reported.[173]

Toxicokinetics of Mercury

The primary route through which the general public is exposed to mercury is the diet, and the primary food is fish and fish products. About 15% of inorganic divalent mercury is absorbed from the gastrointestinal tract in adults and retained in body tissues. Inorganic mercury is nonuniformly distributed after absorption. The highest concentration is found in the kidneys. Mercury and mercury compounds are excreted in urine, feces, and through respiration. The oxidation of inhaled metallic mercury vapor to divalent mercury takes place in red blood cells soon after absorption. Some elemental mercury remains dissolved in the blood long enough for it to be carried to the blood–brain barrier and the placenta. Because of the short transit time from the lung to the brain, about 97% arrives unoxidized. Its lipid solubility and high diffusibility allow rapid transit across the blood–brain barrier and placenta. Oxidation of the mercury vapor in brain and fetal tissues converts it to the ionic form, which is much less likely to cross back over the blood–brain and placental barriers. Results of both human and animal studies indicate that about 80% of metallic mercury vapor is retained by the body, whereas liquid metallic mercury is poorly absorbed in the gastrointestinal tract. Methylmercury in the diet is almost completely absorbed into the bloodstream and distributed to all tissues.[27] The uptake of methylmercury is thought to proceed through a process where it complexes with cysteine in the gastrointestinal tract and is then transported from the blood into endothelial cells by the same amino acid transporter that normally transports methionine. Uptake by the transporter is thought to occur because the methylmercury–cysteine complex is so similar in structure to methionine (Figure 17.7).

Toxicodynamics of Mercury

The toxicity of mercury is mediated through its covalent binding and inactivation of normal cellular functions. Divalent mercury binds to sulfhydryl groups, as well as to carboxyl, amide, amine, and phosphoryl groups.

The systemic toxicity of mercury compounds is attributable to the wide variety of cellular ligands that are potentially affected. For example, divalent mercury ions form coordination or chelation complexes with erythrocytes, causing agglutination and hemolysis. Also, divalent mercury can accumulate in liver lysosomes and, as concentrations increase, the lysosomes rupture and release destructive acid hydrolases.[27,178] The neurotoxic effect seen after exposure to metallic mercury vapor is attributable to the divalent mercury ion formed through oxidation in the brain tissue. One possible mechanism is the divalent ions' interference with enzyme function by binding to sulfhydryl groups. Transport through the cell membrane via the formation of carrier complexes, such as sodium and calcium channels, is also a possibility although this has not been demonstrated.

The inhibition of protein synthesis is one of the earliest detectable biochemical effects of methylmercury in the adult brain. Methylmercury can also react directly with important receptors in the nervous system, as shown by its effect on

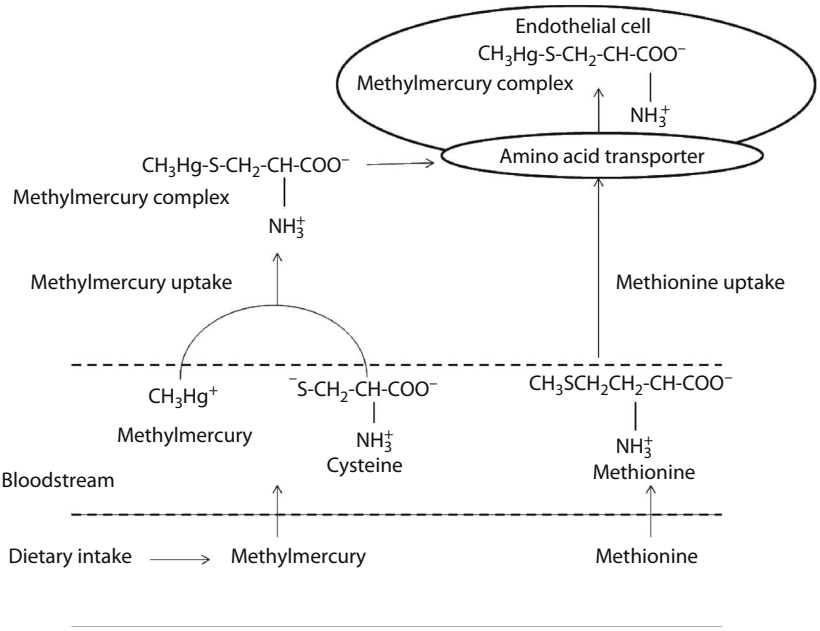

FIGURE 17.7 The complex formed when methylmercury binds cysteine has a similar structure to methionine. One of the ways cellular uptake of the methylmercury–cysteine complex is thought to proceed is through a process where it is transported into the cell by the same amino acid transporter that normally transports methionine.

acetylcholine receptors in the peripheral nerves. It has also been suggested that the carbon–mercury bond in methylmercury undergoes homolytic cleavage to release methyl free radicals that peroxidize lipid constituents of neurons.[27]

Screening and Regulatory Levels of Mercury

The EPA's RfD and the ATSDR's chronic oral toxicity MRL for methylmercury are shown in Table 17.5. Neither agency has derived chronic oral toxicity values for elemental mercury. The EPA's cancer assessment for oral exposure to methylmercury places it in Group C. IARC classifies methylmercury as Group 2B, possibly carcinogenic to humans. IARC places metallic mercury and inorganic mercury compounds in Group 3, not classifiable as to their carcinogenicity to humans.[162] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

OTHER TOXIC METALS WITH RfDs

Aluminum

Sources of Exposure to Aluminum

Aluminum is an abundant element in the earth's crust and occurs in the form of silicates, oxides, and hydroxides, combined with other elements such as sodium and fluorine and as complexes with organic matter. It is not found as a free metal because of its reactivity. It is extracted from bauxite ore. It is highly concentrated in soil-derived dusts from such activities as mining and agriculture, and in particulate matter from coal combustion. Aluminum metal has a wide variety of uses including structural materials for construction automobiles and aircraft and in the production of metal alloys. Compounds of aluminum have a wide variety of uses, including production of glass, ceramics, rubber, wood preservatives, pharmaceuticals, and waterproofing textiles. Natural aluminum minerals, especially bentonite and zeolite, are used in water purification, sugar refining, brewing, and paper industries. Aluminum is released to the environment both by natural processes and from anthropogenic sources. It is naturally present at low levels in most foods, but the primary source of dietary aluminum is from food additives.[27,179] On average, American adults consume 2–25 mg of aluminum daily from food and beverages, with average amounts being 8.2 mg/day for males and 7.1 mg/day for females.[180] Over-the-counter antacids contain large amounts of aluminum hydroxide, and millions of consumers are dermally exposed to aluminum salts from the use of antiperspirants.[181]

Adverse Health Effects of Aluminum

Large oral doses of aluminum results in a toxic syndrome of gastrointestinal tract irritation and interference with phosphate absorption. Industrial exposure to high concentrations of aluminum-containing airborne dusts has resulted in a number of cases of occupational pneumoconiosis. Most of these exposures were chronic and other substances were involved in nearly all instances. It has been hypothesized that aluminum is a risk factor for Alzheimer's disease. However, epidemiologic studies do not show an association between occupational aluminum exposure and the incidence of Alzheimer's disease. Canadian miners, between 1944 and 1979, were exposed to high concentrations of aluminum and aluminum oxide powder preceding each shift as

a prophylactic treatment against silica lung disease. In an initial study of this population, there was no increased incidence of neurological disorders in exposed miners, but there was an increase in neurological impairment as measured by cognitive testing.[182] A follow-up study was conducted to address several methodological weaknesses in the initial study. No statistically significant differences were noted between exposed and nonexposed miners in either neurological disease or cognitive impairment incidence. A more recent study showed no association between occupational exposure to aluminum and the incidence of Alzheimer's disease.[183] Similarly, a methodological improved follow-up study, to an earlier study that found an association between aluminum in drinking water and the incidence of Alzheimer's disease, found no evidence of an association.[184] However, in early investigations, injection of aluminum salts into the brain of rabbits resulted in the development of neurofibrillary tangles, which are indicators of Alzheimer's disease.[185] While a direct causal role for aluminum has not been definitively demonstrated, epidemiological evidence suggests that elevated levels of aluminum in the brain may be linked to the development or progression of Alzheimer's.[186,187] Aluminum is certainly neurotoxic. In renal dialysis patients, excessive parenteral exposure to aluminum can cause a progressive, fatal neurological syndrome known as dialysis dementia.[188]

Toxicokinetics of Aluminum

Aluminum salts are absorbed in small amount from the lung and gastrointestinal tract. The plasma proteins bind and accumulate aluminum. One day postinjection of humans with aluminum citrate, 99% of the aluminum remaining in the circulation was found in the plasma, with 80% of that fraction largely bound to transferrin, 10% bound to albumin, and the remainder associated with lower molecular weight proteins. This plasma protein binding of aluminum is saturable. During dialysis with aluminum-containing dialysate, plasma aluminum levels reach a plateau. Aluminum is widely distributed to the organs, including the lung, liver, bone, muscles, and brain. The highest levels have been found in lung tissue. The marked protein binding of aluminum in plasma leaves only a small fraction of the total aluminum available for filtration in the kidney. Renal clearance of aluminum is approximately 5%–10% of that of urea or creatinine clearance.

Toxicodynamics of Aluminum

In a 1977 study, a positive correlation between increased serum parathyroid hormone and serum aluminum levels in dialysis patients was reported.[189] Studies suggest that aluminum distribution in tissue can be influenced by increasing the concentration of parathyroid hormone. The absorption of aluminum from the gastrointestinal tract of rats was enhanced by injections of parathyroid hormone with increased deposition of the metal in the kidney, muscle, bone, and the gray matter of the brain, but not in the liver or in the white matter of the brain. Thus, the parathyroid hormone exerted a specific effect on the absorption and distribution of aluminum.[190] There is some evidence implicating aluminum as an important factor in downregulation of neuronal protein metabolism. It is hypothesized that aluminum exposure may cause Alzheimer's disease by electrostatically cross-linking proteins, such as the methionine-containing histone H1, and DNA.[191]

Advisory, Screening, and Regulatory Levels for Aluminum

The EPA has not derived an RfD for aluminum but has derived one for aluminum phosphide, 0.0004 mg/kg/day. The ATSDR's chronic oral MRL for aluminum is 1 mg/kg/day.[21] The NIOSH REL for total aluminum exposure is 10 mg/m^3. OSHA's PEL for total aluminum is 15 mg/m^3. The REL and PEL for respirable aluminum are both 5 mg/m^3. OSHA has not established a PEL for aluminum welding fumes; the REL is 5 mg/m^3. Limits are also established for alumina, and soluble salts of aluminum.[20] Neither the EPA nor IARC has fully evaluated the carcinogenicity of aluminum. The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Antimony

Sources of Antimony Exposure

Antimony is a brittle silver-colored metal extracted from ores. Antimony trioxide is primarily used as a flame retardant in rubber, plastics, pigments, adhesives, textiles, and paper. The metal hydride of antimony is a colorless highly toxic gas used in the manufacture of semiconductors.[59] Pentavalent antimonials have been used as a therapeutic for the treatment of leishmaniasis and schistosomiasis.[27]

Adverse Health Effects of Antimony

Ingestion of antimony compounds is associated with gastrointestinal, cardiac, dermatological, hepatic, and neurological toxicity in humans and animals. Acute inhalation exposure to antimony trichloride or antimony pentachloride can cause pneumonitis. Long-term inhalation exposure to antimony can cause benign pneumoconiosis and may raise blood pressure. Dermatological reactions to antimony include eczema and pustules. These effects exhibit signs of an acute inflammatory response but do not appear to be an allergic reaction. The major toxic side effects associated with parenterally administered antimonials, therapeutics used to treat leishmaniasis and schistosomiasis, are cardiotoxicity and pancreatitis.[192]

Both antimony (III) and antimony (V) compounds are generally negative in nonmammalian genotoxicity tests, while mammalian test systems have returned positive results for antimony (III) but not antimony (V) compounds. Assessments of chromosome aberrations have been inconsistent. IARC concluded that animal carcinogenicity data were sufficient for Sb_2O_3 to be classified as possibly carcinogenic to humans.[57,193]

Toxicokinetics of Antimony

The distribution of antimony is not homogeneous within organs or tissues and is dependent on valence state and exposure route. For example, antimony (III) is concentrated in red blood cells and liver, whereas the antimony (V) is primarily concentrated in the plasma. After acute oral, chronic oral, or parenteral exposure to antimony, the highest organ concentrations are found in the thyroid, adrenals, liver, and kidney. Antimony absorbed via the lung may have a longer biological half-time. At the autopsy of deceased antimony smelter workers, high levels were found in the lung and bone tissues but not in liver or kidney. The intestinal absorption of antimony (III) is lower than the pentavalent compound. Both tri- and pentavalent antimonials are excreted in feces and urine.[27]

Toxicodynamics of Antimony

The therapeutic mode of action of pentavalent antimonials is still being investigated. However, there are data that suggest direct involvement of antimony (V). Other data suggest that thiols and ribonucleosides may also participate in the therapeutic mode of action.[194] As with other metals, the mechanisms for the toxic effects of antimony compounds include binding to sulfhydryl groups and inhibiting protein and carbohydrate metabolism. Antimony (III) has also been shown to be a potent inducer of liver and kidney heme oxygenase, the rate-limiting enzyme in heme degradation, in both organs.[27] The cancer mechanisms of action are thought to include production of active oxygen species and indirect interference with DNA repair systems.[57]

Screening and Regulatory Levels for Antimony

The EPA has derived a chronic oral RfD of 0.4 μg/kg/day. The EPA has not derived an RfC, and the ATSDR has not derived either a chronic oral or inhalation MRL (Table 17.5). Antimony has not been evaluated by the EPA for its potential to cause cancer in humans.[19,195] The IARC classification of antimony trioxide is Group 2B, possibly carcinogenic to humans via inhalation and ingestion. The Group 2B classification is based on inadequate evidence of carcinogenicity in humans and sufficient evidence of carcinogenicity in experimental animals.[193] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Barium

Sources of Barium Exposure

Barium is a silvery-white alkaline earth metal and is found in nature in combination with other elements.[196] Barium compounds are used primarily as lubricating agents in drilling muds, but also in the manufacture of paints, bricks, tiles, glass, rubber, and pesticides. Barium sulfate is safely used medically as a contrast agent in x-ray diagnosis because it is not efficiently absorbed across the gastrointestinal lumen. Occupational exposure to barium may occur through inhalation and dermal contact at workplaces where it is produced or used. The general population may be exposed to barium via inhalation of ambient air and ingestion of food and drinking water.[27]

Adverse Health Effects of Barium

The barium ion is highly reactive, and its toxicity is dependent on the solubility of the specific compound, with water-soluble compounds, for example, chloride, hydroxide, and nitrate, being more toxic than insoluble compounds, for example, sulfate and carbonate. Soluble barium compounds may cause local irritation of the eyes, nose, throat, bronchial tubes, and skin. The acute effects of barium ingestion in animals include salivation, nausea, diarrhea, tachycardia, hypokalemia, twitching, flaccid paralysis of skeletal muscle, respiratory muscle paralysis, and ventricular fibrillation.[27,197] High-dose human exposure to barium consistently results in adverse effects that include ventricular tachycardia, hypertension and/or hypotension, and muscle weakness and paralysis. Hypokalemia is reported in a number of individuals exposed to high doses of barium.[198] Ingestion of gram quantities of soluble barium salts may be fatal, with death resulting from cardiac arrest. Various studies have demonstrated the detrimental effect of barium on ventricular automaticity and pacemaker current in the heart. Symptoms of poisoning start with the gastrointestinal muscles leading to nausea, vomiting, and diarrhea. This progresses to the skeletal and cardiac muscles with ventricular fibrillation followed by death due to respiratory muscle paralysis. Occupational exposure to inhaled barium sulfate can cause a benign pneumoconiosis (baritosis), which resolves with cessation of exposure.[27] A dose–response assessment of NOAEL and LOAEL values determined that kidney effects are the most sensitive endpoint for adverse health effects related to chronic soluble barium ingestion in mammals.[16]

Toxicokinetics of Barium

Exposure to barium can occur through the air and diet. About 90% of barium is concentrated in the bone; the remainder goes to soft tissues such as the aorta, brain, heart, kidney, spleen, pancreas, and lung. The highest concentration of barium in soft tissues of humans was found in the large intestine, muscle, and lung. Barium deposition in the bone occurred preferentially in the active sites of bone growth. Inhaled barium can be absorbed through the lung or directly from the nasal membrane into the blood. Barium is eliminated in the urine and feces, the rates varying with the route of administration.[27]

Toxicodynamics of Barium

There is evidence that many of the toxic effects of barium are due to increases in intracellular potassium levels and extracellular hypokalemia. Barium is a competitive potassium channel antagonist that blocks the passive efflux of intracellular potassium, resulting in a shift of potassium from extracellular to intracellular compartments. The intracellular translocation of potassium results in a decreased resting membrane

potential, making the muscle fibers electrically unexcitable and causing paralysis. Barium also possesses chemical and physiological properties that allow it to compete with and replace calcium. This particularly affects functions related to the release of adrenal catecholamines and neurotransmitters such as acetylcholine and norepinephrine.[27]

Screening and Regulatory Levels for Barium

The EPA has derived an oral RfD for barium, 0.2 mg/kg/day. The ATSDR's oral MRL is also 0.2 mg/kg/day. Neither a chronic inhalation MRL nor an RfC is established. Under the EPA's 1986 Guidelines for Carcinogen Risk Assessment, barium is placed in Group D, not classifiable as to human carcinogenicity. Under the proposed guidelines for carcinogenic risk assessment, barium is considered not likely to be carcinogenic to humans following oral exposure, and its carcinogenic potential cannot be determined following inhalation exposure (Table 17.6).[19] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Beryllium

Sources of Beryllium Exposure

Beryllium is an alkaline earth metal and is extracted primarily from beryllium-silicate ore and beryllium-aluminum oxide-silicate ore. The primary uses of beryllium are as a structural metal in lightweight applications, in metal alloys, and in nuclear reactor technology. The most probable human exposure is occupational exposure. Among workers who may be exposed to beryllium are the ore miners, beryllium alloy makers and fabricators, phosphorus manufacturers, ceramic workers, missile technicians, nuclear reactor workers, electric and electronic equipment workers, and jewelers. Occupational exposure to beryllium may be through inhalation of airborne dust and dermal contact with beryllium. Environmental monitoring data indicate that the general population will be exposed to beryllium via inhalation of ambient air and ingestion of contaminated food and drinking water.[27]

Adverse Health Effects of Beryllium

Although beryllium and its compounds can cause contact dermatitis, the primary target organ is the lung. Two types of beryllium-induced lung injury can occur: acute and chronic. Chronic beryllium disease was first reported in 1946 as a delayed pneumonitis. The disease, berylliosis, is characterized by granuloma formation, fibrosis, emphysema, reduction in vital capacity of the lung, and reduced total lung capacity.[199] The chronic disease has two forms: one that occurs during exposure and the other that becomes evident 10 or more years after the cessation of exposure. The mechanism of the delayed onset is not known. The lack of a dose–response relationship between the extent of exposure and development of the disease, long latency period between

TABLE 17.6
Examples of Advisory Chronic Toxicity Values and Cancer Classifications for Toxic Metals

U.S. Toxicity Values for Less Toxic Metals

	Chronic Oral Toxicity		Chronic Inhalation Toxicity		Carcinogenicity		
Metal	MRL (mg/kg/day)	RfD (mg/kg/day)	MRL (mg/m^3)	RfC (mg/m^3)	Oral Slope Factor (mg/kg/day)	Inhalation Unit Risk (μg/m^3)	Cancer Classifications[a]
Antimony	—	0.0004	—	—	—	—	—
Antimony trioxide	—	—	—	0.0002	—	—	2B-IARC
Aluminum	1	—	—	—	—	—	—
Aluminum phosphide	—	0.0004	—	—	—	—	—
Beryllium	0.002	0.002	—	0.00002	—	0.0024[b]	B1-EPA
Uranium-soluble salts	—	0.003	0.00004	—	—	—	—
Uranium	—	—	0.0008	—	—	—	—
Silver	—	0.005	—	—	—	—	D-EPA
Vanadium pentoxide	—	0.009	0.0001	—	—	—	2B-IARC
Nickel-soluble salt	—	0.02	0.00009	—	—	—	—
Nickel refinery dust	—	—	0.00009	—	—	0.00024	A-EPA
Nickel subsulfide	—	—	0.00009	—	—	0.00048	A-EPA
Barium	0.2	0.2	—	—	—	—	D-EPA
Boron	—	0.2	—	—	—	—	—
Strontium	—	0.6	—	—	—	—	—

[a] EPA Group A—known human carcinogen, EPA Group B1—probable human carcinogen (limited human data), EPA Group D—not classifiable as to human carcinogenicity, IARC Group 2B—possibly carcinogenic to humans.

[b] The unit risk should not be used if the air concentration exceeds 4 μg/m^3, since above this concentration, the unit risk may not be appropriate.

exposure and onset, and the low incidence among beryllium-exposed individuals suggest that the disease is immune mediated. Chelation treatment has little effect on the course of the disease whereas corticosteroid treatment has been effective in disease suppression.[27,59]

Beryllium dermatitis is a hypersensitivity reaction that is usually noted 1–2 weeks after exposure to soluble beryllium salts. Patch tests of individuals with soluble beryllium salts provoke a positive response. Beryllium can also induce dermal ulceration if particles of beryllium salts become imbedded in the skin. The ulceration can be long-lasting and surgical intervention may be required to resolve the condition. Beryllium compounds are also carcinogenic.[59] Based on the limited evidence of carcinogenicity in humans exposed to airborne beryllium and sufficient evidence of carcinogenicity in animals, beryllium is classified as a B1 probable human carcinogen.[19]

Toxicokinetics of Beryllium

The lungs and pulmonary lymph nodes retain inhaled beryllium. Ingested beryllium is retained in the bone. Urinary excretion of beryllium occurs slowly. Beryllium in plasma is not filtered by the glomerulus. Rather, it is excreted into the tubule, a process that damages the tubular epithelium.[85]

Toxicodynamics of Beryllium

The pathogenesis of chronic beryllium disease involves a beryllium-specific delayed-type hypersensitivity reaction. It is thought that beryllium acts as an antigen, either alone or as a hapten, to stimulate beryllium-specific CD4+ cells proliferation via the interleukin-2 receptor pathway. Lymphokines are released by activated lymphocytes and may initiate and perpetuate the formation of granulomas and lead to macrophage recruitment. Transport of beryllium itself or beryllium as a hapten to sites outside the lung most likely accounts for extrapulmonary granulomas, possibly caused by local beryllium-specific CD4+ cells. This mechanism would account for the universal finding of pulmonary involvement in beryllium disease, with variable evidence of granulomas outside the lung. Beryllium has been detected many years after the last exposure in tissues, including lung, thoracic lymph nodes, liver, spleen, skin, and muscle.

Advisory, Screening, and Regulatory Levels for Beryllium

The EPA has established an RfD for beryllium of 2 μg/kg/day, an RfC of 0.02 $\mu g/m^3$, and an IUR level of 0.0024 per $\mu g/m^3$. The agency classifies beryllium as a Group B1 probable human carcinogen via inhalation. The EPA states that there are insufficient data to determine the carcinogenicity of beryllium when ingested (Table 17.6). IARC reviewed the available literature and, in 1993, published its findings that there was sufficient evidence in humans and animals for the carcinogenicity of beryllium.[112] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Boron

Sources of Boron Exposure

Boron is a solid element that always occurs in nature bound to oxygen in the form of inorganic borates. Boron enters the environment mainly through the weathering of rocks, boric acid volatilization from seawater, and volcanic activity. Boron and associated compounds have many industrial applications, including the production of borosilicate glass, laundry bleaches, wood preservatives, fire retardants, pesticides, fertilizers, cosmetics, and pharmaceuticals. Anthropogenic sources of boron in the environment include agriculture, wood burning, power generation using coal and oil, glass manufacturing, mining, leaching of treated wood, and sewage disposal.[27,200] People working in related industries are exposed occupationally. The general public's exposure is primarily dietary. Boron is an essential plant nutrient and occurs naturally in fruits and vegetables. In the adult American population, the daily median boron intake is 1 mg/day.[201]

Adverse Health Effects of Boron

The oral lethal dose in adults is reported as 15–20 g, but 80–297 g has been tolerated in a single ingestion.[202,203] The progression of effects of boron toxicity is hemorrhagic gastroenteritis with weakness, lethargy, headache, restlessness, and tremors. Frequently, the skin shows signs of erythema with a boiled lobster appearance and exfoliation.[204,205] Shock syndrome may proceed with cold clammy skin, cyanosis, slow pulse, and low blood pressure. Signs of kidney injury or liver damage may be reported. Death generally occurs several days after ingestion and results from renal toxicity, circulatory collapse, and shock.

Animal experiments indicate that chronic oral exposure to boric acid or borax is toxic to the male reproductive system with testicular lesions being observed in rats, dogs, and mice.[206,207] The available data suggest that the Sertoli cells are the primary site of reproductive toxicity. Boric acid is a developmental toxicant in all three of these mammalian species with the most sensitive endpoints being decreased fetal body weight and malformations/variations of the ribs.[207,208] At present, there are insufficient human data to determine if boron causes male reproductive toxicity in humans, but boric acid is considered a high-priority chemical for study with respect to human reproductive health.[209,210]

Toxicokinetics of Boron

Borates are absorbed from mucous membranes and abraded skin, but not from intact or unbroken skin.[211] Most dietary boron is hydrolyzed within the gut to yield $B(OH)_3$, which, as a neutral compound, is easily absorbed. Borate excretion occurs mainly through kidneys. Pharmacokinetic data indicate that boron, administered as boric acid, is absorbed rapidly and virtually completely from the gastrointestinal tract, as evidenced by recovery of greater than 90% in urine. Regardless of the compound or route of exposure, once in the body, boron forms weak complexes with hydroxyl, amino, and thiol groups.[212]

Toxicodynamics of Boron

The mechanism of boron absorption has not been studied, but a passive, nonmediated diffusion process involving $B(OH)_3$ is likely. Because boron forms labile complexes in aqueous solution, transport is probably as free boric acid rather than a complex.[213] Biochemical and physiological consequences of boron deprivation in humans suggest it affects calcium and magnesium metabolism.[214]

Screening and Regulatory Levels for Boron

Borates have long been known to be essential for plants, but a specific biochemical role remains to be determined.[215] Although boron deficiency has been reported in rats, chickens, and humans, as of yet, no requirement has been established in humans. However, a boron UL was set for adults at 20 mg/day.[8,213,214] The EPA's RfD for boron is 0.2 mg/kg/day. Decreased fetal body weight was considered the critical effect. The ATSDR has not established chronic MRLs for boron (Table 17.6). An NTP 2-year carcinogenesis bioassay found no evidence of carcinogenicity.[216] Under the EPA's draft revised guidelines for carcinogen risk assessment, the data are considered inadequate for an assessment of the human carcinogenic potential of boron.[16] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Nickel

Sources of Nickel Exposure

Nickel occurs naturally in soils, sediments, water, air, plants, and animals. Large amounts of nickel are released into the environment as a result of such natural phenomena as volcanic eruptions or from industrial activities, including mining, alloy production, power plants, and incinerators operations. Human exposure to nickel occurs via inhalation of dust particles, ingestion of contaminated food and water, and dermal contact with nickel-containing materials or soil. Nickel is used in a wide variety of applications, with 80% of the nickel in the United States being used in the production of nickel metal and alloys.[27,59]

Adverse Health Effects of Nickel

Animal experiments have indicated that nickel compounds can be nephrotoxic, hepatotoxic, immunotoxic, and teratogenic. The allergic reaction to nickel is the most common adverse health effect in humans.[27] Allergic asthma is rare but case reports have been published.[59] Acute inhalation exposure to metallic nickel can cause metal fume fever.[217] Nickel carbonyl is a colorless and volatile liquid that is particularly hazardous. It has been estimated that exposure to 30 parts per million nickel carbonyl for 30 min may be lethal in humans.[218] Acute inhalation exposure to this material can cause immediate and delayed toxic effects. Headache, dizziness, and nausea are the immediate manifestations. Ten to thirty-six hours after exposure, substernal pain, coughing, and dyspnea, consistent with chemical pneumonitis, are observed. Recovery is protracted and is characterized by fatigue upon slight exertion. Short-term exposure to 150 parts per billion $Ni(CO)_4$ can cause immediate, but not delayed, symptoms, whereas short-term exposure to concentrations on the order of a few parts per million can cause the more severe delayed-type reactions.[59] Inhalation of nickel compounds can cause lung cancer. Epidemiologic studies show the association between occupational exposure to nickel refinery dust and nickel subsulfide and lung and nasal cancer.[19] The latency period for nickel-induced lung cancer is 13–14 years, and for nasal cancer, it was 15–24 years after first employment.[59]

Toxicokinetics of Nickel

Inhalation, dermal contact, and ingestion are the primary routes of exposure to nickel. Nickel metal is poorly absorbed by the skin, but compounds such as $NiCl_2$ and $NiSO_4$ can penetrate occluded skin.[219] The deposition of nickel particles into the nasopharyngeal, pulmonary, or bronchial regions of the respiratory tract is dependent on the particle size. Water-insoluble nickel compounds enter cells by phagocytosis and are contained in cytoplasmic vacuoles, which are acidified, thus accelerating the dissolution of soluble nickel from the particle.[27]

Toxicodynamics of Nickel

Like the other metal carcinogens, nickel-induced carcinogenicity involves multiple molecular mechanisms. The proposed mechanisms of nickel-induced carcinogenesis include a chain of events where nickel compounds enter the cell, triggering intracellular calcium ion mobilization with the induction of the hypoxia-inducible factor-mediated pathways. Also proposed are pathways where nickel enters the nucleus, directly binds to DNA, forms reactive nickel–oxygen complexes that oxidatize thymine and cytosine, and forms 8-hydroxyguanidine. It is proposed too that the genes located near nickel-damaged heterochromatin are silenced resulting in epigenetic loss of histone acetylation and DNA hypermethylation leading to increased susceptibility to neoplastic transformation. Nickel compounds can also downregulate the p53 tumor suppressor gene, activate the c-Myc proto-oncogene, and induce the AP-1 transcription factor, resulting in cellular proliferation and cancer development.[219]

Advisory, Screening, and Regulatory Levels for Nickel

The essentiality of nickel in humans was assessed in 2001 with a decision not to establish DRI values.[213] However, a UL was established for nickel, 1 mg/day. The only toxicity factor that the EPA has derived for the soluble salts of nickel is the RfD, 20 μg/kg/day (Table 17.6). Neither an RfD nor an RfC is established for nickel subsulfide, nickel refinery dust, or nickel carbonyl. The ATSDR established a chronic inhalation MRL of 0.00009 mg/m^3 for nickel refinery dust and nickel-soluble salts. The EPA classified nickel subsulfide and nickel refinery dust as Group A human carcinogens on the basis of animal and epidemiologic carcinogenicity data. The EPA places nickel carbonyl in Group B2, probable human

carcinogen, and neither a slope factor nor an IUR is established. IARC places nickel compounds in Group 1, carcinogenic to humans, and metallic nickel in Group 2B, possibly carcinogenic to humans.[220] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Silver

Sources of Silver Exposure

Alloys of silver are used in jewelry, tableware, photographic materials, electronics, dental products, and as topical antibacterial agents for the treatment of burn wounds.[59,221] Occupational exposure occurs during mining and silver metallurgy. Occupational exposure to silver may occur through inhalation of dust and dermal contact with elemental silver and silver compounds.

Adverse Health Effects of Silver

Silver is generally low in toxicity. However, silver nitrate is corrosive and a large acute dose can cause severe gastroenteritis, shock, convulsions, and death.[27] Chronic exposure to silver results in argyria, a blue-gray discoloration of the skin, mucous membranes, and eyes. Silver sulfadiazine, used in the management of burn wound sepsis, has resulted in argyria, ocular injury, leucopenia, and toxicity in kidney, liver, and neurological tissues. Silver may affect the immune system and contact dermatitis has been observed following exposure to various silver compounds.[222]

Toxicokinetics of Silver

Elemental silver is absorbed following inhalation, ingestion, or topical application. Excretion from oral, respiratory, or topical exposure is primarily through the gastrointestinal tract. Mucociliary escalator activity accounts for removal of silver following respiratory exposure. Clearance of radioactive silver metal dust in a man who was accidentally exposed illustrated the rapid removal of silver from the lungs primarily by ciliary action, with subsequent ingestion and ultimate elimination in the feces. Radioactive silver was detected in the feces up to 300 days after exposure but was not detected in urine samples.[27,222] The deposition of silver in tissues is the result of the precipitation of insoluble silver salts such as silver chloride and silver phosphate. The highest concentrations of silver are usually found in the liver and spleen and to some extent in the muscle, skin, and brain.[27]

Toxicodynamics of Silver

Toxicity of silver and silver compounds is attributed to the free silver ion interacting with sulfhydryl, amino, carboxyl, and other groups on membrane or enzyme proteins.[221] Light catalyzes the reduction of silver salts deposited in skin to metallic silver. Elemental silver is subsequently oxidized to silver sulfide, which accounts for the gray skin discoloration that is indicative of argyria.[27]

Screening and Regulatory Levels for Silver

Silver is not considered to be a carcinogen or a reproductive or developmental toxicant.[222] The EPA's cancer evaluation is Group D, not classifiable. The EPA has derived an RfD for silver, 5 μg/kg/day.[19] Neither an RfC nor chronic MRLs are derived for silver. OSHA's PEL and NIOSH's REL for silver dust and soluble compounds are both 0.01 mg/m^3.[20,27] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Strontium

Sources of Strontium Exposure

Strontium is naturally occurring and found in rocks, soil, dust, coal, and oil. Naturally occurring strontium is not radioactive. It is estimated that 85% of all strontium consumed in the United States is used in the manufacture of ceramics and glass products, primarily in television faceplate glass and secondarily in ceramic ferrite magnets. Strontium salts are also used in the manufacture of pigments and electrical materials, and strontium salts give a characteristic red color to flames and fireworks. Occupational exposure to strontium may occur through inhalation of strontium compounds at workplaces where strontium is produced or used. The general population may be exposed to strontium via inhalation of ambient air, ingestion of food, and drinking water containing strontium.[27,223]

Adverse Health Effects of Strontium

Acute poisoning from ingestion of strontium compounds results in a sequela of headache, flushing of skin, vomiting, dizziness, marked fall of blood pressure, cyanosis, convulsions, coma, and eventually respiratory paralysis. Death is due to respiratory failure. Young, still growing, humans and animals are more susceptible than adults to the chronic effects of strontium compounds. Widening of the epiphyseal plate is observed at lower levels of dietary strontium. Since strontium metabolism is controlled by calcium, dietary calcium plays a protective role in strontium toxicity. Ingestion of food and water contaminated with strontium nitrate and nitrites is associated with the formation of methemoglobinemia. Reports of adverse effects following inhalations are rare.[27]

Toxicokinetics of Strontium

Estimates for the percentage of ingested strontium that is absorbed from the gastrointestinal tracts of humans vary from 5% to 30%. Intravenously administered ionic strontium forms colloidal or particulate strontium phosphate or binds to plasma proteins to form partly diffusible complexes. The protein complexes are distributed to the skeleton, kidneys, liver, spleen, brain, aorta, and other soft tissues. Strontium is similar to calcium in its chemical reactions and strontium kinetics are controlled by calcium levels in the tissues. Ninety-nine percent

(99%) of the typical body burden of strontium is found in bone, especially the epiphyseal region. In humans, the excretion of absorbed strontium is about 90% through urine. Unabsorbed dietary insoluble salts are excreted in the feces.[27,223]

Toxicodynamics of Strontium

Bone is the target organ following chronic strontium toxicity. High doses inhibit calcification of the epiphyseal cartilage and cause deformities of long bones. Strontium causes these effects by substituting for calcium in the hydroxyapatite crystal during calcification or displacing calcium from existing calcified bone. The metabolic basis of strontium's effect on calcium metabolism is thought to be inhibition of calcium absorption by dietary strontium as a result of a block in the renal synthesis of 1,25-dihydroxycholecalciferol (vitamin D_3) from 25-hydroxycholecalciferol.[190] In contrast to the toxic effects, pharmacologic treatment with low doses of strontium suppresses bone resorption, and strontium ranelate treatment has reduced the risk of vertebral fractures and increased bone mineral density in postmenopausal women with osteoporosis.[224,225]

Advisory, Screening, and Regulatory Levels of Strontium

The EPA has derived an RfD for strontium of 0.6 mg/kg/day. The EPA has not adequately evaluated stable strontium for carcinogenic potential and IARC does not list it. Strontium chromate is a human carcinogen via inhalation, but this is due to the presence of hexavalent chromium, which is a genotoxic carcinogen.[19] ATSDR has developed an intermediate-duration MRL of 2.0 mg/kg/day for strontium, but the agency has not developed chronic MRLs.[21,223] OSHA has not established regulatory levels, and NIOSH has not established advisory levels for strontium and strontium compounds.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Uranium

Sources of Uranium

Uranium is a soft, malleable metal. The primary uranium ores are pitchblende (uranium oxide) and carnotite, a uranium-/vanadium-containing mineral. Uranium is primarily used as nuclear fuel, but there are some minor uses, such as a colorant in ceramics or glass, or as depleted uranium, a by-product of the uranium enrichment process used in armor-piercing projectile. Occupational exposure to uranium may occur through inhalation and/or dermal contact at workplaces where uranium compounds are liberated from mining, grinding, and milling of ores, packaging nuclear fuel rods, smelting operations, or the production of fluorescent glass.[27] The first significant military exposure occurred in friendly fire incidents.[226] The general population may be exposed to uranium via inhalation of ambient air or ingestion of food and drinking water containing uranium compounds.[27,227]

Adverse Health Effects of Uranium

The chemical toxicity of uranium and its salts is primarily manifested as kidney damage. Acute arterial lesions may occur after acute exposures. Soluble uranium, present in plasma as the uranyl ion complexed with bicarbonate, produces systemic toxicity in the form of acute renal damage and renal failure, which may be fatal. The classic signs of renal impairment include albuminuria, elevated blood urea nitrogen, and loss of weight. Renal damage is brought about by filtration of the bicarbonate complex through the glomerulus, bicarbonate reabsorption by the proximal tubule, liberation of uranyl ion, and subsequent damage to the proximal tubular cells.

Toxicokinetics of Uranium

Uranium is absorbed from the intestine or by the lungs, enters the bloodstream, and is rapidly deposited in the tissues, predominantly kidney and bone, or excreted in the urine. The uptake and fate of ingested uranium are controlled by the total quantity ingested and, to a lesser extent, the particular chemical form. In general, the smaller the amount ingested, the greater the fraction absorbed. There are data to indicate that between 12% and 30% of the ingested uranium is absorbed from the intestinal tract into the bloodstream. Of the absorbed uranium, approximately 80% is excreted, 10% goes to the kidneys, and the remaining 10% is deposited in the skeleton.[27] The kidney retention is believed to be brief. Because of efficient renal clearance of circulating uranium, redistribution of uranium deposits is inefficient and the body burden of uranium probably reflects recent dietary intakes.[190] Pulmonary absorption of uranium may be as high as 20% in humans.[27]

Toxicodynamics of Uranium

Oxidation of tetravalent uranium to hexavalent uranium is likely to occur *in vivo*. On entering the body, soluble uranium immediately forms complexes with anions, bases such as bicarbonate, citrate, malate, and lactate. Pentavalent uranium compounds have great affinity for phosphate-containing molecules and tissues, carboxyl and/or hydroxyl groups such as proteins, nucleotides, and bone tissue.[27] Phospholipid constituents of cell membranes that regulate membrane properties and cell membrane interactions and membrane-bound enzymes such as Na-K ATPase are potential sites of uranium action. The effects on lysosomes, mitochondria, and calcium metabolism and the insult to the microvilli of brush border membranes may also be potential sites of toxic action, resulting in reduced sodium and consequently reduced glucose, amino acid, and protein resorption, even before extensive cell damage occurs.[27,59] An implantation study in the rat indicated that urinary mutagenicity might be used as a biomarker to detect exposure to internalized depleted uranium in potentially exposed soldiers.[226]

Advisory, Screening, and Regulatory Levels of Uranium

The EPA developed an RfD for soluble salts of uranium, 3 μg/kg/day. The EPA has not derived an RfC for uranium

or soluble uranium salts.[19] The ATSDR's chronic inhalation MRLs for uranium and soluble uranium salts are 0.8 and 0.04 μg/m^3, respectively. No direct evidence exists that stable uranium is carcinogenic to humans or animals. The EPA's carcinogenicity assessment has been withdrawn, pending further review (Table 17.6).[19,21] NIOSH defines both soluble and insoluble uranium compounds as potentially carcinogenic and has not established RELs. The OSHA PEL for insoluble uranium compounds is 0.25 mg/m^3. The PEL for soluble uranium compounds is 0.05 mg/m^3.[20] Based on economic considerations, the EPA established a drinking water MCL of 30 μg/L. The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Vanadium

Sources of Vanadium

Vanadium is a white to gray common trace metal, which occurs in nature only in combination with oxygen, sodium, sulfur, and chloride. Occupational exposure to vanadium occurs predominately through the inhalation route of exposure and in settings such as mining of vanadium-bearing ores, steel mills where vanadium pentoxide is used, or during the cleaning of boilers fired by oil containing vanadium. The general population can be exposed to vanadium via inhalation of ambient air or by ingesting food or water containing vanadium.[27,228]

Adverse Health Effects of Vanadium

A greenish-black discoloration of the tongue, gastrointestinal symptoms, neurotoxicity, and renal toxicity are reported in workers exposed to vanadium pentoxide. Inhalation of vanadium pentoxide is associated with tracheobronchitis and inflammation. The progression of pathological lung changes with exposure and time is thought to affect the pattern and/or extent of vanadium lung deposition.[229] Oral exposure to vanadium can be toxic to the gastrointestinal, renal, and neurological systems. Vanadium deficiency is known to occur in laboratory animals on a very strict diet, and there is evidence that vanadium helps regulate some phosphoryl transfer enzymes.[213,230] Measurable responses of human subjects to variations in dietary intake of vanadium have been demonstrated. However, dose–response data are absent and the responses are not consistently observed. Thus, data are insufficient to estimate dietary requirements.[213]

Toxicokinetics of Vanadium

Elemental vanadium and vanadium compounds are absorbed from the respiratory tract. Their absorption from the gastrointestinal tract and skin is poor. Within tissues, vanadium (III) and (IV) predominate due to the reducing conditions. However, plasma is high in oxygen and vanadium (V) predominates. Compared with the vanadium (V) compounds, erythrocytes are slower to uptake vanadium (IV) compounds. Vanadium compounds are distributed to internal organs and can reversibly bind to blood transferrin protein. Vanadium is rapidly excreted in feces and urine following the termination of exposure, but there are data indicating that vanadium might accumulate in bone.[228]

Toxicodynamics of Vanadium

Vanadium can reversibly bind to transferrins in the blood and then be taken up into erythrocytes. Vanadium (V) is considered more toxic than vanadium (IV), because vanadium (V) is reactive and is a potent inhibitor of the plasma membranes sodium/potassium-ATPase. Vanadium (V) can also inhibit numerous enzymes that hydrolyze phosphate esters, including ribonuclease and alkaline phosphatases. A decrease in glutathione, nicotinamide adenine dinucleotide phosphate (NADPH), and nicotinamide adenine dinucleotide (NADH) occurs within an hour after intraperitoneal injection of sodium vanadate in mice. It is believed that vanadate requires the cytochrome P-450 components for oxidation to the vanadium (IV). A consequence of this action is the diversion of electrons from the monooxygenase system, resulting in the inhibition of drug dealkylation.[27]

Advisory, Screening, and Regulatory Levels for Vanadium

Although the Food and Nutrition Board has not estimated an EAR or AI for vanadium, it did estimate a UL of 1.8 mg/day. This value is for adults and is based on animal data.[213] The EPA derived an RfD for vanadium pentoxide of 5 μg/kg/day. The EPA has not derived an inhalation RfC for vanadium and has not evaluated the potential human carcinogenicity of vanadium.[19] The ATSDR's chronic inhalation MRL for vanadium is 0.0001 mg/m.[228] The NIOSH ceiling RELs for vanadium dust and vanadium fume are both 0.05 mg/m^3. OSHA's PEL for vanadium pentoxide dust and fumes were vacated by the Eleventh Circuit Court of Appeals in 1993.[20]

Table 17.6 shows the quantitative toxicity values derived by the EPA for the metals listed in this section. Using the RfDs as the criteria, the metals are listed in order of most to least toxic. The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

METALS FOR WHICH REFERENCE DOSES HAVE NOT BEEN ESTABLISHED

Bismuth

Sources of Exposure to Bismuth

Elemental bismuth is a soft, lustrous metal, which can occur naturally or in combined forms in ores. Bismuth is used in low-melting alloys. Compounds of bismuth are used as coloring agents in cosmetics, and in pharmaceuticals, including those used for diarrhea, gastroesophageal reflux, and in

ulcer therapy. Occupational exposure to bismuth occurs via inhalation of airborne dust and dermal contact. The general population is exposed to bismuth primarily through the use of cosmetic products and ingestion of pharmaceuticals containing bismuth compounds such as bismuth subnitrate and bismuth subsalicylate.[27]

Adverse Health Effects of Bismuth

In occupational settings, bismuth is considered one of the less toxic of the heavy metals. However, bismuth absorbed in industrial settings may complicate a diagnosis of lead poisoning, since the dark line in the gums, which is often present in lead poisoning, is also produced by bismuth. Oral exposure to bismuth and its salts can cause mild kidney damage, and toxicity has occurred from its use in medicine. Other toxic effects include malaise, albuminuria, diarrhea, skin reactions, and sometimes serious exodermatitis. Large doses can be fatal.[27] There are data that indicate that bismuth is not carcinogenic. Rats exposed to bismuth oxychloride in the diet for 2 years at doses up to 2.0 mg/kg/day were found to have no increased incidence of tumors.[231]

Toxicokinetics of Bismuth

Insoluble bismuth salts are poorly absorbed orally or dermally. Unabsorbed bismuth compounds are excreted in the feces.[27] Because bismuth induces the production of metallothionein, it is thought that bismuth may bind metallothionein in the blood. The organ distribution of bismuth compounds has been determined from the autopsies of clinically treated patients, and it is similar to that of other heavy metals: the greatest affinity for the kidneys. Bismuth crosses the placental. Newborn animals exhibit the same concentration as their bismuth-treated mothers. Bismuth is also excreted into the milk, but in lower concentration than in the urine.[27,59]

Toxicodynamics of Bismuth

Bismuth subsalicylate is an over-the-counter drug used in ulcer therapy. It does not neutralize gastric acid, but it provides cytoprotection by enhancing secretion of mucous and HCO_3^-, inhibition of pepsin activity, and the formation of bismuth protein complexes that may afford a protective barrier against peptic digestion. Bismuth subsalicylate is also used to treat diarrhea. It is hydrolyzed in the stomach, yielding salicylate and bismuth salts. The salicylate is absorbed. The bismuth salts are not absorbed and interfere with adhesion of bacteria to mucosal cells. Toxic bismuth levels are generally not reached with normal use, although salicylism has been reported following the use of bismuth subsalicylate.[232]

Advisory, Screening, and Regulatory Levels for Bismuth

NIOSH has established RELs for bismuth telluride of 10 and 5 mg/m^3 for total and respirable fractions, respectively. The OSHA PELs for bismuth telluride are 15 and 5 mg/m^3 for total and respirable, respectively.[20] The EPA has not developed an IRIS profile for bismuth compounds. The ATSDR has not developed a toxicity profile or MRLs for bismuth compounds. The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Cerium

Sources of Exposure to Cerium

Cerium is a lanthanum rare earth metal and is used in fireworks and cigarette lighter flints, self-cleaning ovens, and as an abrasive for polishing glass. Cerium is maybe added to the support surface for improved performance of automotive catalytic converters. Adverse effects are reported from occupational exposures to heavy metals, including cerium, which occurred during optical lens manufacturing. Cerium nitrate is used for the topical treatment of extensive burns.

Adverse Health Effects of Cerium

Occupational inhalation exposure has been reported to cause a pneumoconiosis without pulmonary functional impairment.[233] Intravenous administration of cerium chloride produces severe hepatotoxicity in rats.[30,234] There are data that indicate an acute exposure does not cause immune sensitization, but these data do not rule out immune responses after prolonged exposures.[27]

Toxicokinetics of Cerium

Cerium is poorly absorbed by the gastrointestinal tract. Particles of soluble or insoluble cerium ingested as a result of mucociliary clearance from the lung are unlikely to be reabsorbed. Retention in the lung is greater for less soluble forms of cerium, such as the cerium oxides, than for more soluble forms, such as the cerium chlorides. In humans, about 5%–10% of the deposited particles that have intermediate clearance are absorbed into the blood from the nasopharyngeal region and about 50% from the tracheobronchial region. In contrast, only 1% of the deposited particles with slow clearance are absorbed into the blood from these regions. Soluble forms of cerium are rapidly dissolved and absorbed into the circulation. It is estimated that the internal organ transfer rates are the same for cerium chloride and cerium oxide. Cerium in the blood is deposited to the liver, skeleton, and to a lesser extent, spleen and kidney. Deposition to the brain and heart has not been studied after inhalation exposure. About 80%–90% of the initial body burden of slow and intermediate cerium compounds is cleared within 7 days after inhalation.[27,235]

Toxicodynamics of Cerium

The rarity of cerium toxicity is attributable to its low gastrointestinal absorption. However, it is suggested that once absorbed, cerium may exert diverse biological effects due to its resemblance to calcium. Cerium nitrate is used for the topical treatment of extensive burns. In this context, cerium's direct antiseptic effects help prevent postburn sepsis and systemic inflammatory response by fixing burn toxins.[236]

Advisory, Screening, and Regulatory Levels for Cerium

The EPA has not derived toxicity values for cerium. However, the EPA developed an RfC for cerium oxide particles specifically with a mass median diameter of approximately 2 μm and a geometric standard deviation of 1.8–1.9. The use of this RfC for cerium compounds outside these specifications or for compounds other than cerium oxide is not recommended.[19] There are no MRLs developed for either chronic oral or chronic inhalation exposure to cerium.[237] Neither an REL nor a PEL has been developed for cerium.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Gallium

Sources of Exposure to Gallium

Gallium is a relatively rare metal. It is commonly associated with zinc, germanium, and aluminum and primarily in the mineral, germanite. Gallium is used in the manufacture of alloys, and gallium arsenide and gallium aluminum arsenide are used in electronic components of photovoltaics and lasers. Gallium is present in coal. The general public may be exposed to particulate forms of gallium compounds released to the ambient air. Occupational exposure to gallium compounds may occur through inhalation of dust and dermal contact.[27] Medical patients may be exposed to gallium in its use in diagnostic radiology, its use as an antineoplastic agent,[238] or its use in the control of cancer-related hypercalcemia.[239,240]

Adverse Health Effects of Gallium

There are limited indications of occupationally related toxicity. Occupational exposure to GaF_3 fumes resulted in a rash with subsequent reversible neurological effects consisting of muscular weakness. No adverse effects were noted in a reproduction study conducted in male mice.[241]

Toxicokinetics of Gallium

Gallium (III) is the primary oxidation state for gallium compounds. Gallium pharmacokinetics were examined in clinical trials of gallium nitrate. When administered as an intravenous infusion over 30 min, a biphasic gallium urinary excretion pattern was observed. The first half-life was 8–26 min, and the second half-life was 6–196 h. The second phase was attributed to gallium binding to transferrin in the circulation. Sixty-nine to ninety-one percent of the gallium dose administered was excreted in the urine during the first 24 and 48 h.[242,243] In rats, renal toxicity is noted with the formation of precipitates of gallium complexed with calcium and phosphate.[59]

Toxicodynamics of Gallium

The chemical characteristics of gallium suggest that it may interact with cellular processes and biologically important proteins, especially those of iron metabolism. This potential has led to the development of certain gallium compounds as diagnostic and therapeutic agents in medicine especially in the areas of metabolic bone disease, cancer, and infectious disease. Gallium accumulates at the interface of the collagen and mineral components of bone and alters crystal solubility. Significant increases in bone calcium content occur in gallium-treated bone, which also makes bone less likely to be resorbed. In addition, gallium acts by blocking osteoclast activity without affecting the cell's viability. It is thought that the mechanism of antineoplastic activity of gallium is a two-step process in which the first step involves the targeting of transferrin-gallium to transferrin receptor-bearing tumor cells. In the second step, gallium perturbs cellular iron metabolism by interfering with transferrin receptor-mediated uptake of iron. This leads to a condition of tumor cell iron deprivation and inhibition of the iron-dependent function of ribonucleotide reductase, the enzyme essential for deoxyribonucleotide synthesis.[243]

Advisory, Screening, and Regulatory Levels for Gallium

The EPA has not derived toxicity values for gallium.[19] The ATSDR has not developed a toxicity profile or MRLs for gallium.[18] There are no NIOSH RELs or OSHA PELs for gallium or its compounds.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Germanium

Sources of Exposure to Germanium

Germanium metal has a metallic appearance but is very brittle, much like glass. Germanium is not found in the free state but always in combination with other elements such as silver, copper, and arsenic. It is used in the semiconductor industry and was used in the first transistor. Germanium is often used in combination with other materials, such as arsenic and antimony, and alloyed with aluminum, gallium, and indium. It is also used in certain optic applications because the pure metal is transparent to infrared radiation. Industrial exposures to the dusts and fumes of germanium metal occur during extraction from ore and metal fumes from welding operations.[27,59] Germanium oxide and germanium sesquioxide have been used in elixirs for the treatment of cancer and acquired immune deficiency syndrome.[244]

Adverse Health Effects of Germanium

In longer-term oral animal studies, germanium and germanium oxide have been shown to be nephrotoxic, neurotoxic, and myotoxic.[245,246] The potential for germanium to induce lung injury is unclear. In one four-week inhalation toxicity study of germanium powder in rats, histopathologic changes consistent with pulmonary toxicity were present but, a follow-up study using germanium dioxide did not report treatment-related microscopic histopathologic effects in the lung.[247] Germanium does not appear to be carcinogenic. Certain germanium compounds appear to have antineoplastic activity.[248] In a lifetime feeding study in rats, animals receiving 5 parts per million sodium germanate in water had a significantly lower incidence of tumors than the control

animals.[249] To date, there have been at least 31 reported cases of toxicity associated with oral intake of germanium compounds, of which nine were fatal.[244] Nephrotoxicity is the primary manifestation of germanium intoxication.[30,244,250] Germanium compounds are not mutagenic. High doses of germanium may result in an increased embryonic resorption. Malformations have been reported only after administration of dimethyl germanium oxide to pregnant animals.[248]

Toxicokinetics of Germanium

Germanium is well-absorbed; its concentration in human urine is about the same as the concentration in the diet. Germanium in various forms is absorbed from the gastrointestinal tract and excreted in the urine (68%) and the feces (9.7%) within 24 h of administration. It is fairly equally distributed between plasma and red blood cells. Since it leaves the bloodstream in a few hours, it is likely that germanium is transported in the blood unbound to proteins. Germanium is widely distributed in body tissue and not selectively retained by any single tissue. The clearance rate of inhaled germanium particles is reported to be exponential with 52% excreted in 24 h and 18% retained for 7 days postexposure. Inhaled germanium enters the circulation and appears in the kidney and liver 1 h after exposure.[27]

Toxicodynamics of Germanium

An antineoplastic activity of germanium may be attributable to an ability to inhibit the mutagenic activity of other substances.[248]

Advisory, Screening, and Regulatory Levels for Germanium

The NIOSH REL for germanium tetrahydride is 0.6 mg/m^3. OSHA has not derived a PEL for germanium or germanium compounds.[20] The EPA has not developed an IRIS profile, and ATSDR has not developed a toxicity profile or MRLs for germanium or germanium compounds.[18,19] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Gold

Sources of Exposure to Gold

Gold's excellent heat and electrical conductivity and malleability have made it important in industrial applications. Medically it is used either orally or by intramuscular injection to slow the progression of rheumatoid arthritis, but treatment is associated with a high incidence of toxicity.[3] While gold compounds are not commonly used or encountered in the environment, occupational exposure to gold may occur through inhalation and dermal contact with gold at workplaces where gold is processed or used. Environmental monitoring data indicate that the general public may be exposed to gold via inhalation of ambient air, ingestion of food, and dermal contact with gold.[27]

Adverse Health Effects of Gold

Adverse skin and mucous membrane effects, including dermatitis, stomatitis, and pruritus, are most frequent. The incidence and severity of these effects are less for oral as opposed to parenteral treatment.[251] A mild proteinuria is the most common renal effect, but gold-induced nephrosis may occur. Aplastic anemia is relatively rare and has been associated with poor prognosis, which may improve with bone marrow transplantation.[252,253] Although traditionally regarded as inert, gold is being recognized as a common contact allergen.[254,255] In Sweden, it is second only to nickel, and a report from the North American Contact Dermatitis Group ranks it among the 10 most common allergens in the United States.[256] Gold was named Contact Allergen of the Year in 2001 by the *American Journal of Contact Dermatitis*.[257] Eyelid dermatitis was found in 7.5% of patients with a positive gold patch test reaction.[258] Gold allergy is more common in women than men and is linked to nickel and cobalt allergy. Gold hypersensitivity is characterized by late reactions, and failure to monitor the test site for a minimum of three weeks may result in false negatives.[30]

Toxicokinetics of Gold

Gold salts are poorly absorbed from the gastrointestinal tract. After injection of soluble salts, gold is excreted in the urine. Ingested insoluble gold compounds are excreted in the feces. Gold is thought to have a long biological half-life since detectable blood levels can be demonstrated for 10 months after cessation of treatment. Gold (III) binds strongly to metallothionein.[27]

Toxicodynamics of Gold

In vitro studies designed to simulate the oxidative conditions found inside lysosomes suggest that a redox system might be operating. It is postulated that $AuCl_3$ is formed from gold (I) thiomalate during an oxidative burst in phagocytic immune cells. Since $AuCl_3$ is a better oxidant than Au (I), it dominates both the anti-inflammatory and toxic effects of gold salts. Studies of the reduction of $AuCl_3$ and its derivatives by serum albumin and various thiols and thiol-ethers suggested that the reduction occurs over a slower time frame than the hypochlorite oxidation of gold (I).[27]

Advisory, Screening, and Regulatory Levels for Gold

The EPA has not developed an IRIS profile for gold or gold compounds.[19] The ATSDR has not developed MRLs or a toxicology profile for gold or gold compounds.[18] NIOSH has not developed an REL for gold or gold compounds, and OSHA has not developed PELs.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Hafnium

Sources of Exposure to Hafnium

Hafnium is a gray metallic element having a silver-like luster and is found in association with zirconium ores. It has

outstanding corrosion resistance and is used for this characteristic in atomic reactors, electronic components, and alloys. It has been evaluated for its antimicrobial properties as a coating on surgical implants.[93] Occupational exposure to hafnium may occur through inhalation and dermal contact at workplaces where hafnium is produced or used. Monitoring data indicate that the general population may be exposed to hafnium via inhalation of ambient air, ingestion of food, or drinking water.[27]

Adverse Health Effects of Hafnium

Hafnium compounds show moderate toxicity in acute animal tests by several routes of administration.[259] Hafnium is poorly absorbed orally, and the dust is considered to have relatively low toxicity.[260] Workers exposed to 150 mg/m^3 of hafnium- and zirconium-containing dusts showed no adverse effects after 2–6 years.[261] In occupational settings, overexposure to hafnium and its compounds may cause mild irritation of the eyes, skin, and mucous membranes. The NIOSH REL is based on the risk of liver damage, and eye and skin irritation in animals.

Toxicokinetics and Toxicodynamics of Hafnium

Hafnium can be absorbed into the body by inhalation of its aerosol. The absorption following oral administration of various chemical forms of a hafnium radioisotope was found to be between 0.04% and 0.13% of the ingested dose. Absorption was increased by fasting. Studies in rodents indicate that hafnium concentrates in the liver and skeleton.[27] The irritation of eyes and skin is attributed to the chemical reactivity that is characteristic of all metals.

Advisory, Screening, and Regulatory Levels for Hafnium

The NIOSH REL and OSHA PEL, for hafnium and hafnium compounds, are both 0.5 mg/m^3.[20] The EPA has not developed an IRIS profile, and the ATSDR has not developed a toxicity profile for hafnium.[18,19] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Indium

Sources of Exposure to Indium

Indium is not found in the free state, but most commonly, in association with copper, zinc, and sulfur. Indium is used in surface protection of metals, and in many alloys, because of its ability to increase hardness. Indium compounds are also used in the photovoltaic and semiconductor industry. Industrial exposures to indium occur during extraction and purification, in plating and the manufacture of certain electronic instruments.[59]

Adverse Health Effects of Indium

Soluble indium compounds are more toxic than their insoluble indium compounds. The acute lethal dose range for the soluble indium chloride in rabbits, rats, and dogs is 0.33–3.6 mg/kg,[262] whereas the minimum lethal dose for insoluble indium oxide in rats is 955 mg/kg.[94]

Indium compounds are toxic when inhaled. A review of seven cases of occupational exposure found that slightly soluble indium compounds cause interstitial as well as emphysematous lung damage.[263] Copper indium diselenide and indium trichloride, when acutely administered intratracheally to rats, at doses higher than would be expected in an industrial exposure, induced a persistent inflammatory response.[264–266] Copper indium diselenide was only slightly fibrogenic to the lung. This is consistent with its limited solubility.[264] Subchronic inhalation of indium sesquioxide in rats induced a persistent inflammatory response, and no fibrosis was noted. Hamsters were treated once per week for 15 weeks with either indium arsenide or indium phosphide by intratracheal installation and were examined at the end of their lifespan. Adverse histopathologic findings were significantly higher in the treated groups.[267]

Several studies have investigated the reproductive and developmental toxicity of indium compounds. Indium arsenide, administered intratracheally, reduced epididymal sperm counts in rats, but not in hamsters.[268,269] Intratracheal instillation of indium chloride in mice did not affect reproductive performance of either males or females, but it was fetotoxic.[270] Indium trichloride was a developmental toxin in mice and rats when administered intravenously.[271]

Toxicokinetics of Indium

Absorption of indium compounds is highly dependent on form. Insoluble indium compounds are poorly absorbed and distributed, whereas soluble compounds, such as $InCl_3$ and $In_2(SO_4)_3$, are rapidly absorbed and distributed.[59,272] The primary route of indium excretion is also determined by the chemical form administered. Ionic indium is mainly excreted in urine while colloidal indium complexes are primarily excreted in feces. Muscle, skin, and bone constitute the main storage sites.[27]

Toxicodynamics of Indium

Indium chloride exerted inhibitory actions on the enzyme ALA-dehydratase in kidney following acute *in vivo* exposure. It is thought that indium alters the excretion of several other enzymes in the heme pathway in a highly specific manner.[27]

Advisory, Screening, and Regulatory Levels of Indium

The NIOSH REL for indium and indium compounds is 0.1 mg/m^3. OSHA has not published a PEL for either indium or indium compounds.[20] Neither the EPA nor ATSDR has developed toxicological profiles for indium.[18,19] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Lithium

Sources of Exposure to Lithium

Lithium is a silvery-white metal and the lightest solid element. Although it is used in batteries, organic synthesis (Grignard reagent), in the space industry, as a swimming pool sanitizer, and in air conditioners, occupational intoxication has not been reported.[273] Lithium hydride in contact with water releases flammable hydrogen gas. Thus, it must be stored under airtight anhydrous conditions.[30,59]

Adverse Health Effects of Lithium

Inhalation exposure to lithium hydride can cause pulmonary edema, but the hydride rather than the lithium is likely responsible.[217] Signs of lithium toxicity are primarily neurologic and range from fine tremors and muscle weakness, in mild cases, to dysarthria, hyperreflexia, coma, and collapse. Lithium therapy may produce lasting neurologic consequences.[274,275] Renal symptoms of intoxication include polyuria, polydipsia, and renal failure.[276] Lithium therapy during pregnancy has been associated with an increased risk of cardiac anomalies, and there are sufficient animal and human data to indicate lithium can cause developmental toxicity.[30,277]

Toxicokinetics of Lithium

There is no specific antidote for lithium intoxication, and treatment is based on limiting absorption and enhancing excretion.[278] Hemodialysis is used to enhance excretion.[276] There is some evidence that sodium polystyrene sulfonate reduces the half-life of lithium and that it is capable of promoting lithium elimination in chronic intoxications.[279] However, development of hypokalemia may represent a potential limitation in the use of sodium polystyrene sulfonate for the treatment of lithium toxicity.[30,280]

Toxicodynamics of Lithium

Lithium has properties similar to sodium, and substitution for sodium and/or potassium is thought to account for the adverse effects.[281] Oral lithium salts, such as lithium carbonate and lithium citrate, are widely used in the treatment of manic-depressive disorders. Routine serum monitoring is required because of the narrow therapeutic index.[281,282] The same levels of lithium are devoid of psychotropic effects in individuals not suffering manic-depressive disorders.[281]

Advisory, Screening, and Regulatory Levels for Lithium

The NIOSH REL and the OSHA PEL for lithium hydride are both 0.025 mg/m^3.[20] The EPA has not developed an IRIS profile for lithium.[19] The ATSDR has not developed MRLs or a profile for lithium.[18] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Niobium

Sources of Exposure to Niobium

Niobium is a white-colored soft metal found in ores in combination with tantalum and other elements.[59] Niobium is used in alloys and may find use in surgical implants and dental applications.[283–285]

Adverse Health Effects of Niobium

Acute and chronic animal tests have been conducted on several niobium compounds.[286–288] Parenteral administration of niobium pentachloride results in decreased respiration, lethargy, and death. The compound is a moderate to severe skin irritant. Life-term studies of sodium niobate in mice and rats did not show carcinogenicity.[249]

Toxicokinetics and Toxicodynamics of Niobium

Niobium is poorly absorbed from the gastrointestinal tract. The irritation of eyes and skin that results from exposure to niobium is attributed to the chemical reactivity that is characteristic of all metals.[30]

Advisory, Screening, and Regulatory Levels for Niobium

No reports of occupational health hazards from dust or fumes associated with forging or other fabrication techniques of niobium metal and alloys have been recorded.[59] NIOSH has not established a, REL, and OSHA has not established a PEL for niobium.[20] The EPA has not developed an IRIS profile for niobium.[19] The ATSDR has not developed MRLs or a profile for niobium.[18] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Osmium

Sources of Exposure to Osmium

Osmium is found in combination with platinum and nickel-bearing ores. Osmium can form many compounds with oxidation states ranging from 0 to +8, with the most common valances being +2 and +3. Osmium tetroxide is a noncombustible, colorless to pale yellow solid, with a disagreeable chlorine-like odor. An osmium–tungsten alloy has been used for filaments for incandescent lamps. Osmium–iridium alloy is extremely hard and is employed for pen nibs, engraving tools, and watch bearings. Occupational exposure to osmium and osmium compounds may occur through inhalation of dust, and by dermal contact at workplaces where metals are mined or processed or where osmium compounds are produced or used. Since osmium has a low abundance in the environment and limited uses, exposure to the general population is expected to be low.[27]

Adverse Health Effects of Osmium

Metallic osmium and most of compounds other than osmium tetroxide are not considered highly toxic. Osmium tetroxide

has been shown to be toxic in animals and in man. The oral LD_{50} for osmium tetroxide is reported to be 14 mg/kg in the rat and 162 mg/kg in the mouse. The intraperitoneal LD_{50} for the mouse is 14 mg/kg.[59] The reported LC_{50} for the rat and mouse is 400 mg/m^3. In humans, inhalation exposure to OsO_4 can cause irritation of the nose and throat, which can persist for at least 12 h. Industrial exposure to osmium tetroxide concentrations ranging from 0.1 to 0.6 mg/m^3 induced lacrimation and disturbances in vision. Other complaints included conjunctivitis, cough, and headache. Recovery usually occurred within a few days.[30] One human fatality has been reported, resulting from inhalation of osmium tetroxide. Death was attributed to capillary bronchitis and pulmonary edema.[289]

Toxicokinetics and Toxicodynamics of Osmium

Osmic acid was previously used as a local treatment of rheumatoid synovitis. Of the 1% osmic acid solution injected into arthritic joints, most was excreted in the urine with none accumulating in the contralateral knee, the regional lymph nodes, the liver, or the heart.[27] The irritation of the nose and throat following exposure to OsO_4 is attributable to the chemical reactivity that is characteristic of metals.

Advisory, Screening, and Regulatory Levels of Osmium

The NIOSH REL and the OSHA PEL for osmium tetroxide are both 0.002 mg/m^3.[20] The EPA has not developed an IRIS profile for osmium.[19] The ATSDR has not developed MRLs or a profile for osmium.[18] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Platinum

Sources of Exposure to Platinum

Although platinum is relatively rare, it is found both as the pure metal and in combination with nickel, copper, and gold.[59] Platinum is used as a catalyst in the automotive, chemical, and pharmaceutical industries; and its nobility, or resistance to oxidation, makes it important in the manufacture of laboratory equipment.[30]

Adverse Health Effects of Platinum

Metallic platinum is relatively inert. The complex salts are frequent sensitizers producing conjunctivitis, urticaria, dermatitis, and eczema following inhalation and/or dermal exposure.[30,290] A syndrome, formerly known as *platinosis*, is characterized by lacrimation, sneezing, rhinorrhea, cough, dyspnea, bronchial asthma, and cyanosis.[290] *Platinosis* might be taken to indicate pneumoconiosis and fibrosis, but these are not part of platinum allergy syndrome. Thus, the condition is more accurately referred to as an allergy to platinum compounds containing reactive halogen ligands.[291] Cisplatin has been used as a chemotherapeutic agent against various cancers, especially testicular and ovarian tumors, despite nephrotoxicity at therapeutic doses.[290] More recently, carboplatin has been used with comparable efficacy and less toxicity for many types of cancer. In these cases, thrombocytopenia is the major side effect.[290,292] Platinum (IV) is capable of inducing oxidative DNA damage.[293] However, no increased risk of cancer has been reported from occupational exposure to platinum.[30,290]

Toxicokinetics and Toxicodynamics of Platinum

A report on the tissue distribution of a single oral dose of cationic platinum in rats indicated almost all the dose was excreted in the feces. Intravenous administration to rats of small doses of labeled Na_2PtCl_4 showed higher platinum retention in the liver, kidneys, spleen, and muscle than in the bones. About 35% of the dose was excreted within 24 h in urine and feces. The low fecal excretion suggested an enterohepatic excretion pathway.[27] The platinum allergy symptoms are elicited by either an immediate (type I) or delayed (type II, within 24 h) hypersensitivity reaction.[290]

Advisory, Screening, and Regulatory Levels for Platinum

NIOSH has not established an REL, and OSHA has not established a PEL for platinum or platinum compounds.[20] The EPA has not evaluated the toxicity of platinum.[19] The ATSDR has not developed a profile or MRLs for platinum or platinum compounds.[18] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Rhodium

Sources of Exposure to Rhodium

Rhodium is a silver-white hard metal that can form highly corrosive resistant alloys and coatings used in electrical contacts, reflectors, and jewelry.[290] Antimalarial activity is reported for a rhodium–chloroquine complex.[294] Occupational exposure to rhodium and rhodium compounds may occur through inhalation of dust and by dermal contact at workplaces where rhodium is produced or used. The general population may be exposed to rhodium via inhalation of air.[27]

Adverse Health Effects of Rhodium

Only a limited toxicity profile has been developed for rhodium and its compounds. Sensitizing activity of hexachlororhodiate related to occupational exposure is reported.[27] Rhodium trichloride is moderately low in acute toxicity. Intravenously administered rhodium trichloride of approximately 200 mg/kg is lethal in rats and rabbits with death possibly due to CNS depression.[295] Oral rhodium trichloride was low in toxicity with an LD_{50} greater than 500 mg/kg.[290] A chronic feeding study showed slight carcinogenic activity in mice.[296]

Toxicokinetics and Toxicodynamics of Rhodium

There are data to suggest that rhodium is clastogenic through a mechanism involving oxidative damage to DNA.[293] Complexes of rhodium (III) can intercalate into the DNA strand and are photo-oxidants that promote DNA strand cleavage or electron transfer through the double helix.[27]

Rhodium (II) compounds irreversibly inhibit the activity of enzymes that have essential sulfhydryl groups. Enzymes without essential sulfhydryl groups are not affected.[27]

Advisory, Screening, and Regulatory Levels for Rhodium

Neither the EPA nor the ATSDR has developed toxicity profiles for rhodium. Elemental and rhodium compounds are not classifiable as human carcinogens. The NIOSH REL and OSHA PEL for metal fume and insoluble compounds are both 0.1 mg/m^3. The REL and PEL for soluble compounds of rhodium are both a hundred times lower, 0.001 mg/m^3.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Tantalum

Sources of Exposure to Tantalum

Tantalum is a gray hard metal found in ores in combination with niobium and other metals. It is used in electric capacitors, as the carbide for tools, and has a wide range of uses in medical diagnostic and surgical implant applications.[59,297] This includes tantalum gauze used in the repair of hernias, implant plates and screws, and radiographic lung and bone markers.[297]

Adverse Health Effects of Tantalum

Elemental tantalum and its principal oxide are essentially nontoxic *in vitro* and *in vivo*.[285] Furthermore, occupational exposure to tantalum and its oxide has shown no overt adverse health effects.[30] Irritation of skin, eyes, and the respiratory tract and cases of hard metal pneumoconiosis are reported in settings where workers are exposed to a mixture of metals that includes tantalum.[27] There are a few case studies that report allergic responses to tantalum used in surgical implants.[27]

Toxicokinetics and Toxicodynamics of Tantalum

A study of the clearance of radiolabeled tantalum metal powder following its introduction into the respiratory tract of dogs is reported. Results showed a prolonged alveolar clearance phase with a mean biologic removal half-life of greater than 2 years. Studies of humans exposed via inhalation of radioactive contamination indicated that approximately 93% of the activity was eliminated entirely in the feces in 7 days.[27] Irritation of skin, eyes, and the respiratory tract and reports of hard metal pneumoconiosis are attributable to chemical reactivity characteristic of metals.

Advisory, Screening, and Regulatory Levels of Tantalum

The NIOSH REL and OSHA PEL, for tantalum metal and oxide dust, are both 5 mg/m^3.[20] The EPA has not developed an IRIS profile for tantalum.[19] The ATSDR has not developed MRLs or a toxicity profile for tantalum.[298] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Tellurium

Sources of Exposure to Tellurium

Tellurium has a number of industrial uses and is also found in a variety of food products, for example, condiments, dairy products, nuts, and fish, in high concentrations.

Adverse Health Effects of Tellurium

There are no reports of serious illness or death in workers exposed to tellurium and its compounds other than sodium tellurite.[30] Two fatalities occurred after unintentional treatment with 2 g of sodium tellurite by ureteral catheter.[299] The autopsy revealed acute fatty degeneration and edema of the liver. Pneumonitis and hemolytic anemia are prominent features of acute tellurium intoxication.[300] Tellurium hydride has been shown to be highly toxic, causing pulmonary irritation and intravascular hemolysis.[301] Acute oral or parenteral tellurium intoxication resulted in numerous symptoms, with hematuria noted in all animals treated.[300] Weanling rats fed 1% tellurium in the diet developed a peripheral neuropathy characterized by a transient demyelinating/remyelinating event.[302,303]

Toxicokinetics and Toxicodynamics of Tellurium

Absorbed tellurium is slowly metabolized to dimethyl telluride and is excreted in urine, sweat, and breath.[304] Dimethyl telluride is responsible for the *garlic breath* that is associated with tellurium exposure.[305] Ninety percent (90%) of the body stores of tellurium are in bone.[27]

Advisory, Screening, and Regulatory Levels of Tellurium

The NIOSH REL and OSHA PEL is 0.1 mg/m^3 for tellurium and tellurium compounds except tellurium hexafluoride and bismuth telluride. The REL and PEL for tellurium hexafluoride are both 0.2 mg/m^3.[20] The EPA has not developed an IRIS profile for tellurium.[19] The ATSDR has not developed MRLs or a profile for tellurium.[298] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Thallium

Sources of Thallium

Thallium occurs naturally in the environment and is present in nearly all environmental media. Thallium sulfate was widely used as a rodenticide, but its use was banned in the United States in 1972. Thallium is easily taken up by plants through the roots, entering the food chain, and trace amounts of thallium are found in nearly all plant and animal species,

as well as foodstuffs. Historically, it has been used to treat gout, venereal disease, dysentery, ringworm, and tuberculosis. It is currently used in the electronics industry and in the manufacture of prisms, costume jewelry, pigments, low-temperature thermometers, and infrared spectrometers. Occupational exposure to thallium compounds may occur through inhalation and dermal contact at workplaces where thallium and thallium compounds are produced or used or during the handling of pyrites or flue dusts. The general public can be exposed to small amounts of thallium via inhalation of ambient air and ingestion of food and drinking water. Due to the limited commercial applications of thallium compounds, the exposure of the general public to thallium compounds is expected to be small.[27,306] Thallium intoxication from contaminated heroin and cocaine, presumably imported from areas where thallium is still used as a rodenticide, has been reported.[307,308]

Adverse Health Effects of Thallium

The principal clinical features of thallotoxicosis are gastroenteritis, peripheral neuropathy of unknown etiology, and alopecia.[309] Thallium is well-absorbed following oral ingestion and causes severe gastrointestinal symptoms followed by painful paresthesia of the extremities, motor paralysis, and death from respiratory failure. Individuals surviving the acute phase suffer characteristic scalp alopecia about 10 days postingestion.[309–312] Mees' lines are often seen on finger nails 2–4 weeks after exposure.[302] Alopecia combined with a painful peripheral neuropathy are suggestive of thallium poisoning, but a definite diagnosis requires demonstrating elevated thallium levels in hair, nails, feces, saliva, blood, or urine.[313]

Toxicokinetics of Thallium

Thallium ion is absorbed and distributed like potassium ions because it has the same charge and similar ionic radius. Thallium is easily absorbed by the skin as well as by the respiratory and gastrointestinal tracts. The prolonged body retention of thallium is attributed to an enterohepatic recirculation. Following inhalation of thallium oxides and salts, thallium is rapidly absorbed from mucous membranes of respiratory tract, mouth, and lung. After absorption into blood, thallium ion may be absorbed into erythrocyte or transported in the plasma in its ionic form. Thallium ion does not combine with the albumin or other proteins and is rapidly distributed to the tissues. It easily passes through the blood–brain and placental barrier and is excreted in the milk of lactating animals.[27]

Toxicodynamics of Thallium

Because thallium's structure is similar to potassium, it alters potassium-dependent processes. Thallium toxicosis likely involves the substitution of the thallous ion for potassium in the sodium/potassium ATPase pump and/or interference with sulfhydryl enzymes. Presumably, alopecia and Mees' lines result from thallium's interference with the formation of disulfide bonds.[309] Interference with tissue riboflavin, with subsequent effects on metabolic pathways, has also been suggested.[314] Thallous ion accumulation in lens occurs by active transport. An unexpected finding was the high storage of thallium in melanin-containing eye structures. Chelating agents, such as sodium diethyldithiocarbamate, have caused a redistribution of thallium to target organs, with an increase in toxicity.[315] In October 2003, the USFDA approved a Prussian blue formulation for suspected thallium poisoning.[313] Prussian blue's crystal lattice makes it an effective ion exchanger for univalent cations in general, and its therapeutic use depends on the preferential binding of thallium over potassium.

Advisory, Screening, and Regulatory Levels for Thallium

EPA reviewed information, but an RfD for soluble salts of thallium was not estimated due to difficulties in the selection of appropriate endpoints. An RfC has not been derived for thallium. ATSDR did not derive chronic-duration oral or inhalation MRLs. Thallium is placed in Group D, not classifiable as to human carcinogenicity, based on two inadequate negative studies in humans and a lack of animal studies designed to examine carcinogenic endpoints.[19,21] The OSHA PEL and NIOSH REL for soluble thallium compounds are both 0.1 mg/m^3.[20] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Tin

Sources of Exposure to Tin

Tin is a soft white metal, which occurs in combination with other chemicals, for example, chlorine and oxygen.[316] It is alloyed with other metals to make pewter, solder, bronze, and a special cast bronze termed bell metal (up to 24% tin), which is noted for its tonal quality.[59] Most of the tin used in the United States is for plating steel cans. The fluoride is used in toothpaste, and the chloride is used to make frost-free windshields.[317] Organotins function as antimicrobials in agriculture and industry, as stabilizers in polyvinylchloride plastics, and as marine antifouling agents.[59] Although Schwarz et al. reported in 1970 a significant growth effect of dietary tin in weanling rats maintained on purified diets, this has not been independently confirmed, and tin is not considered to be essential.[8,30,318]

Adverse Health Effects of Tin

Soluble salts of inorganic tin are gastric irritants, producing nonspecific signs of nausea, vomiting, and diarrhea. Rats maintained on diets containing 0.3% soluble inorganic tin salts, such as stannous chloride, experienced growth retardation and anemia.[319] Acute inhalation exposure to tin can cause metal fume fever, and chronic exposure can cause a benign pneumoconiosis, stannosis.[217] Organotins, especially the trialkyl derivatives, are highly toxic. The rat acute oral LD_{50} is 10 mg/kg.[320] Triethyltin compounds are skin irritants and potent neurotoxins producing a decrease in myelin content of the CNS and edema of the white matter.[321]

Toxicokinetics of Tin

Inorganic tin compounds are poorly absorbed from the gastrointestinal tract. Rats dosed orally absorbed 2.8% of tin (II) and less than 1% of tin (IV).[322]

Toxicodynamics of Tin

Injected stannous chloride is a potent inducer of rat renal microsomal heme oxygenase, enhancing heme breakdown.[323] Diets supplemented with high levels of iron and copper protected rats from the anemia but did not alleviate growth depression.[324] Tin has adverse effects on the absorption and metabolism of the essential elements iron, copper, and zinc.[319,324,325] Uncoupling of oxidative phosphorylation has been proposed as the mechanism of action.[326] Butyltins have been shown to inhibit human placental cytochrome P450 aromatase activity when measured *in vitro* and to affect male sexual development in rats.[327,328]

Advisory, Screening, and Regulatory Levels for Tin

The NIOSH REL and the OSHA PEL for metallic tin and for other inorganic tin compounds, except the tin oxides, are both 2 mg/m^3. OSHA has not established PELs for the tin oxides. The NIOSH REL for both tin (II) oxide and tin (IV) oxide is 2 mg/m^3. The NIOSH REL and the OSHA PEL for organic tin compounds are both 0.1 mg/m^3.[20] The EPA has not defined toxicity values for elemental tin. The RfD for tributyltin oxide is 0.0003 mg/kg/day; an RfC is not established.[19] The chronic oral MRL for tributyltin oxide is 0.0003 mg/kg/day. The ATSDR has established intermediate and chronic oral MRLs for other forms of tin, including tributyltin oxide and dibutyltin dichloride.[329] The EPA has not evaluated tin and tin-containing compounds for carcinogenicity. The EPA puts tributyltin oxide in its Group D classification, not classifiable as to human carcinogenicity.[19] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Titanium

Sources of Exposure to Titanium

Titanium is a silver-gray-colored metal that can occur naturally in several forms. Titanium is a component of several alloys and is used in surgical implants where it is considered nontoxic.[330] Titanium dioxide, the most common oxide of titanium, is extensively used as a white pigment in paints, plastics, inks, and cosmetics.[59,331] Occupational exposure to titanium primarily occurs in the mining and production of the metal, and in the production and processing of titanium dioxide and carbide. Occupational exposure to titanium compounds may occur through dermal contact and inhalation titanium dioxide dust and inhalation of titanium tetrachloride vapor.[27] For the general population, food is the principal source of exposure to titanium. Dermal exposure may occur with the use of a variety of drug and cosmetic applications. Titanium compounds are used as sunscreens, for treatment of herpes simplex, in dermatological and cosmetic formulations, and in a variety of tablet-coating formulations.[27]

Adverse Health Effects of Titanium

Titanium dioxide is considered to be essentially nontoxic by the oral, dermal, and inhalation routes. A 2-year feeding study of titanium dioxide at maximum doses of 2.5 g/kg/day in rats and 6.4 g/kg/day in mice found no evidence of carcinogenicity. However, the dose–response relationship was statistically significant for thyroid tumors in female rats and keratoacanthomas in male rats.[30,332–334] Toxicity from respiratory exposure to titanium dioxide has been investigated. A 2-year inhalation study was conducted in rats with acceptable results at a level of 10 mg/m^3. The high levels produced squamous cell carcinomas, which are postulated to be the result of saturation of normal pulmonary clearance mechanisms.[335] Epidemiological findings and related information do not conclusively support a relationship between occupational exposure to titanium dioxide and pulmonary fibrosis, cancer, or other adverse health effects.[30]

Toxicokinetics and Toxicodynamics of Titanium

Three percent of ingested titanium is absorbed, and in man, most of the absorbed titanium is excreted in the urine. The highest concentration of the retained dose is found in the spleen and adrenals followed by the striated muscle, liver, and kidney. Titanium has been found in the lungs and kidneys of infants, indicating that it crosses the placenta.[27]

Advisory, Screening, and Regulatory Levels for Titanium

The titanium tetrachloride chronic inhalation MRL is 0.0001 mg/m.[320] The OSHA PEL for titanium dioxide is 15 mg/m^3. NIOSH defined titanium dioxide as a potential occupational carcinogen and for this reason has not established an REL.[20] The EPA has not developed an IRIS profile for titanium.[19]

Tungsten

Sources of Exposure to Tungsten

Tungsten occurs in nature in combination with iron, manganese, and calcium. The major use of tungsten is in cutting and wear-resistant materials.[59] Tungsten-iron shot is also in use as a less toxic replacement for lead shot.[336]

Adverse Health Effects of Tungsten

The bulk of inhaled tungsten oxide is rapidly excreted in dogs.[337] Although exposure to soluble tungsten compounds can be toxic in experimental animals,[338] insoluble tungsten compounds have a low order of toxicity.[336,339,340] Male and female rats given sodium tungstenate in water for 2½ years at

doses of 0.25 and 0.29 mg/kg/day had no significant increase in tumor incidence.[286] Pulmonary fibrosis observed in men with inhalation exposure to cobalt-cemented tungsten carbide has been attributed to cobalt.[59,341] Evaluation of workers with long-term exposure to tungsten or its insoluble compounds showed no development of pneumoconiosis.[30]

Toxicodynamics of Tungsten

The tungsten ion antagonized the normal metabolic action of the molybdate ion and, therefore, can inhibit molybdate-dependent enzymes.[342–345]

Advisory, Screening, and Regulatory Levels for Tungsten

The NIOSH REL for tungsten metal and insoluble compounds is 5 mg/m^3. The REL for soluble compounds is 1 mg/m^3. OSHA has not established PELs for tungsten metal or tungsten compounds.[20] The EPA has not developed an IRIS profile for tungsten.[19] The ATSDR has not developed MRLs or a toxicology profile for tungsten.[298] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Yttrium

Sources of Exposure to Yttrium

Yttrium is a reactive rare earth lanthanide and is silvery white in color.[59] It is used as an alloying agent in stainless steels requiring high resistance to corrosion. When used in combination with zirconium, it improves the strength of magnesium castings. In the electronics industry, it is used as the matrix producing the red color in television screens.[59]

Adverse Health Effects of Yttrium

Yttrium chloride has been reported to cause granulomatous changes in the rat lung following intratracheal instillation. The liver and spleen are reported to be the primary target organs following intravenous injection.[346,347] Despite a long history of industrial use, there are no definitive reports of adverse effects in workers.[59] The LD_{50} for yttrium chloride following intraperitoneal injection in rats is 132 mg/kg body weight.[348]

Advisory, Screening, and Regulatory Levels of Yttrium

The EPA has not derived toxicity values for yttrium.[19] The ATSDR has not developed MRLs or a toxicology profile for yttrium.[18] The NIOSH REL and OSHA PEL for yttrium metal and compounds is 1 mg/m^3.[20] The EPA has not developed an IRIS profile for ytterbium.[19] The advisory, screening, and regulatory values provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

Zirconium

Sources of Exposure to Zirconium

Zirconium is a grayish white element that is produced from two main sources of ore, zircon ($ZrO{\cdot}SiO_2$) and baddeleyite (ZrO_2). Zirconium is used for the cladding in nuclear fuel rods, and zirconium compounds are also used in foundry and sandblasting applications. Industrial exposure occurs during mining and purification operations. A significant percentage of the general population is exposed dermally to aluminum zirconium chlorohydrate complexes in commercially marketed antiperspirant products.

Adverse Health Effects of Zirconium

Zirconium oxide has a low order of toxicity via the inhalation route in animals. Slight toxicity was noted in dogs when exposed to an airborne mist of zirconium chloride at 6 mg/m^3 for 2 months.[349] Zirconium oxide and zirconium chloride exposure at 3.5 mg/m^3 for 1 year had no measurable adverse effect on the animals exposed.[59] Similarly, in most studies of industrially exposed workers, no adverse effects have been associated with inhalation exposure to zirconium fumes or zirconium compounds.[350–352] However, several cases of either fibrotic[353] or granulomatous[354,355] changes in the lung associated with inhalation exposure to zirconium compounds have been reported. Long-term exposure of mice to zirconium sulfate was not associated with increased tumor incidence.[248]

Certain zirconium compounds, such as zirconium lactate, when applied to human skin[59,356–358] or the skin of experimental animals,[359] can produce dermal granulomas of allergic origin. Aluminum zirconium chlorohydrate complexes, used as active ingredients in antiperspirants, do not appear to cause these granulomatous reactions. However, because of risk/benefit considerations, the USFDA and other global regulatory authorities banned the use of these materials in aerosolized drug and cosmetic products.[360]

Toxicokinetics and Toxicodynamics of Zirconium

The absorbed zirconium is either sequestered in skeleton or excreted very rapidly. Less than 1% of daily intake of zirconium of humans is excreted in the urine due to the poor absorption from the gastrointestinal tract. Absorbed zirconium is excreted in human milk. It is thought that a mechanism that maintains zirconium homeostasis is present in man.[27] Zirconium salts are used in the treatment of plutonium poisoning because it displaces the plutonium from its deposition in the skeleton and prevents deposition when treatment is started early.

Advisory, Screening, and Regulatory Levels of Zirconium

The NIOSH REL for zirconium metal and compounds, except zirconium tetrachloride, is 5 mg/m^3. The OSHA PEL is also 5 mg/m^3, but this is for zirconium metal only.[20] The ATSDR has not developed MRLs or a toxicology profile for zirconium.[18] The EPA has not developed an IRIS profile for zirconium.[19] The advisory, screening, and regulatory values

provided herein are revised as new information becomes available. The reader is referred to the responsible agencies/organizations for the most up-to-date values.

QUESTIONS

17.1 How are essential metals distinguished from nonessential metals?

Answer: Quantifying dose–response is fundamental to distinguish essential and nonessential metals. The concept is illustrated by hypothetical dose–response curves. Only essential metals exhibit increasing toxicity with decreasing dose (Figure 17.1a). Thus, only essential metals can generate a U-shaped dose–response curve (Figure 17.1c).

17.2 What are the three fundamental determinants (physical, chemical, or biological) of metal toxicity?

Answer:

a. The strength with which a metal complexes with a molecular ligand. This is a physical–chemical determinant that characterizes nonessential metals' potential to displace essential metals from metalloproteins.
b. The biological significance of the function of the metalloprotein in which the essential metal has been displaced.
c. The potential of a metal to participate in generation of reactive oxygen species.

17.3 Serious side effects are associated with therapy using some of the metal chelators. Newer analogs like the hydrophilic chelator, meso-2,3-dimercaptosuccinic acid, are less toxic because they effectively promote renal metal excretion. What is the limitation of hydrophilic chelators, and what are the two new strategies for addressing this limitation?

Answer: The trade-off for the benefit of efficient excretion is that hydrophilicity limits access to intracellular metals. Newer strategies attempt to address this limitation by (1) combining structurally different chelating agents or (2) coadministering antioxidants (see Figure 17.2).

17.4 Why have zirconium salts been used to treat acute and chronic plutonium poisoning?

Answer: The strategy was to exchange the low metal toxicity of zirconium for the higher toxicity of the plutonium radionuclide. This is possible because the ionic radius of zirconium is similar to plutonium, and this enables zirconium to displace plutonium from its deposition in the skeleton and to prevent the deposition when treatment is started early.

17.5 The toxicity of inorganic lead is well documented. Why has neither an RfD nor chronic MRLs been established for lead?

Answer: The adverse health effects associated with lead occur at blood lead levels so low as to be without a threshold.

KEYWORDS

Adequate intake; Contact dermatitis; Dietary reference intakes; Essential nutrients; Fenton reaction; Ferritin; Mees' lines; Metallothionein; Recommended dietary allowance; Tolerable upper intake level

ACRONYMS

ACGIH	American Conference of Governmental Industrial Hygienists
AI	Adequate intake
ATP	Adenosine triphosphate
CAA	Clean Air Act
CDC	Centers for Disease Control and Prevention
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CNS	Central nervous system
CWA	Clean Water Act
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FDCA	Food, Drug, and Cosmetic Act
IARC	International Agency for Research on Cancer
IOM	Institute of Medicine, a National Academy of Sciences
IRIS	Integrated Risk Information System
LOAEL	Lowest-observed-adverse-effect level
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NIOSH	National Institute of Occupational Safety and Health
NOAEL	No observed adverse effect level
NOEL	No observed effect level
NPDES	National Pollutant Discharge Elimination System
NTP	U.S. National Toxicology Program
OSHA	Occupational Safety and Health Administration
PBPK	Physiologically based pharmacokinetics
PEL	Permissible exposure limit
RCRA	Resource Conservation and Recovery Act
RDA	Recommended dietary allowance
REL	Recommended exposure limit
SDWA	Safe Drinking Water Act
STEL	Short-term exposure limit
TLV	Threshold limit value
TSCA	Toxic Substances Control Act
TWA	Time-weighted average
UL	Tolerable upper intake level
USDA	United States Department of Agriculture

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18 Radiation Toxicity

Roger O. McClellan

CONTENTS

INTRODUCTION

The toxicology of ionizing radiation is a highly specialized area of toxicology of substantial breadth and depth. It is obvious that it cannot be covered in great detail in a single textbook chapter. Nonetheless, recognition that life exists within a *sea of ionizing radiation* of natural origin and that a number of important medical and industrial applications of radiation may result in exposure of humans and other life forms to radiation doses above the background requires that well-educated toxicologists be informed about basic concepts of radiation toxicology. Moreover, many of those basic concepts are also relevant to understanding the toxic effects of nonradioactive agents; to assessing their hazard, exposure, dose, and health risks; and to controlling those health hazards and risks. To meet the needs articulated earlier, the presentation of this chapter is of a survey nature with specific and general references provided that will assist the interested reader in obtaining a more detailed understanding of the subject.

Despite the survey nature of this chapter, it is important to recognize that radiation as a toxic agent has been more extensively investigated than any other single toxic agent or broad class of toxicants. The extraordinary amount of information resulting from those investigations has been reported in detail in innumerable original scientific papers and reviews published in peer-reviewed scientific journals. The vast majority of the papers have been published in English language journals; however, many have also appeared in journals published in the major languages of the world. In addition to the papers published in scientific journals, many nonclassified government reports contain detailed research findings and analyses of research conducted between the 1940s and the 1980s.

In addition to the published original research findings, a number of authoritative scientific organizations have regularly issued integrative reports summarizing current knowledge on the health effects of exposure to radiation and radioactive materials. Most notable are the reports of the National Council on Radiation Protection and Measurements (NCRP), a U.S. organization; the International Commission on Radiological Protection (ICRP); the United Nations scientific Committee on the Effects of Atomic Radiation (UNSCEAR); the International Atomic Energy Agency (IAEA), the Atomic Bomb Casualty Commission (ABCC), now operating as the Radiation Effects Research Foundation (RERF); and various committees on Biological Effects of Ionizing Radiation (BEIR) of the National Research Council (NRC) of the U.S. National Academies.

This chapter is concerned with ionizing radiation, which is energy in the form of waves or particles that during passage through matter transfer energy to the matter. The transfer of energy can remove electrons from the orbit of atoms, leading to the formation of ions, hence the term *ionizing radiation*. There are two general types of ionizing radiation: electromagnetic and particulate radiation. The electromagnetic radiation includes x-rays and gamma rays. The particulate radiation includes alpha particles, beta particles, positrons (positive electrons), neutrons, protons, and heavy ions (such as charged nuclei). This chapter is not concerned with nonionizing radiation such as ultraviolet radiation, radiofrequency radiation such as microwaves, and extremely-low-frequency radiation such as that associated with electric power lines. Suffice it to note, ionizing radiation is far more harmful to living organisms per unit of energy deposited than nonionizing radiation, since ionizing radiation can cause damage to deoxyribonucleic acid (DNA). Ionizing radiation can be measured in air, liquid media, or tissue. Radiation exposure is the absorption of ionizing radiation by an object such as the mammalian body, tissues, or organelles. Radioactivity is the property or characteristic of an unstable atomic nucleus to transform with the emission of energy in the form of rays or particles.

Readers interested in more detailed coverage of the fundamentals of radiation and its effects on living systems will find the textbooks of the following authors useful: Bushong (2013), Hall and Giaccia (2012), Turner (2007), Mettler and Upton (2008), and Till and Grogan (2008). The historical treatise (Stannard and Baalman, 1988) remains a valuable reference on the toxicity of radioactive materials. The classic document on the effects of nuclear weapons by Glasstone and Dolan (1977) provides a historical context for considering the health effects associated with detonating nuclear weapons. Moreover, reading about the effects of nuclear weapons will emphasize the importance of achieving international control of them. In addition, it is important for toxicologists and other scientists to distinguish between concern for the effects of nuclear weapons and the very different issues related to peaceful uses of nuclear energy, radiation sources, and radionuclides. A nuclear weapon and a nuclear reactor are not equivalent.

To aid the reader, some definitions are provided in Table 18.1 for key words and phrases regularly used in radiation science that will augment the "Glossary" at the end of this textbook. Table 18.2 provides a concise summary of the major units used in radiation science and radiation protection. These radiation units evolved as the field of radiation science, and radiation protection developed. The earliest units, termed conventional units, were ultimately superseded by the newer International System of Units (SI), which were most enthusiastically embraced first in Europe and have been accepted much more slowly in the United States. As you read this chapter, take care in identifying the specific units being used since both conventional units and International Units are used consistent with what the authors used in the various original papers and reports. Moreover, it is important to recognize units such as a curie or becquerel (Bq) with different prefixes can be used to describe concentrations of radioactivity spanning many orders of magnitude analogous to going from micrograms to kilograms of chemical mass.

It was a special pleasure for me to prepare this chapter on radiation toxicity since it provided me the opportunity to revisit how my career has been impacted by my personal relationship with the world of radiation, which I note at various places in the chapter. This includes moving in 1944 to Richland, Washington, adjacent to the

TABLE 18.1
Key Definitions in Radiation Science and the Effects of Radiation on Health

ALARA	Principle that states that radiation exposure should be kept as low as reasonably achievable when social and economic factors are taken into account.
Alpha particle	Particle with mass equivalent to a helium nucleus, two protons and two neutrons.
Atom	Smallest particle of an element that cannot be divided or broken by chemical means.
Beta particle	A charged particle of very small mass emitted spontaneously from the nuclei of certain radioactive elements. Physically, a beta particle is identical to an electron moving at high velocity.
Deterministic effect	A biological response whose severity increases with absorbed dose. A dose threshold usually exists.
Element	One of the distinct, basic varieties of matter occurring in nature.
Fission	The process whereby the nucleus of a particular heavy element following the absorption of a neutron splits into two nuclei of lighter elements with the release of substantial energy. Controlled and sustained fission occurs in a nuclear reactor. Fission of ^{239}Pu and ^{235}U is a key component of the detonation of nuclear weapons.
Fusion	The process whereby the nuclei of lighter elements, especially those of hydrogen (deuterium and tritium), combine to form the nucleus of heavier elements with the release of substantial energy. A thermonuclear weapon involves fission followed by fusion.
Gamma rays	Electromagnetic radiation of variable energy originating in atomic nuclei. Physically, x-rays and gamma rays are identical.
Ionizing radiation	Radiation capable of causing ionization.
Irradiated	Referring to matter that intercepts radiation and absorbs part or all of it: exposed.
Isotopes	Forms of the same element having identical chemical properties but differing in atomic mass (due to different numbers of neutrons in their respective nuclei) and nuclear properties (radioactivity).
Linear energy transfer (LET)	Measure of the rate at which energy is transferred from ionizing radiation to tissue. Expressed in kiloelectron volts per micrometer of soft tissue.
Nuclide	An atomic species of an element distinguished by the composition of its nucleus, i.e., the number of protons and neutrons.
Radioactive half-life	The time required for the activity of a given radionuclide to decrease to half its initial value due to radioactive decay.
Radionuclide or radioisotope	An isotope or nuclide that is unstable and will spontaneously decay by emission of alpha or beta particles.
Stochastic effects	Probability or frequency of the biological response to radiation as a function of radiation dose. Disease incidence, such as cancer, increased proportionally with dose, and the absence of a threshold is usually assumed. The severity of the disease is not influenced by dose.
X-rays	Penetrating, ionizing electromagnetic radiation that has a wavelength much shorter than that of visible light. Physically, x-rays and gamma rays are identical.

TABLE 18.2
International Units for Radiation and Radioactivity Compared to Old Units

International Units	Description	Old Units
Becquerel (Bq)	Radioactivity, the spontaneous decay of atomic nuclei 1 Bq = 1 disintegration/s	Curie = 3.7×10^{10} Bq
Gray (Gy)	Dose to tissue 1 Gy = energy uptake of 1 J/kg	Rad[a] = 10 mGy
Sievert (Sv)	Effective dose,[b] dose normalized to effects of gamma radiation by applying a W_R based on the relative biological effectiveness (RBE) of the radiation of interest, to the absorbed dose in an organ or tissue to derive the equivalent dose. The effective dose is usually expressed as millisievert (mSv). 1 mSv = 10^{-3} Sv	Rem[c] = 10 mSv

[a] Rad, originally radiation absorbed dose.
[b] Allows conversion of dose from gamma, beta, or alpha radiation to a standard unit.
[c] Rem, originally based on roentgen equivalent man.

emerging Hanford Nuclear Site; working with scientists at the Hanford Laboratories as a high school and university student beginning in 1950; serving on the staff of the Hanford Laboratories full time in 1960–1964, a special assignment with the Division of Biology and Medicine of the U.S. Atomic Energy Commission (AEC) in 1965–1966; serving as the director of the Inhalation Toxicology Research Institute at the Lovelace organization in Albuquerque, New Mexico, from 1966 to 1988; and serving as the president of the Chemical Industry Institute of Toxicology from 1988 to 1999. In each of these organizations, I had the pleasure of working with exceptionally competent scientists, engineers,

and support personnel who valued the use of a multidisciplinary teamwork approach to address important societal issues. The teamwork approach extended broadly to other research organizations, government agencies, scientific societies, and entities like the NCRPs, which I served on from 1971 to 2001, and the National Academies that were part of a broader collaboration creating scientific knowledge to inform important societal decisions. I hope you will indulge my personal and historical approach to reviewing radiation toxicology from its roots to the present time.

KEY CONCEPTS

This chapter will be useful for the reader to have in mind four overarching concepts or themes that will aid in understanding the health effects of radiation: (1) Radiation and radioactivity are readily measured; (2) radiation and radioactivity are everywhere; (3) exposure–dose–response relationships are of prime concern; and (4) not all doses of ionizing radiation are equally effective in causing effects. It will be evident that these concepts are also important to understand and assess the health hazards and risks of chemicals.

Radiation and Radioactivity Are Readily Measured

The first concept is that both ionizing radiation and radioactivity are readily measured and quantified down to extraordinarily low levels. Indeed, the ease with which radiation or radioactivity can be measured is a great advantage to conduct research on radiation and radioactivity and evaluate potential health risks. However, the ability to measure extraordinarily low levels also presents the challenge of differentiating between quantities of radiation and radioactivity that can be measured at exceptionally low levels and have no associated harm versus measured quantities that may be harmful. A statistically significant measurement of radiation or radioactivity does not automatically equate to a harmful level. It is also important to recognize that units of measured radiation and measured radioactivity are not interchangeable.

Radiation and Radioactivity Are Everywhere

The second concept is that human kind and all living things exist in a sea of radiation and radioactivity. Humans and other organisms can be exposed to ionizing radiation originating from both external and internal sources. The pathways for radioactivity to reach humans and give rise to internal irradiation are illustrated in Figure 18.1.

Sources external to the body include cosmic radiation and radiation coming from naturally occurring radionuclides in the environment, direct radiation from nuclear detonations such as occurred at Hiroshima and Nagasaki and from nuclear weapons tests and external exposure from deposited radioactivity dispersed from testing of nuclear weapons or released from accidents such as occurred at Windscale, Chernobyl, and Fukushima. Many medical diagnostic and therapeutic applications of radiation also involve the use of external sources. The external nature of these radiation exposures is different from the case with chemicals where exposure usually refers to chemicals entering the body. The external exposures to ionizing radiation can be very brief, essentially instantaneous as occurs immediately with detonation of a nuclear weapon or in many medical procedures (Figure 18.2). Protracted external exposures, overextended periods of time, can occur such as from cosmic rays or gamma rays from released radioactivity contaminating surfaces.

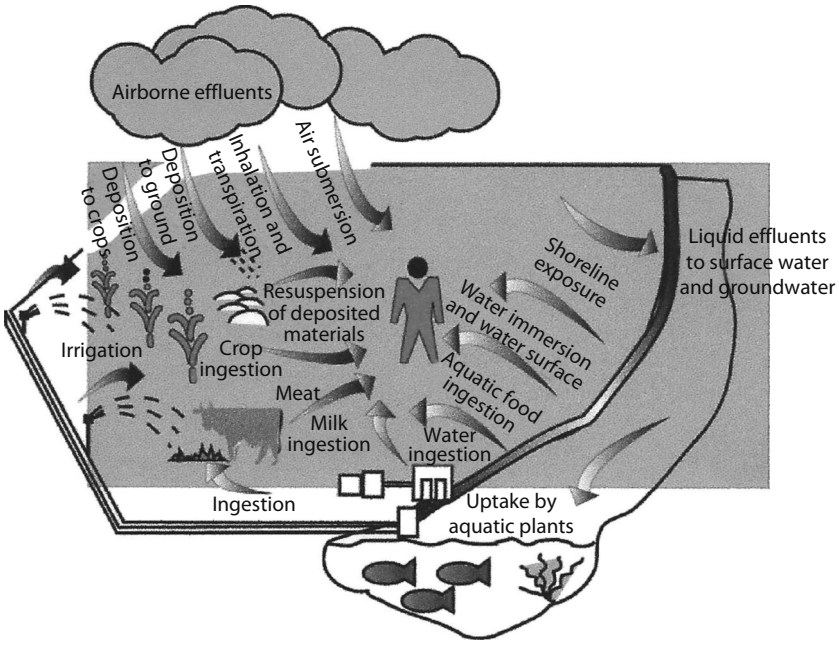

FIGURE 18.1 Schematic representation of pathways for radioactivity to reach humans. (From Till, J.E. and Grogan, H.A., eds., *Radiological Risk Assessment and Environmental Analysis*, Oxford University Press, New York, 2008.)

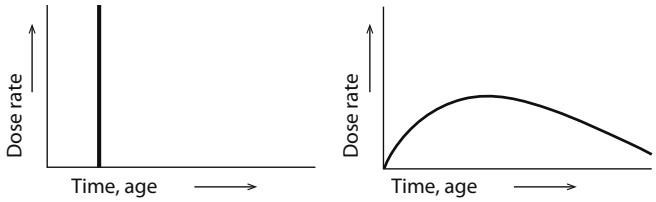

FIGURE 18.2 External brief radiation exposure and resulting tissue dose occur simultaneously while the intake of radioactivity results in a protracted tissue dose.

The situation is quite different for internally deposited radionuclides entering the body via the pathways illustrated in Figure 18.1. Radionuclides of natural origin and man-made may enter the body by ingestion, by inhalation, through wounds, and by absorption through the skin or by purposeful injection in a medical application. Entry of radionuclides taken into the body by these avenues is similar to what occurs with chemicals. However, ionizing energy from internally deposited radionuclides is released over a period of time dependent upon the physical half-life of the radionuclide and the biological characteristics of the radionuclide itself or the chemical compound or matrix in which it is incorporated. The time course for exposure and delivery of the radiation dose is not the same for internally deposited radionuclides.

Soon after the discovery of naturally occurring radioactive elements, their wide distribution in nature became evident. This evidence for the widespread distribution of naturally occurring radionuclides mounted as new and more sensitive radiation detection instruments and measurement techniques were developed. The likelihood of man-made radionuclides, such as those produced by nuclear fission being released and distributed in the environment, was appreciated early in the development of the Manhattan Project for each major production site, such as Hanford and Oak Ridge. The schematic rendering in Figure 18.1 illustrates the pathways for such releases. Because radioactivity can be readily detected and quantified, it can be readily traced in the environment. For example, at Hanford, a large quantity of water passed through the reactors for thermal cooling, was retained briefly in ponds to allow cooling of the water and decay of short-lived radionuclides, and was then released to the Columbia River. Trace quantities of some stable elements in the cooling water were activated to become radioactive. For example, chromate was added to the reactor cooling water to inhibit corrosion. Trace amounts were activated to ^{51}Cr. Trace quantities of phosphorus was activated to ^{32}P. The fuel elements containing uranium occasionally ruptured, releasing fission or activation products such as ^{65}Zn. The radionuclides in the water were available for uptake by aquatic biota, including fish. The fish may then be caught and consumed by people.

The pathway of reactor effluent–river water–fish for human contamination was recognized and studied at the Hanford Nuclear Site. Waterfowl were found to have ingested contaminated aquatic biota and recognized as a source of human contamination. With the initial dissolution and chemical processing of irradiated uranium fuel elements at Hanford, it became apparent that volatile radionuclides such as ^{131}I could be released to the atmosphere and deposited as *fallout* on forage, which would then be ingested by grazing animals. ^{131}I was soon detected in the thyroids of wild rabbits and deer on the plant site and in the thyroids of coyotes that had ingested the contaminated rabbits. Concern was immediately raised for the transfer of ^{131}I to domestic sheep and cattle. The potential was recognized for ^{131}I deposited on forage to be ingested by dairy cows with contamination of milk potentially ingested by humans and resulting in thyroid uptake and irradiation.

It was also recognized that river water contaminated with radioactivity might be used for irrigation. This could be a source of uptake by plant roots or foliar deposition and absorption if sprinkler irrigation were used. Dairy cattle could ingest contaminated pasture or hay with subsequent transfer to milk. This pathway was found to be operative for trace quantities of ^{65}Zn released from the Hanford reactors. As a student, I studied how soil characteristics such as pH influenced the uptake of ^{65}Zn by bean plants. At Hanford, there were also instances in early operations in which radioactive particles were released from the stacks of the fuel-reprocessing facility. The *hot* particles containing ^{106}Ru were readily detected on the plant site and became of concern as a source of skin irradiation if deposited on the skin of humans or animals.

Exposure–Dose–Response Relationships

The third concept that must be addressed to understand the health effects of radiation and radioactivity is the nature of the exposure–dose–response relationship for a range of health endpoints. An overarching conceptual framework for considering the multiple events linking exposure to external radiation or radionuclides with tissue dose, mechanistic events, and health responses is shown in Figure 18.3. This framework shows acute effects, including lethality associated with high levels of external radiation exposure and very large radionuclide intakes over short periods of time that are required to produce the radiation doses required to cause lethality in a matter of days to months. Much greater attention and controversy relate to chronic or late-occurring health effects such as heritable effects in the progeny of irradiated individuals or cancers caused by irradiation with cancers appearing after a latent period of years to decades.

In the case of radiation, it is important to distinguish between exposure and dose. Exposure refers to a measurement of the ionization produced in air, for example, from gamma rays or x-rays. Radiation exposures, that is, ionization in air, can be measured down to very low levels. Exposure also refers to the intake of radioactivity such as by oral intake or inhalation. Dose is the amount of radiant energy absorbed by the body, organ, or specific tissue volume from either external sources or internally deposited radioactivity. The radiation dose can be measured or calculated with a high degree of precision. Thus, in the field of radiation toxicology and radiation protection when reference is made to dose, it has a very specific meaning. This situation is very different

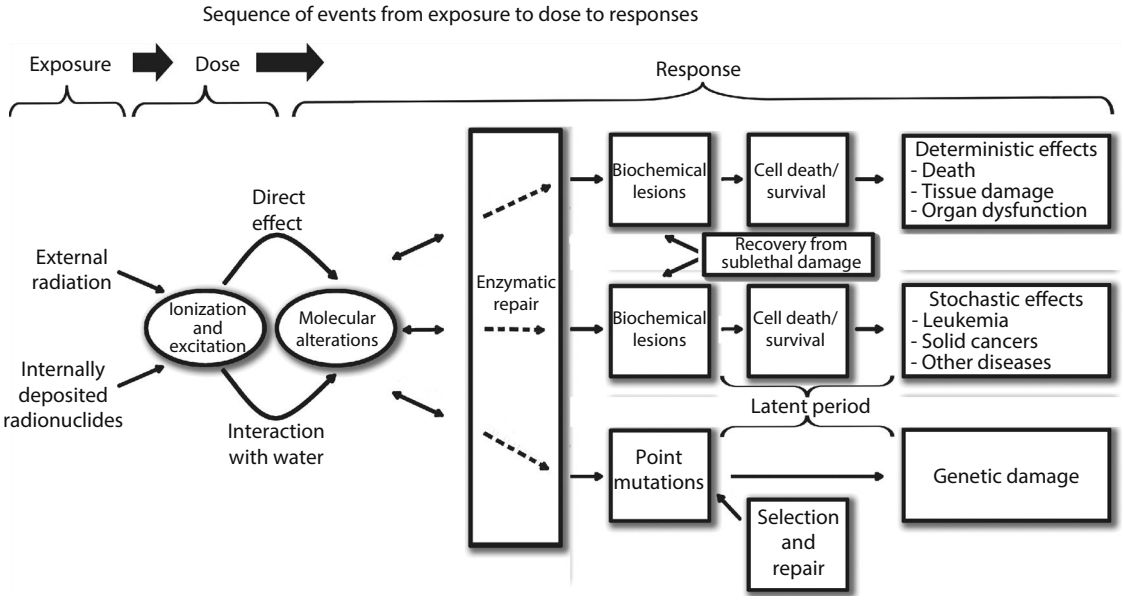

FIGURE 18.3 Conceptual framework linking events from exposure to external sources or internally deposited radioactivity to dose to biological responses. (Adapted from Bushong, S.C., *Radiological Science for Technologists: Physics, Biology and Protection*, 10th edition, Elsevier/Mosby, St. Louis, MO, 2013.)

from the field of chemical toxicology where the words *exposure* and *dose* are frequently used in interchangeable fashion.

A related issue linked to the dose–response relationship is the nature of the responses considered in two broad categories—deterministic effects or stochastic effects, terms commonly used in radiation biology and radiation protection. Deterministic effects or responses occur above some threshold dose of radiation and increase in severity as the radiation dose increases. At lower radiation doses, deterministic effects are not observed. Erythema or reddening of the skin and development of cataracts of the lens are classic deterministic effects. Stochastic effects, occurring at random, are those responses or diseases whose probability of occurrence is a function of radiation dose in the absence of a threshold with the severity of the response or disease being independent of the radiation dose. In general, the initial event leading to a stochastic response is thought to occur in a single cell. Induction of cancer is a classical stochastic effect of radiation.

Paul Henshaw (1941) published one of the earlier and very insightful discussions of the nature of dose–response relationships for different kinds of responses. In this landmark paper, he discussed two fundamental dose–response relationships for radiation: (A) a no-threshold dose–response relationship with the response incidence increasing with dose and (B) a threshold dose–response relationship with a threshold dose below which responses would not be observed (Figure 18.4). He also discussed the various types of injury and responses that radiation exposure produced and which type of curve was applicable to the response. Later the effects associated with curve A would be identified as stochastic effects and those associated with curve B as deterministic effects. This issue has received substantial attention in the field of radiation research and in the development of radiation protection guidance. Ironically,

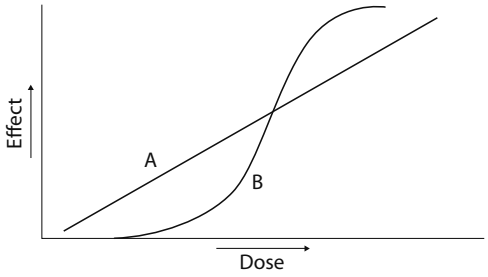

FIGURE 18.4 Schematic rendering of two fundamentally different dose–effect relationships for radiation. (Adapted from Henshaw, P.S., *J. Natl Cancer Inst*., 1, 789, 1941.)

this same issue came to the forefront only decades later in the field of chemical toxicology and in the development of guidance and regulations to protect against chemical hazards. Post–World War II, discussion began of the potential for hormetic effects with low radiation doses.

Not All Radiation Is Equally Effective in Causing Effects

The fourth concept must be considered in understanding the effects of radiation: The effectiveness of a given absorbed dose of ionizing radiation will be influenced by both radiation quality and the rate at which a given dose is delivered. In addition, the biological characteristics of the organ or tissue receiving the radiation dose will influence the nature of the health response. The quality of radiation is a function of the linear energy transfer (LET) of the radiation, for example, low-LET gamma or x-ray photons or beta particles compared with high-LET alpha particles. The influence of radiation quality is

usually assessed by comparing the Relative Biological Effects (RBEs) of a particular radiation with low-LET gamma irradiation or x-irradiation. Another consideration is that the effectiveness of a given absorbed dose of radiation may be influenced by the dose rate at which the dose is delivered (recall Figure 18.2). These latter issues are of particular concern for internally deposited radionuclides, situations that are analogous in some ways to internally deposited chemicals.

As you proceed through the chapter, these three themes will emerge and recur. In the conclusion to the chapter, these themes will be reviewed in the context of radiation as well as the insights they provide for chemical toxicology and risk assessment.

HISTORY OF RADIATION AND RADIOACTIVITY

Discovery of Radiation and Radioactivity to Discovery of Fission

Radiation and radioactivity have been part of the universe from its origin with detailed knowledge of radiation and radioactivity evolving as a result of human curiosity, serendipitous discoveries, and purposeful research. As will be discussed later, human activity has resulted in the creation of some unique, new radioactive elements and many new radionuclides of existing naturally occurring elements. Moreover, human activities have resulted in enhanced concentrations of some naturally occurring radioactive materials (NORM).

The German physicist, Wilhelm Conrad Roentgen, in 1895, discovered a new kind of rays emitted by a gas discharge tube that could blacken photographic films. Roentgen was studying the conduction of cathode rays, now known as electrons, through a glass tube partially filled with gas. This tube, called a Crookes' tube after its founder, was a forerunner of today's fluorescent light bulb. So that Roentgen could better see the light produced in the tube, he darkened the room and covered the tube with paper. By chance, a few feet away was a photographic plate coated with a fluorescent material, barium platinocyanide. When he activated the tube, he noted that the fluorescence plate glowed, and the intensity increased when he moved the plate closer to the tube. Because the rays represented the unknown, he called them x-light or x-rays. Roentgen soon discovered that the x-rays could be used to visualize the skeleton. He published the first medical radiograph, an image of the skeleton in his wife's hand. On January 23, 1896, *Lancet* reported the first medical diagnostic use of x-rays in medicine. The report detailed the finding of a broken part of a knife in a drunken sailor who was paralyzed until the piece of the knife was removed. In 1896, an Austrian surgeon, Leopold Freund, demonstrated that treatment of a hairy mole with x-rays caused the mole to disappear. This represented the first therapeutic medical application of radiation. Today, both diagnostic and therapeutic applications of radiation are an integral part of the practice of medicine.

The second edition of Gmelin's *Handbook of Chemistry*, in 1824, included a description of uranium. By the mid-1800s, it was being used in homeopathic medicine to treat diseases such as diabetes. In 1895, Ernest Rutherford showed that uranium *emanations* had the spectral line of helium. In 1896, Antoine Henri Becquerel discovered radiation emissions from uranium compounds. Pierre and Marie Curie in 1898 isolated polonium and radium from pitchblende ore containing uranium and its decay products and coined the term *radioactivity* to describe these materials as distinct from radiation. Rutherford in 1904 showed that alpha particles are helium atoms and worked out the decay scheme for the uranium series as will be discussed later.

The pitchblende ore and the elements uranium, radium, and polonium used by the early investigators came from mines in Schneeberg and Joachimstal in the Erz Mountains of Central Europe. It was known in the 1400s that miners there suffered a chronic respiratory malady. In 1879, two German investigators, Härtung and Hesse, showed that a majority of the deaths of miners were due to malignant lung tumors. In the early 1900s, the tumors were confirmed to be of bronchogenic origin, and it was suggested that radium and radium emanations had a causative role. Ironically, the Schneeberg/Joachimstal experience would be repeated in the mid-1900s in miners working in uranium mines in the western slope of the U.S. Rocky Mountains, yet later in uranium mines in other countries, and even later in tin mines in China.

It is reported that Becquerel carried a vial of radium in his vest pocket, which inadvertently produced erythema and ulceration in his underlying skin. It is said that Pierre Curie purposefully repeated the experience producing a radiation burn on his forearm and studied the subsequent healing.

The use of x-rays as a medical diagnostic procedure and use of implanted radon needles to treat cancer grew rapidly in Europe and North America and soon gained worldwide acceptance. X-ray machines for diagnostic purposes were considered essential in even small hospitals and clinics. Large x-ray machines with higher-energy x-rays for cancer treatment were found in all major hospitals. During the early 1900s, hospitals in major cities had facilities for loading needles with radon for implantation into cancers. As an aside, I learned that one of my great grandmothers traveled, in 1910, from a small town in Iowa by train to Chicago to be treated for cancer with surgery and radiation.

Soon after Roentgen's discovery of x-rays, research began on the potential, injurious effects of exposure to x-rays. Later, the research would expand to include naturally occurring radionuclides. The results of much of that early research were reviewed by Henshaw (1941). These studies soon demonstrated damaging effects on the reproductive organs and hematopoietic tissue. While studying the effects of x-rays on plants, Atkinson (1898) was bewildered that deleterious effects were not always observed at low doses. This may be the first report of hormetic effects of radiation.

In the early 1900s, gas mantles began to be manufactured using thorium (Thorotrast), which began to be used as a contrast medium in diagnostic radiology.

In the early 1900s, it was discovered that trace quantities of radium and thorium salts could be mixed with phosphors,

which resulted in phosphorescence. Subsequently, young women were employed to paint watch and instrument dials using this mixture. Tipping the fine brushes on their lips resulted in the women ingesting the radium and thorium, which translocated to the skeleton. Later, these individuals would develop hematopoietic anomalies, bone disease, and bone cancer. This tragic experience will be discussed later.

In this same era, it became fashionable to visit spas, especially those with radioactive water and radon in the air, for the alleged therapeutic benefits. Nostrums containing radium and radon soon came on the market. Throughout the 1920s, many individuals were injected with radium or ingested it for a variety of real and fancied disorders. A prominent industrialist regularly ingested sufficient radiothor, a mixture of ^{226}Ra and ^{222}Ra (radiothorium); when he died of radium poisoning, it attracted national attention.

Rutherford, in 1919, observed that bombardment of nitrogen atoms by alpha particles from natural sources produced oxygen. Between 1933 and 1934, Jean Frederic Joliot and Irene Curie (Pierre and Marie Curie's daughter) were the first to produce a new radioisotope by transmutating another element. They bombarded various light metals with alpha particles from ^{210}Po, producing new radioelements. Bombarding boron produced ^{13}N, bombarding aluminum produced ^{30}P, and bombarding magnesium produced ^{27}Si.

In the 1920s, George de Heresy began conducting *tracer* studies of the uptake and translocation of lead in plants, tracing a stable element, lead, by using one of its radioactive decay products. These studies were soon extended to animals.

H.S. Müller (1927) made the seminal finding that exposure to x-rays increased the mutation rate of fruit flies. He irradiated adult flies with high doses of x-rays and then measured the frequency of lethal mutations in the progeny. He reported that the response was linear with increasing dose and did not have a threshold. It is ironic that the Müller (1927) paper is frequently cited for the discovery of the mutagenic effects of radiation. This is actually not the case. Indeed, Müller cites a number of papers discussing the mutagenic effects of radiation, including the Mohr (1919) paper. Of special note is a paper by Oliver (1930), who worked with Müller at the University of Texas. The paper provides quantitative data on the effects of radiation on induction of mutations in *Drosophila*. Because of its historical importance, the Oliver (1930) data are plotted in Figure 18.5. It is noteworthy that the doses were very high, ranging from 1000 to 4000 R. Radiation exposure soon became a favorite approach to inducing genetic alterations, especially in *Drosophila* and many plant species, and a favorite tool of plant breeders developing new and improved commodity crops such as wheat.

In 1932, Ernest Lawrence at the University of California (UC)-Berkeley, invented the cyclotron, a device that created high-speed protons without using high voltages. The first cyclotron was 12 cm in diameter. The accelerated high-speed protons in the cyclotron could be used to bombard an array of different target materials, producing new radioisotopes.

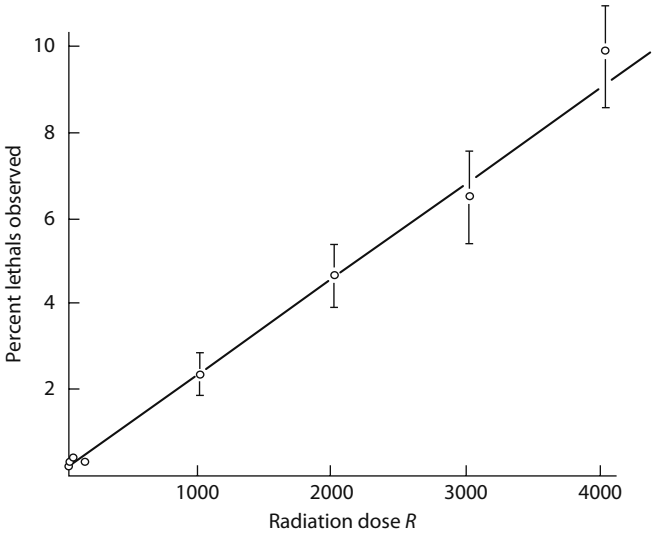

FIGURE 18.5 Dose–response relationship for induction of mutations in x-irradiated *Drosophila*. Note the substantial radiation doses studied. (From Oliver, C.P., *Science*, 71, 44, 1930.)

Ernest Lawrence's brother, John Lawrence, was a physician. This no doubt served as a stimulus in developing many radioisotopes that had potential application in biomedical research and medical practice. With the availability of many new radioisotopes produced by the cyclotron, the opportunities for tracer studies of the uptake and fate of elements in various chemical forms were virtually limitless. John Lawrence, Joseph Hamilton, and others at UC-Berkeley conducted many of the early radiotracer disposition studies in laboratory animals and people as they worked their way through many elements in the periodic table. It had never been so easy to obtain quantitative data on the disposition of different elements in different chemical forms.

The first *radiotracer* studies in domestic animals were conducted at the Agricultural Experiment Station, *The Farm*, at Davis, CA. This was originally a part of the UC-Berkeley system. Dr. Max Kleiber conducted the animal radiotracer studies. Kleiber came to the UC in 1929 to conduct studies on energy metabolism in animals. The story is told that Dr. John Lawrence had some extra ^{32}P, deemed unsuitable for injection into humans, called Kleiber, and offered it for injection into a cow. Kleiber went on to conduct numerous studies in a wide range of animal species with many different radiotracers.

With the availability of ^{59}Fe, it was natural that John Lawrence and his colleagues would conduct some of the earlier radiotracer studies on erythropoiesis in animals and humans. In 1936, Lawrence treated a polycythemia patient with ^{32}P to irradiate the bone marrow. This was probably the first therapeutic use of a man-made radionuclide.

On the threshold of World War II, Henshaw (1941) published a seminal paper that reviewed very succinctly what was known about the effects of exposure to x-rays. This included the various types of injury that had been observed up to that time with considerable focus on what he termed *radiogenetic changes*. He presented two conceptual dose–response

frameworks (Figure 18.4) for radiation exposure and discussed the use of *tolerance dose* in radiation protection, including its limitations for protecting against *radiogenetic effects*.

In December 1938, German chemists Otto Hahn and Fritz Strassmann sent a manuscript to *Naturwissenschaften* (January 6, 1939) reporting that they had detected the much lighter element, barium, after bombarding uranium with neutrons. They shared these results by mail with Lisa Meitner, who had lost her German citizenship because of her Jewish lineage and relocated to Sweden, and her nephew, Otto Frisch, who was visiting her. Meitner and Frisch correctly interpreted the results as being nuclear fission, a splitting of the atom (*Nature*, February 11, 1939). Frisch called the process fission as an analogy to binary fission in biological cells, and the resulting new fission products were called daughter products. The fields of physics and biology were joined!

Events after Discovery of Fission

The discovery of fission had significant consequences. Fission was key to the development of what were initially called atomic bombs, later identified as nuclear bombs or weapons, and yet later the development of nuclear power reactors. The United States, early during World War II, created the Office of Scientific Research and Development to coordinate research aligned with national defense, including research on nuclear materials. This office sponsored Enrico Fermi's research to demonstrate that a self-sustaining chain reaction of nuclear fission in natural uranium could be produced and that uranium could be transmutated to higher atomic number elements. Fermi, using space under the Stagg's field grandstand at the University of Chicago, assembled a *pile* of highly purified graphite to moderate the neutrons from natural uranium. Frank Spedding at Iowa State University produced the high-purity uranium. On December 2, 1942, Fermi and his crew demonstrated that a sustained chain reaction producing neutrons in the uranium pile could be started and stopped by introducing neutron-absorbing materials such as boron.

In early 1943, the responsibilities of the Office of Scientific Research and Development were transferred to the U.S. Army Corps of Engineers and a new secret entity created, the Manhattan Engineer District. The history of the Manhattan Project has been chronicled by Hewlett and Anderson (1962), Rhodes (1986), and Kelly (2007). The Manhattan Project had the specific purpose of developing an atomic bomb as soon as possible, anticipating the use of atomic bombs in ending World War II. Creation of atomic bombs involved the production of sufficient quantities of two highly purified fissile materials, ^{235}U and ^{239}Pu, and development of a means to assemble them into a weapon. A fissile material is capable of sustaining a chain reaction of nuclear fission with thermal neutrons. The project, under the direction of General Leslie R. Groves, involved activities at multiple sites, including several universities, most notably the University of Chicago, Iowa State University, and UC-Berkeley. Three specialized sites were created de novo to meet the unique needs of the project. A laboratory site at Los Alamos, New Mexico, under the direction of J. Robert Oppenheimer, was created to design, fabricate, and test the first atomic bomb and manufacture additional atomic bombs. The UC was the prime contractor.

A site at Oak Ridge, Tennessee, would house unique facilities to separate ^{235}U from a mixture of ^{235}U and ^{238}U and an experimental pile or reactor. Natural uranium only contains about 0.72% ^{235}U by weight; devise methods for concentrating the ^{235}U to 2–5% and higher to support a continuous nuclear chain reaction in a carbon/water-moderated reactor and to substantially higher concentrations and purity for use in a bomb. The Oak Ridge facility used a combination of thermal diffusion followed by gaseous diffusion and electromagnetic isotope separation to provide material for a bomb. The ^{235}U was shipped to Los Alamos for fabrication into one type of bomb. The Union Carbide Company, a diversified chemical company, was the prime contractor for the Oak Ridge site. A decision was made that the population of the combined population of the Oak Ridge, Tennessee area and nearby Knoxville, Tennessee was too large to consider using it as a site for large nuclear reactors and chemical separation facilities required to produce kilogram quantities of ^{239}Pu for constructing a second type of bomb.

Thus, it was determined that a third large remote site would be needed for a *plutonium production complex*. The site had to be remote from any significant population, accommodate a large *hazardous manufacturing area*, and have an abundant supply of clean water and electrical power. In January 1943, General Groves, acting on the advice of Colonel Franklin T. Mathias and DuPont engineers, selected a site in southeast Washington for what was initially called the Hanford Engineering Works (HEW). The U.S. government exercised *eminent domain* and quickly displaced about 1500 residents of three small farming communities (Hanford, White Bluffs, and Richland, Washington) in the area. Hanford was the name of the community that would become the construction camp used until the reactors and fuel-reprocessing facilities were completed. Then the town site was abandoned. The E. I. DuPont de Nemours and Company was selected as the initial prime contractor for the Hanford site based on the company's unique capabilities to design, construct, and operate facilities producing highly hazardous materials. Recall that the DuPont Company was originally founded as a gun powder mill and evolved into a diversified chemical company. A workforce that soon exceeded 40,000 individuals began constructing (1) three uranium-fueled *piles*, (2) a fuel fabrication facility, (3) a chemical separation complex to separate ^{239}Pu from irradiated fuel elements, (4) support facilities, and (5) the vastly enlarged Richland community to provide permanent housing for plant workers. Findlay and Herly (2011) provide a historical account of the development of the Hanford project.

The *piles* or reactors each contained 1100 tons of graphite with transverse aluminum tubes loaded with 180 tons of uranium fuel and cold water flowing through the tubes to cool the reactor. Radiation safety was of paramount concern.

A team led by the late Herbert M. Parker, who would become a legend in the radiation protection field, assumed the responsibilities for it and related research activities. In the fall of 1944, I moved to Richland, Washington, to join my parents who were employed in the construction phase of the HEW. My father would later spend most of his work career in the chemical separations plant operations of the HEW.

In September 1944, the 100-B reactor at Hanford went critical with Enrico Fermi presiding over the start-up operation. After an initial glitch, some changes were made, and the neutron flux increased and stabilized. The uranium fissioned just as it had in the carbon black and uranium pile Fermi and his colleagues had built at the University of Chicago. The sustained and controlled chain reaction created a plethora of fission products, including ^{131}Te that quickly decayed to ^{131}I and large quantities of ^{90}Sr and ^{137}Cs. A small portion of the ^{238}U atoms transmutated to ^{239}Np, which then decayed to ^{239}Pu. In December 1944, *green* spent fuel rods began to be processed at Hanford using the methods developed by Glenn Seaborg and his team at UC-Berkeley and the University of Chicago. Many decades later, Glenn Seaborg visited the Lovelace laboratories in New Mexico where we were conducting research on the toxicity of inhaled plutonium. He related that he shipped some of the first plutonium he isolated at the University of Chicago to his colleague, Joseph Hamilton, at UC-Berkeley, to study its disposition in rats. The descriptor *green* was used for fuel elements that were processed shortly after an extended period of neutron irradiation and, thus, still contained large quantities of short-lived radionuclides.

The chemical separations process at Hanford yielded small quantities of ^{239}Pu, lots of fission products, and of course, residual uranium. By February 1945, ^{239}Pu was being shipped to Los Alamos, New Mexico; by July, enough was available to fabricate the first atomic fission bomb. As an aside, ^{239}Pu would be the principal product of the Hanford site for more than three decades. Ultimately, nine nuclear reactors were built and operated at Hanford. Most were shut down by 1971, and the last, a dual purpose (plutonium production and generation of electricity), was shut down in 1987. The radioactive fission products and other waste stored in underground tanks remain as a legacy. The Hanford cleanup continues today. Dalton et al. (1999) provide an interesting account of the cleanup issues for the Hanford site compared and contrasted with those at the Russian Mayak site to be discussed later.

Atomic Bomb

On July 16, 1945, at the Trinity Site in central New Mexico, the first atomic bomb with ^{239}Pu as the fissile material was detonated under the watchful eye of Robert Oppenheimer, who headed the bomb design and fabrication team at Los Alamos. Oppenheimer was reported to have quoted a passage from Bhagavad Gita: "If the radiance of a thousand suns were to burst at once into the sky, that would be like the splendor of the Mighty one … I am become Death, the shatter of worlds." The world would never be the same!

Downwind from the Trinity detonation, some cattle, sheep, and burros were grazing in the fallout field (Szasa 1984). Within weeks, the backs of some dark-skinned Hereford cattle and burros were showing white spots, a result of beta radiation skin burns from radioactive fallout particles. Soon, the U.S. government started to buy some of the cows and burros and ship them to Manhattan Project laboratories at Los Alamos and later to Oak Ridge, Tennessee. This probably qualifies as the first U.S. government radiation injury indemnification program!

On August 6, 1945, a previously untested ^{235}U bomb called Little Boy was dropped on Hiroshima, Japan, and on August 9, 1945, a ^{239}Pu bomb called Fat Man, similar to the device detonated at the Trinity Site, was dropped on Nagasaki, Japan. Within a short period of time, Japan surrendered, ending World War II. The extensive follow-up studies on the Japanese atomic bomb survivors will be discussed later.

Radioactive Fallout from Nuclear Weapons Testing

After World War II ended, the United States continued the development, manufacture, and testing of nuclear weapons. On July 1 and 25, 1946, the United States detonated nuclear fission bombs at Bikini Atoll in the Pacific Ocean.

On January 1, 1947, the activities of the Manhattan Project were transferred to a newly created civilian agency, the U.S. AEC, ostensibly a shift from military to civilian control of nuclear weapons and energy. The history of the AEC has been reviewed in depth by Hewlett and Anderson (1962) and Hewlett and Duncan (1969). The AEC was responsible for continuing the development and production of nuclear weapons based on *orders* from the military, developing nuclear reactors to generate electricity and propel naval vessels and, in general, advancing peaceful uses of nuclear energy through basic and applied research. Nuclear weapons testing in the Pacific by the United States continued in 1948.

Later, in 1974, the activities related to oversight of commercial nuclear reactors for generating electrical power were delegated to a newly created U.S. Nuclear Regulatory Commission with other activities continued under a newly created Energy Research and Development Agency (ERDA). In 1977, ERDA became the U.S. Department of Energy (DOE).

As World War II came to a close, the Union of Soviet Socialist Republics (USSR) accelerated its efforts to develop a nuclear bomb. Aided by intelligence on the U.S. nuclear efforts and the British nuclear efforts, which were closely coordinated with those of the United States, the USSR followed a similar path in building reactors and separation facilities at the Mayak Production Association (MPA) site in Russia to produce ^{239}Pu. Soon after the USSR began processing irradiated fuel elements in 1949, the U.S. Air Force detected airborne concentrations of fission products. It has been said that the detection of ^{131}I in the atmosphere stimulated U.S. efforts to establish the relationship between atmospheric ^{131}I concentrations and the release of ^{131}I from separation facilities processing *green fuel*. This led to the December 1949 *Green Run* test conducted at Hanford, which

will be described later. On August 29, 1949, the USSR detonated a *Fat Man*–type ^{239}Pu device. Ironically, one of the earlier measurements of fallout radionuclides from the Soviet test was made at Hanford. The nuclear arms race was soon public and accelerating! The Cold War began.

In December 1950, President Harry Truman established the Nevada Test Site (NTS) for continental nuclear tests. On January 27, 1951, a 1 kton warhead was dropped from an airplane at the NTS. This was the first of 126 atmospheric tests the United States conducted between 1951 and 1962.

The nuclear tests continued in the Pacific in 1951 and 1952 when the first hydrogen fusion devices were detonated. The testing of a very large hydrogen fusion bomb in 1954 resulted in contamination of a number of Pacific Atolls (Conrad et al. 1980). A Japanese fishing boat, the Fifth Lucky Dragon, was exposed, and one of the crew died of radiation sickness. The population of the Marshall Islands was exposed to high levels of radiation with an increased incidence of thyroid cancers observed later. The Partial Test Ban Treaty signed in 1963 forbade the testing of nuclear weapons in the atmosphere and under water. Additional tests resulting in the release of fission products to the atmosphere were conducted at the NTS through mid-1963. Other tests were conducted underground, in some cases with leakage of fission products to the atmosphere. Other countries also conducted nuclear weapons tests as they developed nuclear weapons. The United States and other nuclear powers signed the Comprehensive Test Ban Treaty in September 1996, making a legal commitment to never again test nuclear weapons.

Under the auspices of the AEC, a number of national laboratories (NLs) were created: the Los Alamos NL; Sandia NL; Lawrence Berkeley NL; Argonne NL; Pacific Northwest NL; which grew out of Manhattan Project activities at Hanford; and later Brookhaven NL and Lawrence Livermore NL. These labs were all built with U.S. government funds and operated by contractor organizations. In addition, specialized laboratories were created at a number of locations, including the University of Rochester, University of Chicago, University of Utah, UC at Los Angeles, UC at Davis, and the Lovelace Foundation for Medical Education and Research. These laboratories augmented by numerous scientists working in university and private laboratories with financial support from the AEC, ERDA, and DOE would make major contributions to advances in nuclear science and, most significantly, understanding the biological and health effects of radiation and radioactivity. To a large extent, these advances predated major developments in chemical toxicology. Many of the major advances in physics, chemistry, biology, medicine, and, I would include, the fields of toxicology and risk assessment made in the last half of the twentieth century are traceable to the research programs that grew out of the Manhattan Project and later were supported by the AEC, ERDA, and DOE (Hewlett and Anderson 1962; Hewlett and Duncan 1969). Some of the roots of my own special areas of interest, inhalation toxicology and the health effects of airborne materials, are clearly traceable to concern over the health effects of radiation and radioactive materials and research conducted at the NLs and in universities and special laboratories under the auspices of the AEC. Many of these developments are reviewed by Stannard and Baalman (1988). My own personal career was strongly influenced by a temporary assignment in the Division of Biology and Medicine of the U.S. Atomic Energy Commission (1965–1966). I gained much from working with many seasonal professionals, and especially from sharing an office with the late Paul Henshaw (recall his 1941 classic paper).

Nuclear Reactor for Electrical Generation and Propulsion

Electricity was first produced by thermal energy from a nuclear reactor on December 21, 1951, at the National Reactor Testing Station located at a remote site in southern Idaho and operated by Argonne NL. That site, now the Idaho NL, was initially created as a test site for reactors that could be used to generate electricity or propel naval vessels or even aircraft. The first nuclear-powered naval vessel, a submarine, the USS Nautilus, went to sea on January 17, 1955. Many other nuclear-powered vessels, both submarines and surface, followed. Later, the Los Alamos NL would provide leadership for developing nuclear-powered rockets for space exploration.

The nuclear reactor designs developed for naval propulsion by General Electric Company and Westinghouse Company as AEC contractors served as the basic design for the first commercial reactors used to generate electrical power. The IAEA (2012) estimated that nuclear reactors contributed 5.7% of the world's energy in 2011. Today, nuclear power generates about 20% of the electrical power used in the United States. Nuclear power generates a significant portion of electrical power in France and Japan, countries with limited fossil fuel reserves.

In the early 1970s, a major program was initiated in the United States to develop a breeder reactor that would both use ^{239}Pu as a nuclear fuel and produce additional fuel. Similar efforts were conducted in other countries, most notably France and Japan, and later in Russia. The U.S. program was discontinued during the administration of President Carter when the United States also decided to discontinue reprocessing irradiated nuclear fuel. This occurred for multiple reasons, including concern for how to handle and store the fission product waste and the processed fuel components and for the misuse of ^{239}Pu. Other countries such as Japan, France, and Russia continued to process irradiated fuel and explore how ^{239}Pu could be used as a reactor fuel, that is, mixed oxide (uranium and plutonium) fuel. France has continued to reprocess irradiated fuel elements, recovering uranium for reuse as a fuel and storing the waste. The Lovelace research team published an early risk assessment for a breeder reactor system (Cuddihy et al. 1977). An ironic conclusion of that study was that conventional coal-fired power plants of that era emitted greater quantities of naturally occurring radioactivity than the estimated alpha particle-emitting materials postulated to be released from the ^{239}Pu-fueled breeder reactor system, assuming it was designed and operated as specified.

In the United States, large quantities of irradiated and unprocessed uranium fuel rods continue to be stored, awaiting resolution of the long-term nuclear waste storage dilemma. A recent paper by North (2013) details the many trials and tribulations of the United States in developing a high-level nuclear waste site as a geological repository. A site at Yucca Mountain in Nevada was thoroughly researched for more than three decades as a possible site and then abandoned. Ironically, the Waste Isolation Pilot Plant (WIPP), located in a salt bed geological structure near Carlsbad, New Mexico, has been operated successfully for more than two decades as a repository for low- and intermediate- level radioactive waste originating in U.S. nuclear defense activities at locations such as Hanford and Los Alamos. Unfortunately, legislation restricts WIPP from accepting radioactive waste from the civilian nuclear program.

Radiation, Radionuclides, and Medicine

From the time of their discovery to the present, the use of radiation and radionuclides in medicine has continued to grow. Bushong (2013) has noted that for seven decades after Roentgen's discovery of x-rays in 1895, diagnostic radiology continued to evolve as an essential part of modern medicine. During this time, there were many technological advances and marked increases in the use of radiation and radioactive materials to diagnose disease. More recently in the last quarter of the twentieth century, marked advances in radiation instrumentation joined with new computer technology led to new machines and procedures. These advances include the routine use of multislice helical computed tomography (CT), computed radiography, digital radiography, digital fluoroscopy, and magnetic resonance imaging.

Other diagnostic modalities, such as positron emission tomography using radiolabeled pharmaceuticals, have been developed and widely used. These medical applications have included the use of short-lived technetium-99m, thallium-201, rubidium-82, fluoride-18, oxygen-15, and nitrogen-13. Numerous medical centers now have cyclotrons to produce the short-lived radionuclides that are an essential part of modern nuclear medicine. With the introduction of new instrumentation and procedures, the diagnostic information available has increased, and the radiation dose to individual patients has decreased.

In recent years, CT has been shown to be a convenient, accurate, and rapid diagnostic procedure with over 70 million CT scans performed annually in recent years in the United States (Brenner 2010). Compared with conventional x-ray examinations, the organ dose from a CT scan is typically 100 times higher (on the order of 5–100 mSv). This has raised concern over both the radiation dose to individual patients and the collective population dose from the increased use of CT scans. In my opinion, it is important, when considering diagnostic procedures, to recognize that these procedures are being conducted to obtain diagnostic information of value to the patients. There is a clear benefit to the individuals receiving the radiation dose.

In the last half of the twentieth century, substantial advances have been made in the use of radiation to treat cancer. These advances involved the development of improved devices for delivering precise doses of ionizing radiation to well-defined volumes of cancerous tissue while minimizing the radiation dose to adjacent normal tissue. For some specific cancers, such as prostate cancer, radioactive sources have been implanted to deliver the radiation dose to cancerous tissue much like the use of radon needles implanted into tumors more than a half century earlier.

SOURCES OF RADIATION EXPOSURE AND DOSE

In this section, the sources of radiation dose to populations will be briefly reviewed. Table 18.3 shows the radiation dose from different sources, providing perspective for the discussion of individual sources that follows. Note that natural background radiation and man-made or anthropogenic radiation sources contribute about equally to the average radiation dose.

Natural Environmental Radiation

Natural environmental radiation comes from three sources: cosmic radiation, terrestrial radiation, and internally deposited radionuclides. Cosmic radiation is that originating in galactic space and the solar systems and impinging on the earth's environment. It consists of an array of high-energy

TABLE 18.3
Relative Contribution of Different Radiation Sources to the Average Radiation Dose Received by the U.S. Public for 2006

Radiation Source	Millisieverts (mSv)	Percent
Background		
Space radiation	0.31	5
Internal radionuclides	0.31	5
Terrestrial dose	0.19	3
Radon and thoron	2.29	37
Background	3.10	
Anthropogenic		
Computer tomography (CT)	1.49	24
Nuclear medicine	0.74	12
Interventional fluoroscopy	0.43	7
Conventional radiography/ fluoroscopy	0.31	5
Consumer products	0.12	2
Occupational	<0.1	<0.1
Industrial	<0.1	<0.1
Anthropogenic	3.10	

Source: National Council on Radiation Protection and Measurements (NCRP) Report 160; Ionizing Radiation Exposure of the Population of the United States, Bethesda, MD, 2009.

particles such as neutrons, protons, electrons, mucons, and pions and high-energy nuclei and gamma photons. The flux is sufficient that it is of concern for astronauts in space. Cosmic radiation intensity decreases as it nears the earth and is greater in high-flying aircraft than at the earth's surface. Terrestrial radiation exposure comes from deposits of uranium, thorium, and other radioactive sources in the earth.

Three series of naturally occurring radionuclides are important: (1) the uranium series extending from ^{238}U (a half-life of 4.468 billion years) to stable ^{206}Pb, (2) the actinium series extending from ^{235}U (a half-life of 704 million years) to stable ^{207}Pb, and (3) the thorium series extending from ^{237}Th to stable ^{208}Pb. In each series, multiple elements and radionuclides of varied radioactive half-life are involved with emissions of alpha particles, beta particles, and gamma rays of varied energy. The uranium and actinium series are of special interest because they begin with very long-lived radionuclides of U. Uranium ore, which contains 99.72% ^{238}U, 0.72% ^{235}U, and traces of ^{234}U, has been mined and extracted to provide various decay products and, most importantly, as a source of ^{235}U to fuel nuclear reactors. The uranium series is of special interest because it includes radon (^{222}Rn), which is a noble gas found in high concentrations in uranium mines, some other types of mines, in some homes, and other structures in geographic areas with high concentrations of uranium.

The earliest work on the association between working in uranium mines and increased risk of lung cancer considered radon gas itself to be the causative agent. John Harley, in his doctoral thesis research, demonstrated that the linkage was more complex and involved both radon and its decay products (Harley 1953). Radon decays by alpha emission to ^{218}Po, which, in turn, decays by alpha emission to ^{214}Bi, which decays to ^{210}Pb, which has a half-life of 21 years. Exposure via inhalation to ^{222}Rn inevitably involves exposure to ^{218}Po and ^{214}Bi, which in the air aggregate with other particles. Using today's nomenclature, these particles would be considered nanoparticles. The doses from the alpha emissions of ^{218}Po and ^{214}Bi are the significant contributors to the radiation dose from ^{222}Rn and its decay products, which with exposure for sufficient duration and concentration can increase the risk of lung cancer (Harley and Pasternack 1982).

When field studies were conducted in the 1950s and 1960s to evaluate the exposure of uranium miners to radon and daughter products, the approach used measurements of total airborne alpha activity. The results were expressed as a working level (WL)—an equilibrium mixture of the decay products of the alpha decay products of radon equal to 100 pCi/L (3700 Bq/m^3). Exposure of a miner for 170 h at this concentration is identified on a working level month (WLM). Later, analyses such as those of BEIR VI (NRC 1999) had to convert exposure measurements expressed in WLM units to SI units (Bq/m^3) and then an even more uncertain extrapolation to absorbed dose (Gy).

Other naturally occurring radionuclides that can enter the body are also of interest as contributors to radiation exposure from natural and unavailable sources. All potassium contains ^{40}K, which has a half-life of 1.248×10^9 years and decays by beta emission. It is the largest source of internal radiation exposure for humans and other mammals next to the exposure from inhaled Rn and its decay products (Table 18.3). It is estimated that the adult human body contains about 150 g of K. The ^{40}K content in the adult human body is estimated to yield 4400 disintegrations per second. ^{40}K decays to ^{40}argon, and thus, the ratio of ^{40}K to ^{40}Ar can be used for radio-dating the age of some specimens.

Another naturally occurring radionuclide is ^{14}C present in carbon. It decays by beta emissions with a half-life of 5740 years and is constantly being replenished in the atmosphere. Since carbon is an essential component of all living things, ^{14}C is present in all living things. As trees sequester C, they sequester ^{14}C in proportion to what is in the atmosphere. When trees die, the ^{14}C present continues to decay. The ratio of ^{14}C to C can then be used for dating the age of wood specimens.

It is important to recognize that the radiation dose received by individuals from natural sources will vary dependent upon where they live. The elevation of their location will influence the radiation dose of cosmic origin. The local geologic sources will influence the radiation dose from naturally occurring radioactivity. The greatest variation in radiation dose to individuals will be associated with the dose to the respiratory tract from inhaled radon and daughters. This is the case since the concentration of natural uranium, the source of radon gas, varies widely across the United States and around the world.

Nuclear Weapon Detonations

As discussed elsewhere, the potential for atmospheric release of large quantities of radioactivity with detonation of a nuclear bomb was anticipated before the first nuclear weapon was tested at the Trinity Site in New Mexico. With detonation of the fissile ^{239}Pu device at the Trinity Site, that potential was fully realized with the release of a wide array of fission product radionuclides that were carried downwind and deposited as *fallout*. Over the next several decades with atmospheric testing of many nuclear weapons, the distribution of these fallout radionuclides through the ecosystem, uptake by humans, and their health effects would be studied extensively. Although many different radionuclides are released by fissioning of ^{235}U and ^{239}Pu in nuclear weapons, attention has been focused most intensely on ^{131}I (half-life, 8.05 days), ^{90}Sr (half-life, 28 years), and ^{137}Cs (half-life, 30 years). This was the case because these radionuclides are produced in abundance in a nuclear detonation and enter the agricultural ecosystem with passage via forage, ingestion by cows, transfer to milk, and ingestion by humans. When ingested, the radionuclides are avidly retained. Just like stable iodine, the ^{131}I is taken up by the thyroid and irradiates the thyroid tissue. Stable strontium and ^{90}Sr behave metabolically like Ca and concentrate in bone, and the decay of the ^{90}Sr and its daughter ^{90}Y irradiates the skeleton and bone marrow. Stable cesium and ^{137}Cs behave like K; thus, ^{137}Cs distributes throughout the body like potassium, resulting in essentially total body

TABLE 18.4
Radionuclide Releases from Reactor Accidents (Amount in Terabecquerels [TBq] = 10^{12} Bq)

Radionuclide	Half-Life	Windscale (United Kingdom) (October 10, 1957)	Three Mile Island (United States) (March 28, 1979)	Chernobyl (Ukraine) (April 26, 1986)	Fukushima (March 11, 2011)
^{133}Xe	5.3 days	12,000	—	6,500,000	17,000,000
^{131}I	8.05 days	740	1.	~1,760,000	~200,000
^{137}Cs	30 years	22	0.0015	~85,000	~30,000
^{134}Cs	2.0 years	—	—	~54,000	—
^{90}Sr	28 years	—	0.0022	~10,000	—

irradiation. The detonation of hydrogen fusion bombs, developed later, resulted in the release of a generally similar array of radionuclides. This is the case because the fissioning of fissile material is used to trigger the fusion.

Nuclear Reactor and Other Accidents

In addition to concern for radionuclides released in nuclear explosions, there has been concern from the beginning of operation of nuclear reactors for accidental release to the environment of U fuel, fission product radionuclides, and the transmutated radioactive elements such as ^{239}Pu. It is important to recognize that the spectrum of fission products released from the instantaneous explosion of a nuclear weapon is different from the spectrum of those radionuclides released from a reactor or a chemical plant processing irradiated fuel. The reactors and irradiated fuel elements contain fission products produced from fissioning of the uranium, plutonium, and thorium fuel and activation of nuclides formed over the extended time course of irradiation of the fuel, which may be several years in duration. One key difference between the sources is that detonation of nuclear weapons results in the release of both ^{131}I and numerous iodine radionuclides or precursors that have much shorter half-lives than ^{131}I. The shorter-lived radioiodines are not present in abundance in releases from a reactor. A second difference is that after a relatively short period of operation, a reactor core will contain ^{134}Cs in addition to ^{137}Cs. A nuclear detonation produces very little ^{134}Cs. The ^{134}Cs has a half-life of 2.0652 years.

Nuclear reactor accidents with the release of radioactivity have occurred in England, the United States, Ukraine, and Japan (Table 18.4). The Windscale accident involved a reactor designed to produce ^{239}Pu and later modified to also produce tritium (^{3}H) for use in fusion weapons. Unlike most other nuclear reactors, the Windscale reactor was air-cooled, posing special issues for its control.

The Three Mile Island reactor in Pennsylvania was part of a commercial nuclear electrical generating complex. The accident involved a meltdown of a substantial portion of the reactor core. Fortunately, the operating system and containment operated as planned, and the radionuclide releases were remarkably small.

Although used to generate electricity, the Chernobyl reactor, located in the Ukraine near Kiev, was similar in many respects to the ^{239}Pu-producing reactors that operated at the Hanford site. This included the absence of the kind of containment vessel used to house commercial reactors providing thermal energy for generation of electrical power in the United States and other countries outside the sphere of the former USSR. The substantial release of radioactivity in the Chernobyl accident (Table 18.5) was not unexpected in view of the very ineffective containment system. A reactor of this type would not have been constructed and could not have been licensed for commercial use in the United States, Western Europe, or Asia. Radioactivity from Chernobyl was injected over a number of days into the atmosphere. The releases from Chernobyl were first reported to the public from Sweden. The released radioactivity deposited in readily measurable levels across Europe and distributed in trace levels around the globe.

The particulate nature of some of the radionuclides released from Chernobyl and the associated *fallout* is evident from the autoradiograph (Figure 18.6) of a leaf I collected in a park in the fall of 1986 in Kiev, Ukraine, about 80 km south of Chernobyl. The surface area of tree leaves collected substantial amounts of *fallout* radioactivity. My colleagues and I also analyzed fallout particles collected from a shipping crate obtained from a site slightly closer to Chernobyl. We found that some of the fallout particles contained not just fission product radionuclides, but also uranium, clear evidence that

TABLE 18.5
Radionuclide Releases from Chernobyl Reactor Accident (Amount in Pbq, a PBq = 10^{15} Bq)

Radionuclide	Half-Life	Core Inventory (PBq)	Amount Released (%)	Activity (PBq) Released
^{33}Xe	5.3 days	6500	100	6500
^{131}I	8.0 days	3200	50–60	~1760
^{134}Cs	2.0 years	180	20–40	~54
^{137}Cs	30.0 years	280	4–6	~85
^{90}Sr	28.0 years	200	4–6	~10
^{106}Ru	1.0 years	2100	>3.5	>73
^{144}Ce	285.0 days	3300	3.5	~116
^{238}Pu	86.0 years	1	3.5	0.035
^{239}Pu	24,400 years	0.85	3.5	0.03

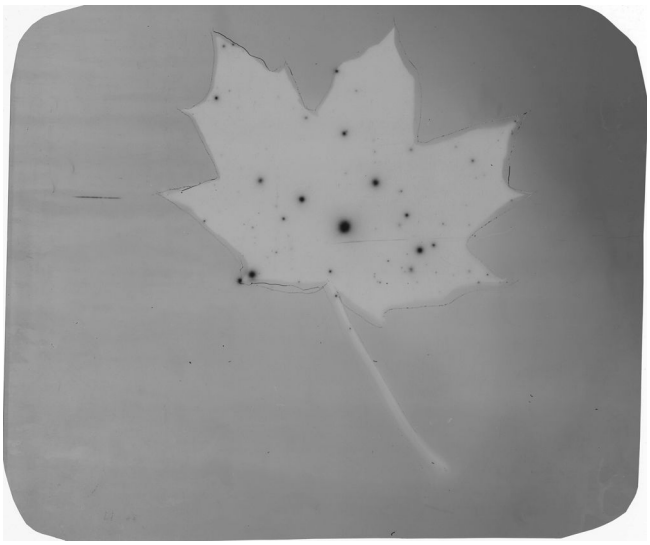

FIGURE 18.6 Autoradiograph of a tree leaf collected in Kiev, Ukraine, in the fall following the Chernobyl Reactor Accident. The darkened spots are related to radioactive particles deposited on the leaf.

TABLE 18.6
Representative Radiation Quantities from Various Diagnostic Procedures

Examination	Entrance Skin Dose (mGy)	Mean Marrow Dose (mGy)	Gonad Dose (mGy)
Traditional radiographs			
Skull	2.0	0.10	<1
Chest	0.1	0.02	<1
Cervical spine	1.5	0.10	<1
Lumbar spine	3.0	0.60	2.25
Abdomen	4.0	0.30	1.25
Pelvis	1.5	0.20	1.50
Extremities	0.5	0.02	<1
Computed tomography			
Head	40.0	0.20	0.50
Pelvis	20.0	0.50	20

Source: Reprinted with permission from Bushong, S.C., *Radiological Science for Technologists: Physics, Biology and Protection*, 10th edn., Elsevier/Mosby, St. Louis, MO, 2013, Table 37-1.

fuel was released from the core of the Chernobyl accident. The most significant sources of internal irradiation to the public were from ^{131}I, ^{137}Cs, and ^{134}Cs. The ^{134}Cs and ^{137}Cs deposited everywhere and were also a source of external radiation exposure.

The Fukushima, Japan, accident was triggered by a series of earthquakes and a tsunami impacting multiple nuclear reactors that were part of a large nuclear electricity-generating station built on the seashore at a low elevation. The successive *monster* ocean waves hitting the Fukushima site destroyed even their backup systems. The fact that the releases of radioactivity from the Fukushima reactors were not greater was a testimonial to the containment systems that remained largely effective. The most substantial release was of the noble gas, ^{133}Xe, which is not metabolized. It serves as an external radiation source when individuals are immersed in a cloud containing ^{133}Xe. The ^{131}I and ^{137}Cs were the major sources of internal irradiation. Details of the exposures from the reactor accidents will be discussed later.

It is important to note that highly sensitive radiation monitoring instruments and methods can detect extraordinarily low concentrations of radioactivity in air, water, tissues, and other samples. Radioactivity injected into the atmosphere by nuclear explosions, burnup of space systems with radioactive (^{238}Pu) power sources, and nuclear reactor accidents (such as Chernobyl) have been followed around the globe. Ultralow concentrations of ^{239}Pu, either from Pu not consumed during detonation of ^{239}Pu weapons or produced during the detonation of nuclear weapons, have been detected around the globe.

External Radiation Exposure and Dose

Human populations can be exposed to external radiation in multiple ways (Table 18.3). This includes cosmic and terrestrial radiation of natural origin. In addition, populations may be exposed to radiation from deposited radioactivity from nuclear weapons testing, nuclear reactor accidents, mishandling of nuclear waste, and, potentially, terrorist activities. I mention terrorist activities for completeness. However, in my opinion, it is very unlikely that terrorist devices would disperse sufficient radioactivity to be of concern for producing health effects either as a distributed external radiation source or from inhalation or ingestion of dispersed radioactivity. However, even small quantities of dispersed radioactivity of any kind can still create public alarm and require a substantial cleanup effort.

While all members of populations can receive external radiation exposure from natural sources, it is important to recognize that individuals can receive significant external radiation exposures from medical applications (Tables 18.3 and 18.6). The numbers of purposeful radiation exposures to obtain diagnostic information vary among individuals. Some individuals may never have medical situations that ever involve radiation exposure from a diagnostic procedure. On the other hand, some individuals may have medical conditions that require the use of repeated diagnostic or interventional procedures involving external radiation exposure. In recent years, the use of CT scans has increased dramatically. Concern has been raised as to the radiation exposure received by patients that have received multiple CT scans (Hall and Brenner 2008; Brenner 2010).

Radionuclide Exposure Pathways and Dose

As evident from Figure 18.1, exposure to radionuclides of natural origin or associated with human activity can occur in multiple ways. The most significant exposure pathway for naturally occurring radionuclides involves inhalation of radon and its daughter products. For the average individual in the United States, inhalation of radon and daughter products

results in an annual dose of just over 2.0 mSv. The exposure from radon and its daughter products across the population is quite variable. Individuals living in low-radon areas or even individuals living in high-radon areas, but having well-ventilated homes and workplaces, will receive limited doses of radon. Individuals in areas with high levels of radon from high levels of uranium in the soil and living in poorly ventilated homes may receive radon and associated daughter products, leading to irradiation of the conducting airways of the respiratory tract that are substantially above the national average.

From 1946 through 1963 when numerous atmospheric tests of nuclear weapons were conducted, individuals around the globe were exposed to fallout radionuclides. Individuals living near test sites, such as those living downwind of the NTS, received the highest exposures to fallout radionuclides and the associated highest radiation doses. The dose to individuals from inhalation of the airborne radioactivity was quite low. The highest radiation doses were those from radionuclides such as ^{131}I, ^{137}Cs, and ^{90}Sr moving through the agricultural food chain. The most significant of these pathways is that involving contamination of pasture or hay, ingestion of the contaminated pasture grass or hay by cows, secretion in the milk, and ingestion of the milk by people. Table 18.7 presents some representative values for secretion of ^{90}Sr, ^{131}I, ^{137}Cs, and ^{144}Ce in cow's milk.

Because ^{131}I is concentrated in the milk and, when ingested, concentrated by the thyroid, ^{131}I is of special concern. The extent to which the thyroid concentrates ^{131}I is apparent from the distribution data shown in Table 18.8. Because ^{131}I has a radioactive or physical half-life of 8.05 days, it is of primary concern soon after a nuclear detonation or release from a reactor or nuclear fuel processing plant. In 40 days, the amount of ^{131}I will have undergone five half-lives of decay, and only 1/32th of the original ^{131}I will remain.

When the original ^{239}Pu-producing reactors in the United States, and later in the USSR, were started up, there was a desire to quickly process the irradiated fuel and separate out the ^{239}Pu for use as a fissile material in bombs. As a result, *green* fuel elements, which had recently been irradiated and, thus, contained high concentrations of ^{131}I, were processed. Later, it became standard practice to allow the irradiated fuel to *cool* and age, thereby reducing the content of short-lived radionuclides like ^{131}I before the fuel was processed. Ironically, the largest release of ^{131}I from the Hanford complex occurred on December 2, 1949, with an experiment code named *Green Run*. It was designed to simulate what occurred in late 1944 and early 1945 at Hanford and what was presumably happening earlier in 1949 in the USSR when *green* fuel was processed. Soon after the uranium fission reactors began operation and fuel reprocessing was initiated, the United States and USSR were eager to recover ^{239}Pu for the manufacture of nuclear weapons. Recovery involved processing irradiated fuel that had only been cooled for a few weeks. The *Green Run* experiment involved processing of *green* fuel and anticipated the gathering of extensive data on meteorological conditions and ^{131}I concentrations from the site to hundreds and, indeed, thousands of miles downwind. The goal was to create an algorithm to relate the ^{131}I concentrations in the air to the amount of irradiated uranium fuel being processed, including the ^{239}Pu content. It was thought that these data would be useful in interpreting data on airborne ^{131}I originating in the USSR and estimating the Soviet production of ^{239}Pu. Unfortunately, meteorological conditions changed abruptly soon after processing of the fuel began, resulting in stagnant conditions and fumigation of eastern Washington with ^{131}I. This release of 8000 curies of ^{131}I and the associated population exposures were the largest in the history of the Hanford operations. An epidemiological study conducted later did not detect an increase in thyroid cancer in the exposed population (Davis et al. 2004). An alternative interpretation has been provided by Hoffman et al. (2007).

The release of ^{131}I from the Hanford operations and concern over fallout of ^{131}I from nuclear weapons testing stimulated the conduct of an extensive research program on numerous studies of ^{131}I metabolism and toxicity in sheep and swine at Hanford, including long-term studies of the effects of daily ingestion of ^{131}I by sheep (McClellan and Bustad 1964; Bustad et al. 1965a; McClellan 1995). As a high school student, I helped care for an off-site flock of control sheep for this study. Regular monitoring of the thyroids of the sheep maintained off the Hanford site revealed increased levels of ^{131}I associated with recent nuclear weapons tests. Fallout ^{131}I traveled around the globe.

In the early 1960s, Hanford scientists conducted a multifaceted study to simulate a single brief release of ^{131}I in order to

TABLE 18.7
Fraction of Orally Administered Dose of ^{90}Sr, ^{131}I, ^{137}Cs, and ^{144}Ce[a] Secreted in Milk of Dairy Cows

Radionuclide	Oral Dose Secreted in Milk (%)
^{90}Sr	0.08–2
^{131}I	5–10
^{137}Cs	~10
^{144}Ce[a]	<0.01

[a] ^{144}Ce is poorly absorbed from the gastrointestinal tract.

TABLE 18.8
^{131}I Concentrations in Blood, Milk, Thyroid, and Other Tissues

Organ, Tissue, Secretion, or Excretion	Dose (%)/kg or Liter
Blood	0.17
Muscle	0.02
Spleen, pancreas	0.08
Kidney, liver	0.14
Ovary, salivary glands	0.10
Feces	0.50
Milk	0.50
Thyroid	320,000

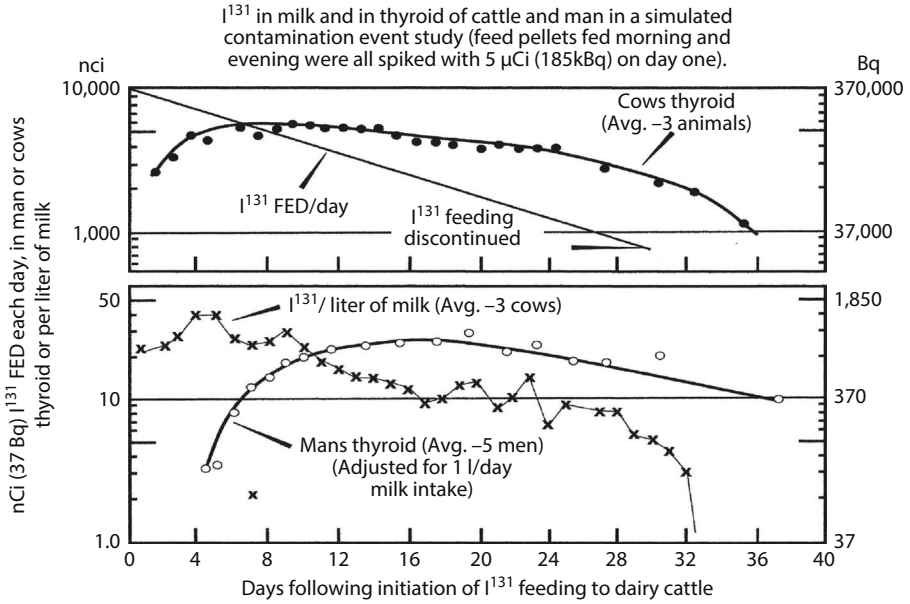

FIGURE 18.7 Interrelationships for radioiodine (^{131}I): intake by cows, concentration in cow's thyroid and milk, and human thyroid. (Adapted from Bustad, L.K. et al., The significance of radionuclide contamination in ruminants, in: *Physiology of Digestion in the Ruminant*, ed. Dougherty, R.W, Butterworth, Washington, DC, pp. 131–143, 1965.)

understand the feed–cow–milk–human intake–thyroid burden relationships (Bustad et al. 1963, 1965a). The results are shown in Figure 18.7. A large number of feed pellets were spiked with ^{131}I on the first day of the study and fed each day to the cows. The radioactive decay of the ^{131}I (half-life of 8.05 days) resulted in the cows receiving less ^{131}I each day. The thyroids of the cows were regularly monitored along with the ^{131}I content of the milk. Human volunteers ingested aliquots of the milk containing trace amounts of ^{131}I each day, and their thyroids were monitored. These data clearly show the quantitative relationship between ^{131}I in the cow's thyroid, cow's milk, and human thyroid. It was very valuable information in interpreting the impact of the ^{131}I releases in the Chernobyl and Fukushima accidents. Indeed, it is understood that these data were used to set limits on the release of ^{131}I-contaminated milk for human consumption and used to guide agricultural practices (shift of cattle from pasture to stored feed) in order to limit the ^{131}I exposure of cows with the goal of reducing the ^{131}I milk contamination and human intake.

The use of oral stable iodine administered shortly before or soon after exposure to ^{131}I has been advocated as a means of releasing the radiation dose to the thyroid by blocking or reducing ^{131}I uptake. It is important to note the intake of large quantities of stable iodine is hazardous to some individuals who are sensitive to stable iodine intake. In my opinion, the most effective means of reducing the thyroid dose from ^{131}I is not to drink contaminated milk. The potential dose from inhaling ^{131}I is quite low compared with the radiation dose from ingested ^{131}I. As an aside, administration of stable iodine orally to dairy cows did reduce the ^{131}I dose to the cow's thyroid and modestly reduced the ^{131}I content of the milk.

Two other radionuclides, ^{90}Sr and ^{137}Cs, are of concern due to their presence in fallout from the detonation of nuclear weapons. ^{90}Sr has a radioactive half-life of 29.1 years and decays by beta emission to ^{90}Y, which decays by beta emission with a half-life of 64 h. Because of the short half-life of ^{90}Y, equilibrium is quickly established between ^{90}Sr and ^{90}Y. ^{90}Sr behaves metabolically like calcium in ecosystems and in the mammalian body. Thus, it is relatively readily absorbed from the gastrointestinal tract, translocated to, and incorporated into bone where beta particle decay of the ^{90}Sr and its short-lived daughter ^{90}Y irradiates bone and bone marrow. With ^{90}Sr, as with ^{131}I, there is concern for the forage–cow–milk–child pathway. Because of the long radioactive half-life, the ^{90}Sr will remain in the ecosystem for years. This raises concern for ingestion by cows of ^{90}Sr-contaminated pasture and hay. Because of the abundance of ^{90}Sr in weapons fallout, the toxicity of ^{90}Sr was the subject of substantial research beginning in the late 1940s.

^{137}Cs has a radioactive half-life of 30.17 years, decaying to ^{137}Ba with a half-life of 2.6 min. ^{137}Cs is metabolized like K and other alkali metals and is readily absorbed from the gastrointestinal tract, distributed in all the soft tissues of the body, and secreted in milk. As with ^{131}I and ^{90}Sr, there is concern for the forage–cow–milk–child route of exposure for ^{137}Cs. Table 18.9 provides data on the tissue distribution of ingested ^{137}Cs in sheep and goats and illustrates the relatively uniform concentration of ^{137}Cs in soft tissues of the body. These data indicate the need to consider the forage–meat–human consumption pathway in addition to the pathway via cow's milk when evaluating human exposures.

The NCRP (2007) reviewed the literature on ^{137}Cs in the environment, including transfer through foodstuffs to humans. For transfer from livestock feed (daily intake) to milk (concentration per liter), the report cited values of 0.0048–0.01 with a best estimate of 0.01. For transfer from livestock feed (daily intake) to meat (concentration per kg), the report cited values of 0.004–0.06 with a best estimate of 0.05.

TABLE 18.9
Distribution of Cesium-137 in Blood, Femur, and Soft Tissues Following Ingestion

	Cesium Concentrations Sheep	Daily Dose (%)/kg Tissue Goat
Muscle	8	20
Kidney	10	36
Heart	8	—
Brain	—	5
Ovary	—	13
Testis	9	—
Blood plasma	0.5	1
Femur	1	—

The importance of the forage–meat–milk pathway was also recognized when studies were conducted on native populations in northern Alaska that depended on caribou as a source of meat (Hanson and Palmer 1965; Hanson 1967). It was soon apparent that high concentrations of ^{137}Cs in caribou meat and then in people were traceable to deposition of fallout ^{137}Cs on lichens. The lichens grow very slowly and avidly take up minerals, including ^{137}Cs from the atmosphere. The caribou graze in lichen *pastures* in a cyclic fashion over several years, allowing for accumulation of the ^{137}Cs in the lichens. Detailed studies established quantitatively the linkage between ^{137}Cs content in lichens, caribou meat, and the body content in the native individuals as well as wild carnivores. The ^{137}Cs concentrations increased by about a factor of 2 at each successive trophic level of the food chain from lichens to caribou to man.

^{134}Cs is a shorter-lived radionuclide of Cs that decays with beta and gamma emissions and a half-life of 2.0652 years. It is present in quantities about equal to ^{137}Cs in the fuel core of a typical nuclear reactor that has been operating for some time. Fallout from nuclear weapons detonations does not contain significant amounts of ^{134}Cs. Thus, ^{134}Cs received little attention as a source of human exposure during the nuclear weapons testing and fallout era. Of course, its behavior in the environment and body of animals and humans can be predicted from knowledge of ^{137}Cs when corrections are made for the differences in radioactive half-life. The accidents at Chernobyl and Fukushima resulted in substantial releases of ^{134}Cs (Table 18.4).

Following the Chernobyl accident, some of the radioactive plume moved north and northwest over Scandinavia. Not surprisingly, $^{134–137}$Cs was detected on pastures, including the lichen pastures in the area that used to be called Lapland and is now called Sami Land. $^{134–137}$Cs was soon detected in the carcasses of sheep and reindeer. Norway and Finland set relatively high limits on the concentrations of $^{134–137}$Cs, that is, 6000 Bq/kg, that would be allowed in sheep or reindeer meat intended for human consumption. In these countries, only a small portion of the reindeer and sheep carcasses exceeded these limits. On the other hand, Sweden set quite low limits, initially at 300 Bq/kg, and then raised them to 600 Bq/kg as the allowable levels of $^{134–137}$Cs. As a result, thousands of reindeer carcasses contaminated with $^{134–137}$Cs were confiscated and frozen in Sweden and stored awaiting a decision on their potential use. I participated in a meeting in Stockholm the winter after the Chernobyl accident to provide advice as to what to do with the thousands of stored carcasses contaminated with trace quantities of $^{134–137}$Cs. I reasoned that the Sami population warranted special consideration because they had the potential for regularly eating reindeer meat. It would be desirable for them to shift from reindeer meat in their diet to noncontaminated beef or mutton. For most non-Sami people, the reindeer meat was a delicacy eaten only a few times a year. My recommendation was that it was *safe* to allow release of the frozen reindeer carcasses for sale to the occasional consumer. It is my understanding that, unfortunately, the carcasses were destroyed.

With $^{134–137}$Cs released in a nuclear reactor accident, there is concern beyond the forage–cow–milk and meat pathway for human intake. That concern is for external gamma radiation exposure of people in the contaminated area from the deposition of $^{134–137}$Cs on soil, vegetation, roadways, and buildings. This has proved to be a major source of exposure following the Chernobyl and Fukushima accidents although the radiological impact was much greater for Chernobyl.

INTRODUCTION TO RADIATION EFFECTS: AN INTEGRATIVE PARADIGM

The effects of radiation on living systems begin with interaction at the atomic and molecular level (Figure 18.3). The interactions take the form of ionization or excitation of orbital electrons and result in deposition of energy in tissue. The ionizations can directly affect biological molecules, principally DNA. In addition, there may be indirect action of radiation mediated by free radicals that can diffuse far enough to reach target molecules. The latter is noteworthy since water constitutes about 80% of the human body. When atoms are ionized, their chemical-binding properties change. The ionization may result in breakage of molecules or relocation of atoms within the molecule. These changes, in turn, can alter cell function, including causing cell death. Ionization of water or biological molecules is an initial step in a complex multistep process illustrated in Figure 18.3. All of these mechanistic steps have been investigated in extraordinary detail over the past half century and reinvestigated as more sophisticated cellular, biochemical, and molecular methods have been developed. Many of the papers describing this research were identified within the 100 most cited papers published in the journal *Radiation Research* (RRJ 2012). The textbooks by Bushong (2013), Hall and Giaccia (2012), Mettler and Upton (2008), and Turner (2007) all provide an in-depth coverage of the vast literature on mechanisms of action of ionizing radiation.

The ionization in cells can result in cell death, mutations, and other alterations in cell function. Cell killing and functional alterations in surviving cells can alter organ function and with sufficiently high doses lead to death of the individual. The most common form of cell death from radiation is mitotic death. The irradiated cells die as they attempt to divide because of

severe damage to their chromosomes. The cell-killing properties, which were recognized soon after the discovery of x-rays, are the basis of the effectiveness of ionizing radiation in cancer therapy. The goal of radiation therapy is to kill cancerous cells and minimize damage to adjacent normal tissues.

In the 1950s, rapid advances were made in culturing cells in vitro. With a keen interest in radiation effects, it is not surprising that many investigators started to study the effects of x-rays and other forms of radiation on cultured cells. Puck and Markus (1956) were among the first to report the results of such experiments. They initially studied the now very famous Hela cells, a human cell line that originated from a patient at Johns Hopkins University (Figure 18.8). Experiments of this type have been conducted innumerable times with normal and malignant cells of all kinds from humans and laboratory animals. The results have been remarkably similar, a survival curve with a shoulder followed by a slope that is a straight line on a log-linear plot. The size of the shoulder is quite variable. For some cell lines, the curve is not straight but bends continuously so a linear–quadratic (LQ) function is a better fit and then has no meaning. The D_o value for the survival curve (i.e., the reciprocal of the slope) is defined as the dose required to reduce the surviving cells to 37%. The D_o of the survival curves for most x-irradiation cell lines cultured in vitro is typically in the range of 1–3 Gy. Some cells that have impaired DNA repair capacity may have D_o values as low as about 0.5 Gy. If the radiation dose is fractionated with sufficient time between fractions for repair to occur, the survival curve shifts to the right.

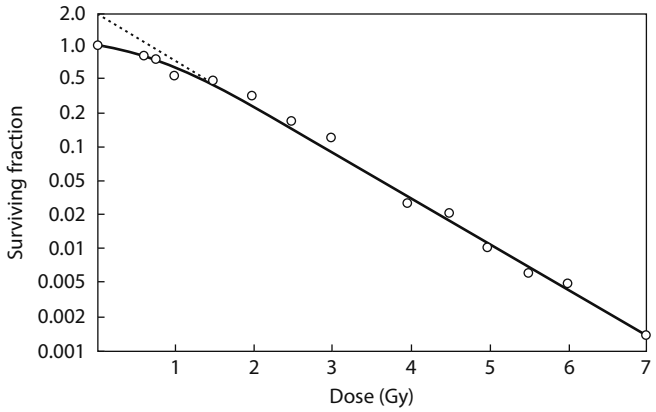

FIGURE 18.8 Survival curve for Hela cells in culture exposed to x-rays. (From Puck, T.T. and Markus, P.I., *J. Exp. Med.*, 103, 653, 1956.)

Gray (1965) based on studies in irradiated mice with leukemia as the endpoint called attention to the role of cell killing and induction of transformed cells in producing leukemia as illustrated in Figure 18.9. In his studies with mice, he observed a bell-shaped dose–response relationship for leukemia. He attributed this to a dose-related increase in the proportion of normal cells transformed to a malignant state and a dose-related decrease in the probability that transformed cells survive radiation exposure. While the interpretation may not be quantitatively precise, it does draw attention to the critical interplay between cell survival and mutational events and subsequent cell transformation in the induction of cancer in irradiated individuals. The role of these two events is explicitly incorporated in the classical Moolgavkar–Vinson–Knudson model of carcinogenesis (Moolgavkar and

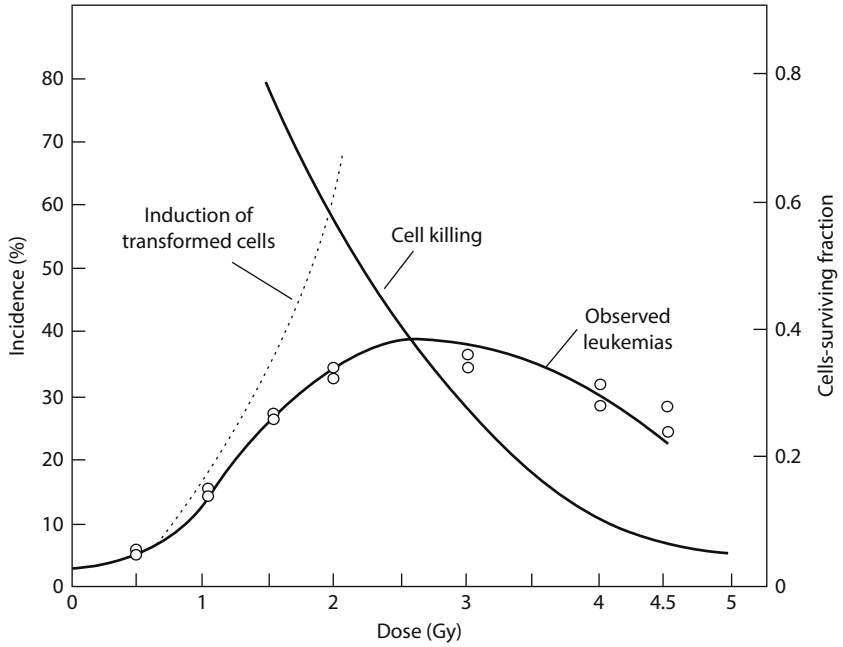

FIGURE 18.9 The postulated role of cell killing and induction of cell killing in producing leukemia in irradiated mice. (From Gray, L.H., Radiation biology and cancer, in: *Cellular Radiation Biology: A Symposium Considering Radiation Effects in the Cell and Possible Implications for Cancer Therapy: A Collection of Papers*, eds. M.D. Anderson and Tumor Institute, Lippincott, Williams and Wilkins, Philadelphia, PA, pp. 8–25, 1965.)

Venzon 1979; Moolgavkar and Knudson 1981; Moolgavkar 1986; Moolgavkar et al. 1988, 1999; Dewanji et al. 1989).

The dose–response relationships for induction of chromosomal aberrations in somatic and germ cells vary with the quality of the radiation, cell type, and species studied. It is obvious that for mutations to be induced and passed to subsequent generations of cells, the irradiated cells must survive. Thus, for x-ray or gamma rays, the induction of mutations can only be studied at relatively low radiation dose with brief exposures. Of course, if the radiation dose is protracted, higher total radiation doses can be studied because of the dose-sparing effect of dose protraction (recall Figure 18.2). With internally deposited radionuclides, the radiation dose rates are relatively low, so higher total doses can be studied. The results of studies by Brooks (2013) on the induction of chromosomal aberrations in vivo in liver cells in Chinese hamsters are summarized in Figure 18.10. The base case is the effects of acute ^{60}Co gamma radiation.

At the lowest radiation doses, the relationship between radiation dose and aberrations per cell is linear and then at higher doses sweeps up, reflecting the interactions associated with high radiation doses. At low total doses, chronic gamma irradiation (<5 Gy/day) and acute gamma irradiation are equally effective in producing aberrations. This would equate to an RBE of 1.0. The RBE factor has classically been used to adjust measurements of dose for radiation at different kinds (gamma rays or x-rays as a base and alpha and beta particles and neutrons) to equal effectiveness in producing biological effects. At higher total radiation doses, the relationship between dose and aberration frequency is essentially linear. It is noteworthy that when the radiation dose is delivered by internally deposited ^{144}Ce–^{144}Pr, which decays by emission of beta particles, the dose–response relationship is remarkably similar to protracted external gamma irradiation. This is to be expected since both are low-LET radiation. The nature

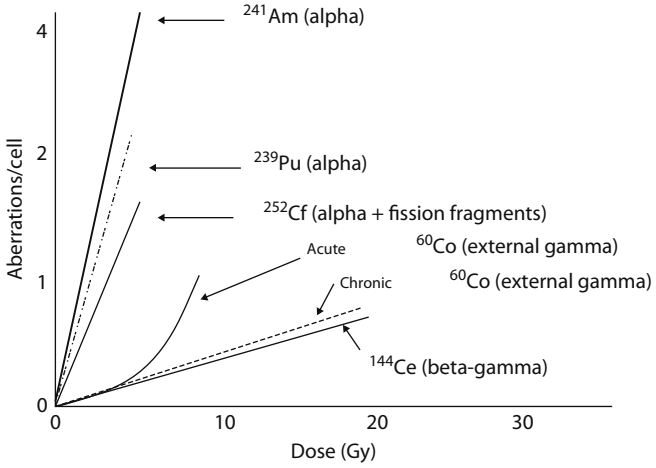

FIGURE 18.10 Dose–response relationship for in vivo induction of chromosome aberrations in liver cells of Chinese hamsters by high LET (alpha particles from ^{241}Am, ^{239}Pu, and ^{252}Cf) and low LET (^{60}CO gamma rays and ^{144}Ce-^{144}Pr beta particles). (From Brooks, A.L., *Health Phys*., 105, 407, 2013.)

of the dose–response relationship for the internally deposited alpha-emitting radionuclides (^{241}Am, ^{239}Pu, and ^{252}Cf) is quite different. The alpha radiation, which has a high LET, is much more effective in producing aberrations than is the low-LET radiation. As will be described later, the alpha-emitting radionuclides are also highly effective in inducing cancer.

The induction of mutations in somatic cells increases the probability of cancer occurring after a variable latent period, over and above the background incidence. The induction of mutations in germ cells is key to induction of genetic effects in the progeny of the irradiated parents.

The health impact of any radiation dose, delivered either from external exposure or internally deposited radionuclides, is a function of (1) the quality of the radiation, (2) the rate at which the dose is delivered, (3) the total dose delivered to critical tissues, and (4) the time elapsed from when the radiation dose was delivered. These factors all influence the probability of cells being killed, mutations being induced, cells being damaged, damage in cells being repaired, and surviving cells having mutations that manifest in their progeny. It is important to recognize that these radiation-induced events all occur in company with the similar events occurring naturally or induced by other factors. Irradiated cells do not respond in isolation.

One of the most recent developments in radiation biology has been the discovery of a bystander effect. It has been recognized for some time that targeted cytoplasmic irradiation can result in mutational changes in the nuclein of hit cells. It is now known that cells not directly hit by alpha particles or other higher-energy ions also contribute to the response of bystander ells (Wu et al. 1999). The clinical significance of the finding is still being discussed. Multiple authors (Lorimore et al. 2003; Azzam and Little 2004; Blyth and Sykes 2011) have provided a comprehensive review of current knowledge of radiation-induced bystander effects, including their relevance to human exposures.

A critical difference between time dimensions of delivery of the radiation dose from external sources and internally deposited radionuclides was illustrated earlier in Figure 18.2. With external exposure, the dose is typically received over a very short time period with the exception of low-level exposure from natural background. Exposure and delivery of the dose occur at the same time. While the radiation dose to various portions of the body may vary, the radiation dose among the tissues of the body is relatively uniform unless some portion of the body has been purposefully irradiated and other portions shielded. Of course, it is possible for an individual to receive multiple brief exposures.

The situation is quite different for internally deposited radionuclides. An individual may be briefly exposed to a radioactive aerosol at an industrial setting. The radiation dose to the respiratory tract and other body tissues will be dependent on the particle size, the chemical characteristics of the material inhaled and its solubility, and obviously the physical half-life and emission characteristics of the radionuclide(s). The time course for exposure, for example, by inhalation or ingestion, versus the time course for delivery of the radiation

dose is different. Moreover, the radiation dose rate and cumulative radiation doses received by different organs and, indeed, various cell types and tissues within a given organ may be markedly different, dependent on the distribution of the radionuclide within the organ.

A measure of the rate at which energy of differing quality is transferred to tissue, LET is expressed in units of kiloelectron volts of energy transferred per micrometer of track length in tissue (keV/μm). The LET of typical diagnostic x-rays is about 3 keV/μm. These x-rays have an RBE of 1.0 serving as a base for considering the RBE of other radiation. Beta particles will typically have an RBE of about 1.0, while alpha particles have an RBE of about 20 as was shown in Figure 18.10 for induction of chromosomal aberrations. As the LET of the radiation increases, the RBE also increases. After reaching a maximum, the RBE for radiation with an LET above 100–200 keV/μm decreases due to overkill.

The penetrating ability of x-rays and gamma rays originating outside of the body is a function of the photon energy. High-energy photons can penetrate to considerable depths in tissue, and a substantial portion may pass through the body, the basis for diagnostic x-ray radiographs. Gamma emissions from decaying radionuclides within the body may, with sufficient energy, result in the gamma photons leaving the body. Thus, the body may serve as a radiation source. Gamma photons such as those from the decay of internally deposited $^{134-137}$Cs deliver radiation dose to both the organ containing the radiocesium as well as adjacent organs (Boecker 1972).

Gamma photons and beta particles in fallout particles when deposited on the skin irradiate the underlying dermal cells. The vast majority of the radiation dose to the underlying tissue is typically from the beta particles. The distance that the beta particles travel in tissue is dependent upon their energy. Beta particles from the decay of internally deposited radionuclides effectively irradiate the tissue in which the radionuclide is deposited, such as lung, liver, or bone. Beta-emitting radionuclides, when present at high concentrations such as in lung-associated lymph nodes, may result in irradiation of adjacent organs such as the heart and lung. The range of alpha particles is quite short such that all of their energy is released as they traverse only a few cells.

The sensitivity of tissue to radiation damage, especially for radiation below 10 keV/μm, is influenced by its degree of oxygenation. Well-oxygenated tissue is more sensitive to radiation damage than when the tissue is anoxic, the oxygen effect. The oxygen enhancement ratio is a ratio of the dose necessary under anoxic conditions to produce a given effect in comparison with the dose necessary to produce the same effect under aerobic conditions. The oxygen enhancement ratio is on the order of 3.0 for x-rays and gamma photons with an LET of 0.1–0.5 keV/μm decreasing to an oxygen enhancement ratio of about 2.0 for 5 keV/μm radiation and an absence of any oxygen enhancement for radiation with an LET of greater than 50 keV/μm.

Protraction and fractionation of the radiation dose have a profound effect on the effectiveness of the irradiation, whether it is originating externally or from internally deposited radionuclides. If the length of time over which a given dose is delivered, that is, the dose rate is decreased, the effectiveness of a given total dose is decreased. The same reduction in effectiveness is observed if the same total dose is delivered in equal fractions over a number of days.

The situation for internally deposited radionuclides is complicated by multiple factors: the nature of the exposure (a brief intake versus chronic intake), the route of exposure (inhalation, ingestion, or a wound), the biological characteristics of the radioactive material (e.g., its solubility and elemental characteristics), how it distributes in the body, the radiation emissions, and the physical half-life of the radionuclide(s). With a brief intake, the effective half-life (a function of both biological and physical half-life) will determine how the dose rate decreases over time postexposure. For example, a short-lived radionuclide, such as ^{90}Y with a half-life of 64 h, will result in a dose delivered at a steadily decreasing dose rate with essentially all of the dose delivered in a matter of 10 days or so. In contrast, a long-lived radionuclide, such as ^{90}Sr, with a physical half-life of 28.8 years and an effective half-life in the body of several hundred days will deliver a steadily decreasing dose rate such that the total dose will be delivered over several years. Multiple intakes of a radionuclide will result in a steady increase in the amount of internally deposited radioactivity in the tissues with a corresponding increase in the radiation dose rate. Dependent upon the duration of intake and the decay half-time of the radionuclide, an equilibrium relationship may be reached between intake and body burden. Every exposure to internally deposited radionuclides results in protracted radiation exposure. The reduced effectiveness of radiation dose protraction from internally deposited radionuclides, especially for long-lived radionuclides, is well documented as will be discussed later.

EFFECTS OF EXTERNAL RADIATION EXPOSURE

Acute Effects of External Exposure

Acute effects of external radiation exposure, including acute radiation-induced lethality, have been studied in detail in many species. As will be discussed later, the early literature refers in a generic manner to early deaths being due to multiple acute radiation syndromes, while more recent literature uses the term *acute radiation sickness* (ARS; Mettler and Upton 2008). The descriptor *acute* relates to deaths occurring within a few months of external exposure to radiation or a very substantial intake of radioactive material and delivery of substantial radiation doses over a relatively short period of time. The term *syndromes* refers to the predominant underlying tissues that are injured—neurovascular, gastrointestinal, hematopoietic, and cutaneous—and involved in death at various absorbed radiation doses. This division into distinct phases and tissues may be misleading because at doses above a few gray, many tissues are damaged, and the condition of the subject results from interactions among multiple mechanisms in multiple organs and tissues producing a range of clinical signs and symptoms. Thus, the more modern term, ARS, is more appropriate.

Many thousands of individuals died of ARS following the bombings of Hiroshima and Nagasaki, and, as will be discussed later, many thousands of individuals survived the bombings, including many who had experienced ARS. However, the catastrophic nature of these bombings limited the amount of detailed clinical data that could be acquired on the victims. It is important to recall that the Japanese victims received very brief, essentially instantaneous, exposures to gamma and x-ray photons and, at Nagasaki, some neutrons (Recall Figure 18.2). These individuals were also impacted by the blast wave and thermal radiation exposures coming from the detonation of the weapons. It is estimated that for a typical nuclear detonation, the distribution of energy released is as follows: (1) blast (pressure shock wave)—50%, (2) thermal radiation (including light)—35%, and (3) nuclear radiation (both prompt and delayed)—15% (Glasstone and Dolan 1977). It should not be surprising then that most of the awesome destructive impact of a nuclear detonation is associated with the blast wave and thermal radiation. The impact of the blast and thermal energy released was dominant close to the point of detonation or epicenter and caused injury and death of thousands of individuals. The radiation exposures and resulting tissue doses are of concern at greater distances from the point of detonation where the blast overpressure and thermal energy are lower. Inhalation and ingestion of radioactive materials did not have a substantial role in the deaths observed following the Japanese bombings and was not a significant source of long-term radiation exposure.

The most useful information on ARS is based on the subjects included in registries, especially that maintained by the IAEA, which includes about 400 cases (IAEA 1998). About one-fourth of the cases died of ARS, and about three-fourths survived despite having some signs and symptoms of ARS. The number of survivors provides valuable insights into the management of acute radiation exposure cases. As an aside, about one-third of the cases are from the Chernobyl accident, either employees at the reactor when the accident began or first responders. Essentially all ARS cases are attributed to external radiation exposures. One fatality has been documented from medical misadministration of ^{198}Au, several cases related to combined external exposure from an internal intake of ^{137}Cs, and one case attributed to intake of ^{210}Po, a short-lived alpha emitter, in a purposeful poisoning case.

The IAEA (1998) has carefully analyzed all of the cases and drawn some general conclusions that are presented in Tables 18.10 through 18.12. The tables are adapted from the IAEA (1998) report, which provides an extensive discussion of the findings on which these summary tables are based. These tables are also discussed in Mettler and Upton (2008), which I view as the most recent comprehensive text on the

TABLE 18.10
Prodromal Phase of Acute Radiation Sickness

	Degree of ARS and Approximate Dose of Acute WBE (Gy)				
Symptoms and Medical Response	**Mild (1–2 Gy)**	**Moderate (2–4 Gy)**	**Severe (4–6 Gy)**	**Very Severe (6–8 Gy)**	**Lethal[a] (>8 Gy)**
Vomiting onset	2 h after exposure or later	1–2 h after exposure	Earlier than 1 h after exposure	Earlier than 30 min after exposure	Earlier than 10 min after exposure
Percentage of incidence	10–50	70–90	100	100	100
Diarrhea	None	None	Mild	Heavy	Heavy
Onset	—	—	3–8 h	1–3 h	Within min or 1 h
Percentage of incidence	—	—	<10	>10	Almost 100
Headache	Slight	Mild	Moderate	Severe	Severe
Onset	—	—	4–24 h	80	1–2 h
Percentage of incidence	—	—	50	May be altered	80–90
Consciousness	Unaffected	Unaffected	Unaffected		Unconsciousness (may last s/min)
Onset	—	—	—	—	s/min
Percentage of incidence	—	—	—	—	100 (at >50 Gy)
Body temperature	Normal	Increased	Fever	High fever	High fever
Onset	—	1–3 h	1–2 h	<1 h	<1 h
Percentage of incidence	—	30–80	80–100	100	100
Medical response	Occupation observation	Observation in general hospital, treatment in specialized hospital if needed	Treatment in specialized hospital	Treatment in specialized hospital	Palliative treatment (symptomatic only)

Source: Adapted from International Atomic Energy Agency (IAEA), Diagnosis and treatment of radiation injuries, Safety report series No. 2, IAEA, Vienna, Austria, 1998.

WBE, whole-body exposure.

[a] With appropriate supportive therapy, individuals may survive for 6–12 months with whole-body doses as high as 12 Gy.

TABLE 18.11
Latent Phase of Acute Radiation Sickness

	Degree of ARS and Approximate Dose of Acute WBE (Gy)				
	Mild (1–2 Gy)	**Moderate (2–4 Gy)**	**Severe (4–6 Gy)**	**Very Severe (6–8 Gy)**	**Lethal (>8 Gy)**
Lymphocytes (G/L) (days 3–6)	0.8–1.5	0.5–0.8	0.3–0.5	1.0–0.3	0.0–0.1
Granulocytes (G/L)	>2.0	1.5–2.0	1.0–1.5	≤0.5	≤0.1
Diarrhea	None	None	Rare	Appears on days 6–9	Appears on days 4–5
Epilation	None	Moderate beginning on day 15 or later	Moderate, beginning on days 11–21	Complete earlier than day 11	Complete earlier than day 10
Latency period (days)	21–35	18–28	8–18	7 or less	None
Medical response	Hospitalization not necessary	Hospitalization recommended	Hospitalization necessary	Hospitalization urgently necessary	Symptomatic treatment only

Source: Adapted from International Atomic Energy Agency (IAEA), Diagnosis and treatment of radiation injuries, Safety report series No. 2, IAEA, Vienna, Austria, 1998.

G/L, gigaliter; WBE, whole-body exposure.

TABLE 18.12
Findings of Critical Phase of Acute Radiation Sickness Following Whole-Body Exposure

	Degree of ARS and Approximate Dose of Acute WBE (Gy)[a]				
	Mild (1–2 Gy)	**Moderate (2–4 Gy)**	**Severe (4–6 Gy)**	**Very Severe (6–8 Gy)**	**Lethal (>8 Gy)**
Onset of symptoms (days)	>30	18–28	8–18	<7	<3
Lymphocytes (G/L)	0.8–1.5	0.5–0.8	0.3–0.5	0.1–0.3	0.0–0.1
Platelets (G/L)	60–100	10–60	25–35	15–25	<20
	10%–25%	25%–40%	40%–80%	60%–80%	80%–100%[b]
Clinical manifestations	Fatigue, weakness	Fever, infections, bleeding, weakness, epilation	High fever, infections, bleeding, epilation	High fever, diarrhea, vomiting, dizziness and disorientation, hypotension	High fever, diarrhea, unconsciousness
Lethality (%)	0	0–50	20–70	50–100	100
		Onset 6–8 weeks	Onset 4–8 weeks	Onset 1–2 weeks	1–2 weeks
Medical response	Prophylactic	Special prophylactic treatment from days 14 to 20; isolation from days 10 to 20	Special prophylactic treatment from days 7 to 10; isolation from the beginning	Special treatment from the first day; isolation from the beginning	Symptomatic only

Source: Adapted from International Atomic Energy Agency (IAEA), Diagnosis and treatment of radiation injuries, Safety report series No. 2, IAEA, Vienna, Austria, 1998.

[a] 1 Gy = 100 rad.

[b] In very severe cases, with a dose >50 Gy, deaths precede cytopenia.

medical effects of ionizing radiation. All of the acute clinical responses discussed would be considered as deterministic effects.

Associated with the Chernobyl accident, 134 patients were considered to have the ARS (Gusev et al. 2001; Mettler et al. 2007; Mettler and Upton 2008). This group comprised 28 individuals who died, including 20 deaths among 21 individuals who received 6.5–16 Gy (very severe ARS), 7 deaths of 22 patients who received 4.2–6.4 Gy (severe ARS), and 1 death of 50 patients who received 2.2–4.1 Gy (moderate ARS). In retrospect, three of the deaths were believed to have occurred unnecessarily as a result of inappropriate bone marrow transplantation. The transplant surgeon participating in the treatment of these cases was very experienced with treating patients purposely exposed to known quantities of radiation with precisely calculated and delivered bone marrow doses before receiving a bone marrow transplant. The situation is quite different in an accident when the actual radiation exposure and dose received by the subject must be estimated from clinical signs and symptoms. Treatment of individuals with doses below 9 Gy only worsened the ARS results due to complications associated with immunological rejection of the bone marrow transplant. Mettler and Upton (2008) suggest that only a small percentage of radiation accident

TABLE 18.13
Representative Lethal Dose 50% (30 Days) Values for Various Mammalian Species

			$LD_{50/30}$ Days Absorbed Dose at Midline	
Species	**Type of Radiation**	**Air R**	**Rad**	**mGy**
Mouse	250 KVP x-ray	443	638	6380
Rat	200 KVP x-ray	640	796	7960
Guinea pig	200 KVP x-ray	337	400	4000
Rabbit	250 KVP x-ray	805	751	7100
Monkey	250 KVP x-ray	760	546	5460
Dog	250 KVP x-ray	281	244	2440
Swine	1000 KVP x-ray	510	247	2470
Goat	200 KVP x-ray	350	237	2370
Sheep	200 KVP x-ray	250	237	2050
Burro	~1.1 MeV gamma	651	256	2560

victims would benefit from bone marrow transplantation, those patients that received sufficiently high radiation doses to completely destroy their original marrow cells, but sufficiently low to have minimal other radiation-induced effects.

Mettler and Upton (2008) have estimated the radiation dose required to kill 50% of individuals within 60 days in the absence of medical intervention as 3.5–4.0 Gy, air or surface dose. This would correspond to a midline tissue dose of about 2.7 Gy. As noted earlier, numerous studies have been conducted on acute radiation effects in species other than man. Some representative lethal doses (50% deaths within 30 days) are shown in Table 18.13. The range of values is remarkably narrow from about 2.5 to 7.5 Gy. Caution should be exercised in interpreting differences in the $LD_{50/30}$ values between species as being true interspecies differences. The studies were conducted at different times with different radiation sources and subjects that were in different environments on different diets and with different internal microbial flora.

Heritable Effects

Observations by Müller (1927), Oliver (1930), and others that exposure to high doses of x-rays caused a readily observable dose-dependent increase in mutations in the fruit fly, *Drosophila melanogaster*, galvanized both scientific and public concern for an increase in *genetic* disease in the progeny of irradiated individuals. Other studies would follow showing the effect varied for different stages of meiosis (Marshak 1935). Today, it is recognized that the term *heritable effects* is more descriptive in recognizing that the cancer induction by radiation exposure is a *genetic* effect, a fact that was not well recognized and accepted until much later. Concern for radiation-induced heritable effects was a dominant factor in the early setting of the maximum permissible exposure limit for radiation workers. In the 1950s, based on the results of studies with x-irradiated fruit flies, it was thought that exposure to 50–150 R (0.5–1.5 Gy) would double the spontaneous mutation rate. It was also thought that effects of radiation exposure on heritable effects was cumulative over multiple generations and would increase the burden of heritable disease for the human race. It is also important to recognize that in the 1940s and 1950s, the cancer-inducing ability of radiation was not as apparent as it is now. Early results from the studies of the Japanese atomic bomb survivors indicated an increase in leukemia (Folley et al. 1952; Ishimaru et al. 1971; Richardson et al. 2009); however, an increase in solid cancers had not yet been observed in the 1950s. Thus, it was reasonable then to view radiation induction of heritable effects as a greater concern than cancer induction.

Two avenues of research would produce results that would temper and, indeed, change the early conclusions and result in later radiation protection standards being increasingly dominated by concern for cancer induction rather than heritable effects. A major influence came from the research of W. L. (Russell 1963, 1965) Russell and his colleagues at Oak Ridge NL who used about seven million inbred mice to investigate the influence of radiation exposure on seven specific locus mutations. These studies showed different radio sensitivities for the mutation rates of different loci. In the mouse, they also found a substantial dose rate effect: dose protraction resulted in fewer mutations than a single acute dose. Recall the results in Figure 18.10 for the induction of chromosomal aberrations in Chinese hamsters given acute versus chronic gamma irradiation. Moreover, Russell and colleagues (1963) also found that increasing the interval between irradiation and conception markedly reduced the effectiveness of a given dose. However, those results had to be tempered by recognition that the oocytes of female mice were very sensitive to radiation and were killed by quite low radiation doses.

The fourth BEIR Committee (NRC 1990) and the UNSCEAR (1988) estimated the doubling dose for inducing heritable mutations in humans was at least 1 Gy for low-dose-rate, low-LET irradiation. Sankaranarayann and Chakraborty (2000) and Neel (1998) discussed this shift in view. A report edited by Neel and Schull (1991) summarizes a wealth of information obtained from studies on the children of the atomic bomb survivors.

An important line of evidence for external radiation exposure inducing heritable effects has come from the study of the cohort of 31,150 children born to parents who received significant exposure from the Japanese A-bombings (Neel et al. 1990). A range of indicators of heritable effects were evaluated. The difference in measured markers of heritable effects between the children of parents proximally or distally exposed was in the direction to be expected if there were a heritable effect of radiation. However, none of the findings was statistically significant. Schull et al. (1981) in an appraisal of the data on the children of Japanese atomic bomb survivors estimated that the doubling dose could be 1.56 Sv for the acute instantaneous external radiation exposure of the parents from detonation of the A-bombs. Neel et al. (1990) indicate that considering all heritable outcomes, the best estimate of the doubling dose for humans is between 1.7 and 2.2 Sv. These results suggest that humans are not as sensitive to the

genetic effects of radiation as mice (Neel 1998). UNSCEAR (2005) provides a comprehensive review of the hereditary effects of ionizing radiation.

Hall and Giaccia (2012) noted that the concern of the ICRP for heritable effects as part of the total radiation detriment has shifted over the decades. Heritable effects were viewed as the dominant controlling effects for radiation protection purposes in 1955 and earlier; this was reduced to 25% in 1971 and further reduced to 18% in 1991. By 2007, it was viewed as only 4% of the total radiation detriment. Over this total time period, heritable effects of radiation continued to be viewed as increasing as a linear function of dose without a threshold. The BEIR V (NRC 1990) estimates that the heritable risk of radiation is about 0.2% per Sv for the general population and about 0.1% per Sv for a working population. Thus, a dose limit for radiation workers of a maximum of 50 mSv/year is viewed as adequately protective of potential heritable effects.

Late-Occurring Effects of External Radiation Exposure

A very large body of information exists on the late-occurring effects of external radiation exposure, and Mettler and Upton (2008) have discussed this literature in detail. Shore (2013) has recently provided a succinct summary of the epidemiological findings on radiation and cancer. The International Agency for Research on Cancer (IARC 2000) published a monograph on reviewing the cancer hazards of x-irradiation and gamma irradiation and neutrons. Unfortunately, the review focused on cancer hazard and did not rigorously consider the quantitative relationship between dose and cancer outcome. The fact that ionizing radiation is a cancer hazard is well established. The issues today are concerned with dose–response relationships, especially at low dose rates and low total doses.

Some of the major populations studied to evaluate the effects of external radiation exposure are listed in Table 18.14. The best information available on humans has been obtained from detailed studies, still ongoing, of the survivors of the atomic bombings of Hiroshima and Nagasaki and, to a lesser extent, from individuals accidentally or purposefully exposed, as in the case of patients receiving either diagnostic or therapeutic x-irradiation. In all but a few of these situations, the radiation exposures were instantaneous as with the atomic bomb exposures or were quite brief, high-intensity exposures as illustrated earlier in Figure 18.2. It is important to recognize that very little of the information on late effects of human exposures to external radiation involves well-characterized absorbed doses from chronic external radiation exposure.

TABLE 18.14
Human Populations Studied Following External Exposure to X-Radiation or Gamma Radiation

Population	Effect	Key Reference
Atomic bomb survivors	Cancer and other diseases	Ozasa et al. (2012)
Prenatal irradiation	Leukemia Cancer	Doll and Wakeford (1997)
Ankylosing spondylitis patients	Cancer	Court Brown and Doll (1965)
Radiologists	Leukemia	Matanowski et al. (1975) Smith and Doll (1981)
Thymic enlargement	Thyroid cancer	Shore et al. (1993)
Tinea capitis	Thyroid cancer	Shore et al. (2003) Ron et al. (1989)

An extraordinarily large body of information exists on late-occurring effects observed in a number of laboratory animal species such as rats, mice, dogs, and burros following brief, fractionated, or protracted whole-body exposure to external radiation. The information obtained in the laboratory animal studies complements the human experience and is especially valuable in interpreting and extending the findings in exposed human populations. I emphasize populations since the effects of primary concern are largely stochastic effects, with the emphasis on the occurrence of cancer, whose probability of occurrence increases with dose, and the severity of the effect is independent of dose. Thus, relevant information can only be obtained from studying populations of laboratory animals. Indeed, the probability of radiation exposures in excess of background resulting in an increased occurrence of disease, including cancer, over and above the background incidence is so low that quite large populations must be studied to obtain statistically significant results. To provide perspective, assume the background incidence of an endpoint, such as a specific kind of cancer, is 1%. It will be necessary to have a group size of 400 to detect a statistically significant increase in the endpoint over background incidence with 95% confidence. The statistically significant increase would be an incidence of 5% corresponding to a relative risk (RR) of 5.0. It is apparent that laboratory animal bioassays as well as studies of small human populations are *blunt* approaches for detecting increases in late-occurring, relatively infrequent diseases. It is important to note that the clinical course and morphological characteristics, both gross and microscopic, of the radiation-induced cancers are the same as those occurring spontaneously and of unknown etiology. A radiation-induced cancer does not carry a special unique signature related to its radiogenic origin.

Although the results of the long-term studies of external radiation in laboratory animals are extraordinarily elegant, those data have not generally been viewed as suitable for developing quantitative dose–response estimates of human cancer risks. The situation might have been different if data on exposed human populations were not available. However, when data are available from human studies, they will always, and in my view, appropriately *trump* the use of animal data in developing quantitative dose–response estimates for humans and in developing radiation protection standards and guidance. The primary use of results from animal studies is to aid in understanding the results of the studies of exposed human populations and in extrapolating the human data to unique

exposures such as with different radiation quality. The utility of data from in vitro cellular- and molecular-level studies, especially using cells from species other than humans, is frequently treated in the same manner.

Contrary to the perception of most of the public and many scientists in other fields, and even some radiobiologists, radiation is a rather weak carcinogen. Remarkably high levels of radiation exposure are required to identify statistically significant increases in cancer incidence over background levels. It is useful to recall that in countries having well-developed economies and long-lived populations, about one in three individuals (estimates of 40% or 42% have also been given) will develop cancer over their life span and about one in four individuals will die with a cancer. Throughout this chapter, the word *cancer* will typically be used in its broadest definition, malignant solid tissue tumors and leukemias, unless otherwise noted.

Before proceeding to discuss radiation-induced cancer, it is appropriate to briefly discuss another late-occurring radiation-induced effect, cataracts. Radiation induction of cataracts has typically been viewed as a deterministic effect with a dose threshold and for which the severity increased and latency decreased as the radiation dose increased above the threshold. The ICRP (2007) estimated that brief exposures with doses of at least 0.5–2 Sv are required to cause detectable lens capacities, and doses of over 5 Sv are required to produce vision-impairing cataracts. Shore et al. (2010) analyzed the dose–cataract response relationship for 3994 A-bomb survivors. They reported a best dose-threshold estimate of 0.1 Gy (confidence interval [CI]: <0, 0.8 Gy). However, the data were compatible with there being no dose threshold or a threshold of up to 0.8 Gy but not higher. Detailed observations over their lifetime of large populations of individuals irradiated at doses substantially above the background may reveal that for late-occurring diseases, the distinction between deterministic and stochastic effects may become less distinct than currently thought.

Radiation-induced cancers were observed quite soon after the discovery of radiation and radioactivity. The association between radiation exposure and the occurrence of clinical injury and cancer was obvious on a case-by-case basis. That these cases were observed so soon, with a very short latent period between radiation exposure and the clinical manifestation of the cancers, is a reflection of the extraordinarily high repeated radiation exposures and resultant very large tissue doses these individuals received. The total doses received were likely very high because the repeated, sometimes daily, radiation exposures and tissue doses were sufficiently low so as to avoid acute serious injury. For example, both Marie and Irene Curie are thought to have died of leukemia. Many physicians, dentists, and x-ray technicians who were early users of x-rays also developed skin cancers.

Studies on a number of populations irradiated during medical procedures have given valuable insights into the quantitative relationships between external exposure and the resultant tissue dose and the occurrence of cancer. Pre-1950, it was common practice to use x-rays to treat children with

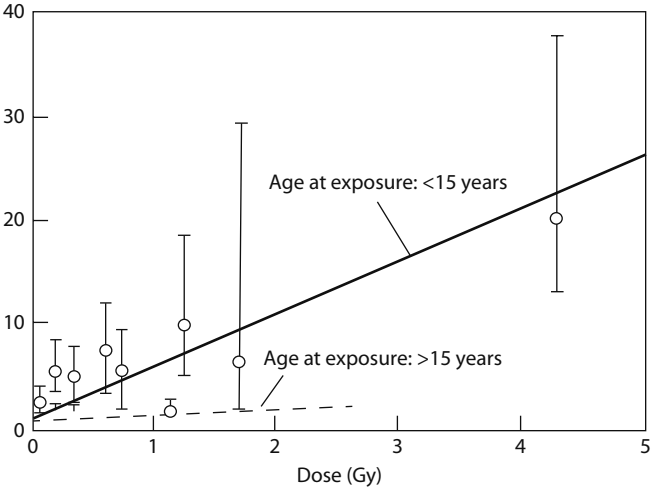

FIGURE 18.11 RR of thyroid cancer after exposure to acute doses of external low-LET radiation. (From Ron, E. et al., *Radiat. Res.*, 141, 259, 1995.)

enlarged thymus glands, diseases of the tonsil and nasopharynx, or tinea capitis (ring worm). A follow-up of these populations revealed a dose-related increase in thyroid cancer (Ron et al. 1995) (Figure 18.11). The cancers occurred in the thyroid because the thyroid glands were in the radiation exposure field since the x-ray beams were not as well collimated as today. The excess relative risk (ERR) was estimated to be 7.7% per Gy for the individuals irradiated at less than 15 years of age. The excess risk for individuals irradiated over 15 years of age was substantially reduced. Radiation-induced tumors develop slowly and often can be successfully treated by surgery or with radioiodine. Thus, the mortality rate for thyroid cancer is estimated to be about 5% (Hall and Giaccia 2012).

Dose-related increases in breast cancer have been observed in a number of populations (Boice et al. 1979). The populations studied included women treated with x-rays for postpartum mastitis and patients who were repeatedly examined fluoroscopically following the induction of pneumothorax to treat pulmonary tuberculosis. The dose–response data for breast cancer were considered to be reasonably well fit by a linear function.

By far the most comprehensive data on the effects of external radiation exposure have come from the detailed follow-up of the survivors of the atomic bombings of Hiroshima and Nagasaki. A cooperative effort by the governments of Japan and the United States was initiated soon after World War II to follow these populations and assist in coordinating their medical care. The initial effort was carried out by the ABCC, which became the RERF. The U.S. DOE and its predecessor organizations provided government funds for both entities via the U.S. National Academy of Sciences (NAS). This cooperative effort continues today. The goals of the program include care of the populations and scientific studies to gain insights into the long-term effects of brief external exposure to radiation. There are three ABCC/RERF study groups. The Life Span Study (LSS) includes about 120,321 individuals,

82,214 in Hiroshima and 38,107 in Nagasaki. Information from this group provides valuable insights into radiation-induced cancer incidence and cancer mortality. The in utero cohort includes about 3300 individuals who received in utero exposure as a result of their mother's exposure. Valuable information from this population has been obtained on the incidence of malformations, growth retardation, microcephaly, and mental retardation. The 77,000 children born to survivors of the atomic bombings, the so-called F_1 generation, are the third population group. Observations in this group provide valuable data for assessing heritable effects (Neel et al. 1990). It is important to note that radiation exposure and organ doses for each individual in the ABCC/RERF cohort has been carefully estimated taking into account precisely where the individuals were when the bombs exploded and any shielding between them and the detonation (Cullings et al. 2006; Young and Bennett 2006).

Ozasa et al. (2012) have published an overview of cancer and noncancer disease mortality for the atomic bomb survivors from 1950 through 2003. This report of Ozasa et al. (2012) is the 14th in a series on the mortality of the LSS cohort with the first report published in 1962 (Beebe et al. 1962). The reports contain detailed information on dosimetry, medical follow-up, and the statistical methods used to analyze and model the data. No other large population exposed to any other toxic agent has been studied as exhaustively over more than a half century, and continuing today, as the population of Japanese atomic bomb survivors.

The efforts of the ABCC/RERF scientists and staff and the cooperation of the atomic bomb survivors and their children have been remarkable! The observed and excess deaths for the LSS are summarized in Table 18.15. In addition to the 86,611 individuals shown in this table by dose level, another group of individuals who were not in the cities at the time of the bombings has been followed. Some individuals have viewed that population as a control group; however, the RERF scientists have taken an alternative approach in analyzing the data as discussed in the following. The summary data reported by Ozasa et al. (2012) extend through 2003. Thus, if an individual had been a year old at the time of bombing in 1945, he or she would have been 59 years old in 2003. Other individuals in the surviving population would have been older in 2003.

When considering the findings from the studies of the Japanese survivors, it is important to recall that they include some 624 individuals with very high radiation exposures (doses over 2 Gy). Recall the earlier discussion of acute radiation diseases. If those individuals with radiation doses of over 2 Gy had received only slightly higher radiation exposures, some would have died from acute radiation injury. By analogy to animal bioassays, the individuals in the LSS cohort in this highest dose range received maximum tolerated doses.

It is of interest to note that of the 86,601 individuals alive in 1950, a total of 46,614 individuals or 53.8% of the individuals had died by 2003 with 46.2% surviving. The 10,929 cancer deaths represent 23.4% of the deaths to date. The RERF scientists have *not* estimated the number of excess deaths attributed to radiation by comparing the number of deaths in the irradiated groups with that in a control population. Alternatively, they have calculated the number of excess cases by modeling using a linear dose–response model with effects modification. Table 18.15 shows the number of excess cases for cancer and noncancer diseases in each dose interval as well as the fraction of cases attributed to radiation exposure. The portion of radiation-attributable cases increases in each dose group, reaching 56.5% for cancer and 16.3% for noncancer effects in the highest-dose group (over 2 Gy). As expected, most of the excess cases estimated to be attributed to radiation exposure are in the highest radiation dose intervals. Some readers may be surprised to note the relatively modest number of estimated excess cancer cases. As noted earlier, despite popular opinion to the contrary, radiation is a relatively weak carcinogen. For the Japanese A-bomb LSS cohort through 2003, 527 cancer deaths and 353 deaths from

TABLE 18.15
Observed and Estimated Excess Deaths in Cancer and Noncancer Diseases in the ABCC/RERF LSS Cohort

			Solid Cancer			Noncancer Diseases[a]		
Colon Dose (Gy)	Number of Subjects	Person-Years	Number of Deaths	Number of Excess Cases[b]	Attributable Fraction (%)	Number of Deaths	Number of Excess Cases[a]	Attributable Fraction (%)
<0.005	38,509	1,465,240	4621	2	0	15,906	1	0
0.005–	29,961	1,143,900	3653	49	1.3	12,304	36	0.3
0.1–	5974	226,914	789	46	5.8	2504	36	1.4
0.2–	6356	239,273	870	109	12.5	2736	82	3.0
0.5–	3424	129,333	519	128	24.7	1357	86	6.3
1–	1763	66,602	353	123	34.8	657	76	11.6
2+	624	22,947	124	70	56.5	221	36	16.3
Total	86,611	3,294,210	10,929	527	4.8	35,685	353	1.0

Source: Ozasa, K. et al., *Radiat. Res.*, 177, 229, 2012.

[a] Nonneoplastic blood diseases were excluded from noncancer diseases.

[b] Based on the ERR as defined from the linear model with effect modification: $\gamma_o(c,s,b.a)[1 + \beta_1 d \cdot \exp(\tau\, e + \upsilon\, 1n(a)) \cdot (1\, \sigma\, s)]$.

diseases other than cancer were estimated to be attributable to radiation exposure. Over half of the estimated excess deaths were individuals that received doses in excess of 0.5 Gy. To provide perspective, recall that Mettler and Upton (2008) estimated a midline tissue dose of 2.7 Gy would result in 50% of the irradiated individuals dying within 60 days.

Of the 86,611 subjects with estimated doses for 15 organs, 50,620 (58%) had died during the 1950–2003 follow-up period. This included 99.6% of those who were 40 years or older when exposed; if alive in 2003, they would have been 98 or more years old. About 80% of those less than 20 years of age in 1945 were still alive. Twenty-two percent of the deaths were due to solid cancers, 1.4% to lymphoid and hematopoietic malignancies, 71% to nonneoplastic diseases, and 5% to external causes. The estimated excess risk values for major causes of death are shown in Figure 18.12. The ERRs were

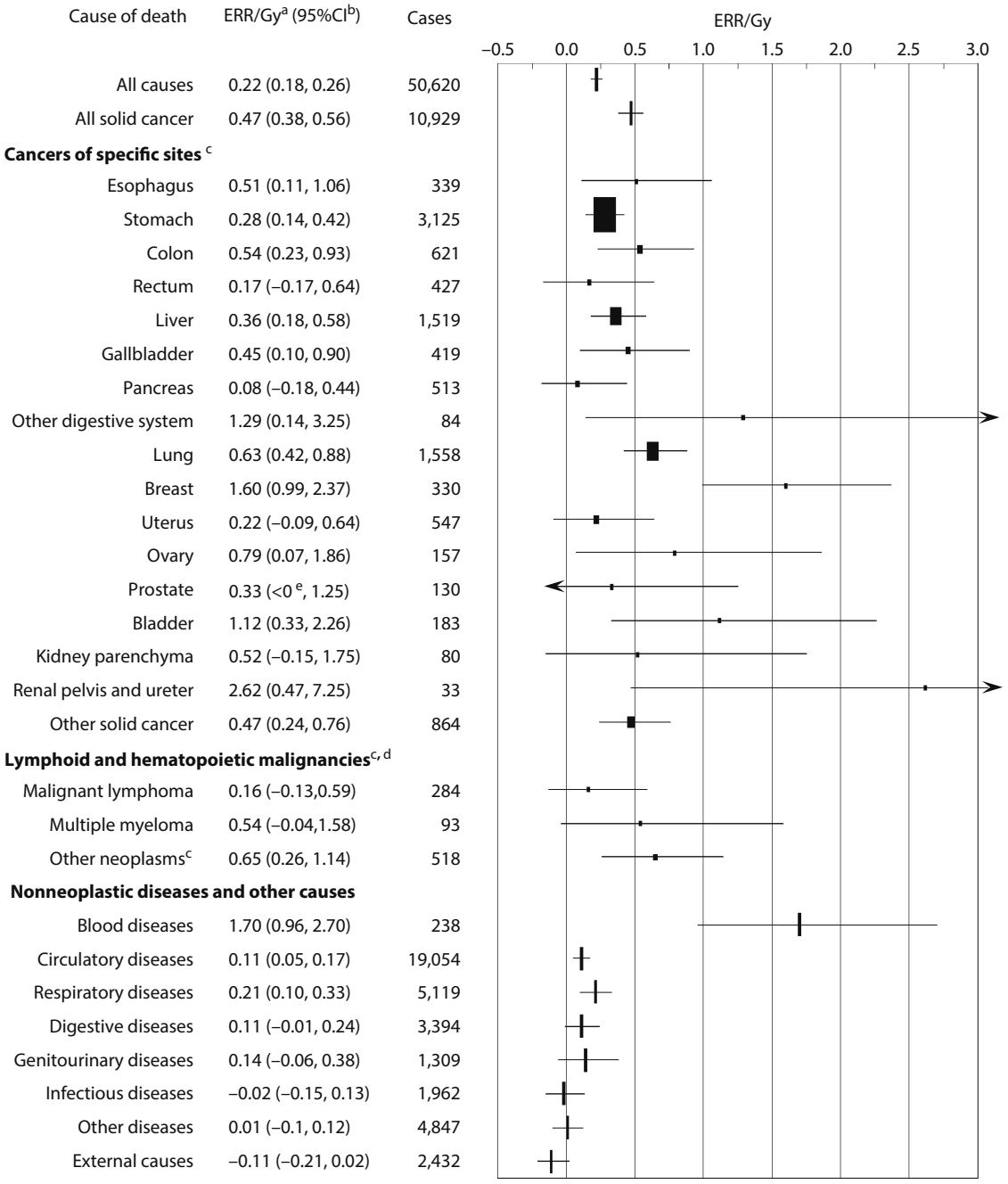

FIGURE 18.12 Estimates of ERR per gray and 95% CI for major causes of death in the Japanese A-bomb survivors. [a]ERR was estimated using the linear dose model, in which city, sex, age at exposure, and attained age were included in the background rates, but not allowing radiation effect modification by those factors. [b]Confidence interval. Horizontal bars show 95% CIs. [c]The size of plots for site-specific cancers was proportional to the number of cases. [d]ERR (95% CI) of leukemia was 3.1 (1.8, 4.3) at 1 Gy and 0.15 (−0.01, 031) at 0.1 Gy based on an LQ model with 318 cases (not displayed in the figure). [e]The lower limit of 95% CI was lower than zero, but not specified by calculation (1950–2003). (From Ozasa, K. et al., *Radiat. Res.*, 177, 229, 2012.)

calculated using the simple linear statistical dose–response model. As expected, the most stable estimate was found for the cancers of specific sites with the largest number of cases such as stomach, liver, and lung cancer. Ozasa et al. (2012) also reports how the estimates of effect modification of ERR were influenced by sex, age at exposure, and attained age.

The sex-averaged ERR per gray for all solid cancers was 0.42 (95% CI; 0.32, 0.53). The lowest dose range with a significant ERR for all solid cancers was 0–0.20 Gy with an estimated ERR of 0.56 Gy^{-1} (95% CI; 0.15, 1.04, P = 0.01) and included 74,444 persons with 9063 solid cancer deaths. An estimated 97 excess solid cancer deaths were in this group. The authors noted as follows: "The maximum likelihood estimate of a dose threshold was 0.0 Gy (i.e., no threshold with an estimated upper bound of 0.15 Gy for 95% CI as determined by minimizing the deviance" (Ozasa et al. 2012). The ERR per gray was highest for those exposed at 10 years of age or younger and decreased with each decade of age at exposure and for all ages decreased with attained age.

Both linear and LQ models were evaluated. Figure 18.13 shows the ERR for solid cancers using alternative models. The authors reported as follows: "The linear dose–response relationship provided the best fit to the solid cancer data across the entire dose range in this study, but significant upward curvature was observed over the truncated dose range of 0.2 Gy."

In considering the data presented in Ozasa et al. (2012) on lymphoid and hematopoietic malignancies, it is important to note that most excess cases of leukemia occurred shortly after the atomic bombings and before the LSS was assembled (Folley et al. 1952; Ishimaru et al. 1971; Richardson et al. 2009).

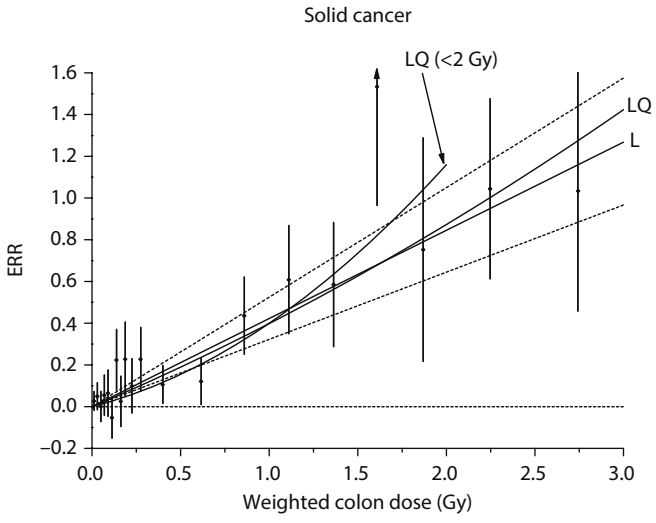

FIGURE 18.13 ERR for all solid cancers in relation to radiation exposure. The black diamonds represent ERR, and the bars are the 95% CI for the dose categories together with trend estimates based on linear (L) with 95% CIs (dotted lines) and LQ models using the full dose range and LQ model for data restricted to dose <2 Gy. (From Ozasa, K. et al., *Radiat. Res.*, 177, 229, 2012.)

The data presented in Ozasa et al. (2012) have already stimulated considerable discussion. The data have been reanalyzed and an alternative interpretation provided by Doss (2013). They reanalyzed the atomic bomb survivor data using a more flexible model that allowed the line of best fit to cross the Y-axis at any location and would also allow for negative predicted ERRs (Figure 18.14). They note, "The flexible model does not show a monotone increase in ERR from zero dose, but it becomes monotone and increases only after the dose reaches 0.27 Gy. Also, the point-wise 95% CI are below zero for most doses below 0.49 Gy. This does not prove there is a threshold effect at 0.27 Gy or 0.49 Gy, but it does demonstrate that there is too much variability in the data to suggest that the threshold for the harmful effects of radiation is zero." Thus, the debate over excess risks of low-level radiation exposure continues.

As noted in Figures 18.12 and 18.13, the risks were elevated for some noncancer diseases, resulting in an estimated 353 excess cases, the attributable fraction representing about 1% of the total noncancer deaths. The elevated risk of diseases of blood and blood-forming organs may be genuinely due to radiation or to possible misdiagnoses of hematopoietic malignancies as nonneoplastic diseases at early time period.

The risk of circulatory diseases was significantly higher. This is of special note since deaths due to circulatory diseases are the leading cause of death in developed countries and typically occur late in life. Shimizu et al. (2010) have reported a more detailed analysis of the relationship between radiation exposure and circulatory disease in the LSS cohort for 1950–2003. This included 19,054 deaths—9622 from

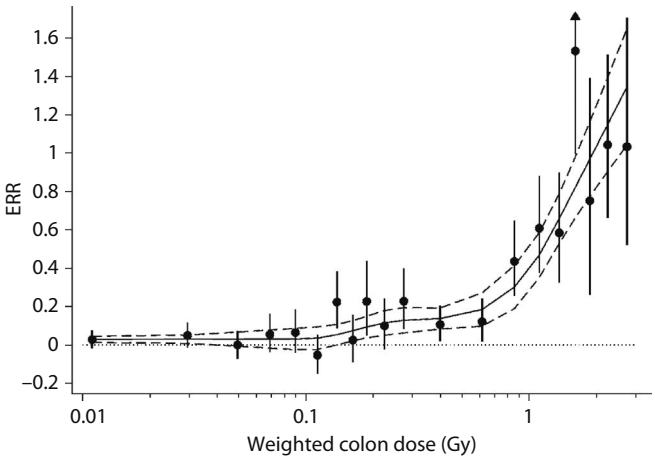

FIGURE 18.14 ERR for all solid cancer in atomic bomb survivors in relation to radiation exposure. The black circles and error bars represent ERR and 95% CIs for the dose categories. Data from (Ozasa et al., 2012). Solid line—fit to the ERR data using a multiple linear regression in which weighted log colon dose was entered into the model using a restricted cubic spline transformation with five knots. Regression weights were equal to the inverse of the variance of the point estimates. Dashed lines are 95% CI of the fit. Figure from performing analysis equivalent to Doss et al. (2012) with the corrected data in Ozasa et al. (2013). Figure provided by Brian L. Egleston. (From Doss, M., *Dose Resp J.* 11, 480, 2013.)

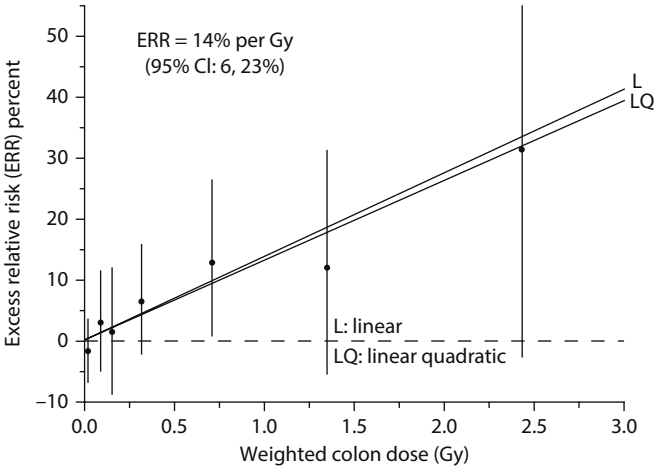

FIGURE 18.15 Heart disease mortality in the Japanese A-bomb survivors, adjusted for gender, age at exposure, attained age, diabetes, obesity, and other factors. (From Shimizu, Y. et al., *Br. Med. J.*, 340, b5349, 2010.)

stroke; 8463 from heart disease; and 969 from other circulatory diseases. They estimated that the excess risk over the full dose range was 11% Gy (95% CI 5%–17%, $P < 0.001$) with an estimated 210 excess deaths from radiation exposure. "For strokes, the estimated excess risk per Gy was 9% (95% CI 1%–17%, $P = 0.02$) on the basis of a linear dose–response model, but an indication of possible upward curvature suggested relatively little risk at low doses. For heart disease, the estimated ERR per Gy was 14% (6%–23%, $P < 0.001$); a linear model provided the best fit, suggesting excess risk even at lower doses. However, the dose–response over the restricted dose range of 0–0.5 Gy was not significant" (Figure 18.15).

The meta-analysis (Little et al. 2009) of circulatory disease from exposure to low-level ionizing radiation supports the results of Shimizu et al. (2010). These recent findings on the possible relationship between radiation exposure and circulatory disease are of keen interest. They suggest that for late-occurring diseases, the distinction between deterministic and stochastic effects may not be as distinct as previously thought.

The risk of respiratory deaths was also increased with influenza and pneumonia accounting for 63% of the respiratory deaths. At early time periods, they were associated with acute epidemics. More recently, these deaths are associated with terminal diseases among the elderly.

It is important to recall that in 2003, 42% of the atomic bomb survivors were still alive 58 years after the bombings of Hiroshima and Nagasaki. They included 80% of those individuals 20 years and younger when irradiated in 1945. Based on the normal pattern of age-related mortality in the general population, most of these individuals would ultimately be expected to die from noncancer diseases of the circulatory system, cancer, and respiratory system, the most common causes of death in the elderly. From 1950 through 2003, deaths from these three causes represented 70% of the total deaths (circulatory, 38%; respiratory, 10%; and cancer, 20%).

The brief description of the latest results from observations on the LSS cohort does not do justice to this remarkable study. Individuals interested in a more in-depth understanding of the late-occurring effects of radiation exposure, and other agents that may cause cancer, are encouraged to read the Ozasa et al. (2012) paper and earlier reports on the LSS series. I encourage the interested reader to go to the RERF site (http://www.temple.edu/newtechlab/TRACES/radeffect.html) where all published reports and papers are available online. As noted elsewhere, I am not aware of any other extended retrospective study in a large population that matches the rigor of the studies of the LSS cohort with regard to estimation of individual doses, medical follow-up, and analysis. As will be discussed later, the LSS cohort studies are especially important because these findings with radiation-induced cancer have been generalized to other cancer-causing agents, especially with regard to the nature of the dose–response relationship. As an aside, most long-term epidemiological studies of human populations exposed to chemicals use an exposure metric, such as exposure intensity or cumulative exposure. It has rarely been possible to estimate a tissue dose metric for large populations exposed to chemicals.

Two additional populations exposed to external radiation warrant discussion: the Russian Techa River Cohort and the 15-Country Collaborative Study of Nuclear Industry Workers. The Techa River population is of interest because they received protracted low-dose-rate radiation exposures and modest total radiation doses. The cohort consists of 29,370 individuals who lived between 1950 and 1960 along the Techa River in the southern Ural Mountains in Russia. Between 1949 and 1956, approximately 10^{17} Bq of uranium fission products was released into the Techa River from the MPA. Subsequently, individuals living along the river received external gamma radiation exposure from contaminated river sediments and floodplain soil and internal radiation exposure from ^{90}Sr, ^{137}Cs, and other radionuclides from the consumption of contaminated water, milk, and food products. The book by Dalton et al. (1999) compares and contrasts the MPA operation and the Techa River population with the Hanford Operations and associated population. The book suggests a degree of similarity that I do not think exists based on my own experience growing up near Hanford. One of the difficulties with the book is it never relates the population exposures for the two populations and focuses on the quantities of radioactive waste at the two sites. Fortunately, the Hanford waste was placed in storage tanks, and, unfortunately, in the early days, the MPA waste was released directly to the environment.

A study of the long-term health effects of the Techa River population was initiated in 1967 (Kossenko et al. 2005). The results of extensive efforts to reconstruct the radiation doses received by the individuals have been reported (Degteva et al. 2006; Tolstykh et al. 2011). An increase in the incidence of leukemia (follow-up from 1953 to 2005) and solid cancers (follow-up from 1956 to 2006) has been reported (Krestinina et al. 2007, 2010). The most recent report (Schonfeld et al. 2013) documents the solid

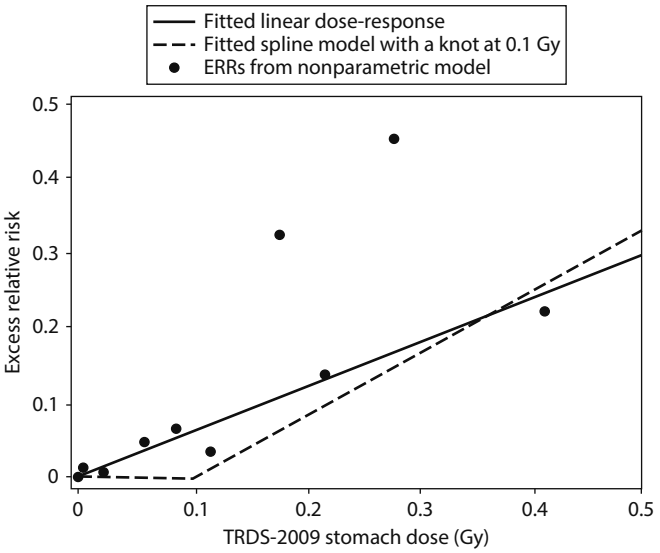

FIGURE 18.16 Solid cancer mortality dose–response relationship in the Techa River Cohort based on TRDA 2009 stomach doses. Circles represent ERR by dose categories from a nonparametric model. Fitted linear dose–response (solid line) and fitted spline model with a knot at 0.1 Gy (dashed line) are also shown. (From Schonfeld, S.J. et al., *Radiat. Res*., 179, 183, 2013.)

cancer mortality for the Techa River Cohort from 1950 to 2007. A total of 2303 solid cancers were observed with 927,743 person-years of follow-up. It was estimated that 2253 cases were background, and 50 cases, about 2% of the total, were excess cases attributed to radiation exposure (Figure 18.16). The linear ERR per gray was 0.61 (95% CI 0.04–127, P = 0.03). This potency estimate is remarkably similar to that observed for the Japanese atomic bomb survivors (Ozasa et al. 2012). The figure also shows the results for a fitted spline model with a knot at 0.1 Gy. Thirty of fifty estimated excess cancer cases were related to 71,537 person-years of follow-up for individuals estimated to have received over 0.1 Gy. The authors concluded as follows: "The data were consistent with a linear dose–response model, but could also be described by models in which the risk at low doses (<0.1 Gy) is less than predicted by the linear model."

The largest epidemiological study of radiation exposure and cancer risk is the 15-Country Collaborative Study of cancer risk among radiation workers in the nuclear industry that is coordinated by the IARC (Cardis et al. 2007; Thierry-Chef et al. 2007; Vrijheid et al. 2007). This study includes 407,391 nuclear industry workers who had been monitored individually for external radiation exposure. Some individuals in the cohort started working in the nuclear industry as early as 1943 at Oak Ridge and 1944 at the Hanford site. As an aside, my father (now deceased) and I are both in the Hanford subcohort. The nuclear industry for this study is used to refer to facilities engaged in the production of nuclear power, the manufacture of nuclear weapons, the enrichment and processing of nuclear fuel, or reactor or weapons research. Uranium mining is not included.

The last report (Cardis et al. 2007) was based on 407,392 nuclear workers with 5,192,710 person-years of follow-up. The overall average cumulative recorded dose was 19.4 mSv with a total recorded collective dose of 7892 Sv. There were 18,993 deaths from all causes and 5233 cancer deaths, about 27% of the all-cause mortality. There was statistically significant association between radiation dose and all-cause mortality (ERR of 0.42 per Sv, 90% of CI, 0.07, 0.79). This was mainly attributed to a dose-related increase in all cancer mortality with an ERR/Sv of 0.97, 90% CI, 0.28, 0.77. The authors noted that stratification on duration of employment had a large effect on the ERR/Sv, reflecting a strong, healthy worker effect in these cohorts. The authors note that the ERR for all cancers, excluding leukemia (0.97 per Sv), is higher than, but statistically compatible with, the estimate derived from male adult atomic bomb survivors (0.32 per Sv) and the BEIR VII estimate of 0.26 per Sv for exposure at ages of 30 and above and attained age of 50. Most of the 32 cancer types or groupings of cancer studied had little or no association with radiation dose. Lung cancer was the only cancer to show a statistically significant rise in ERR. The authors note that smoking confounding may be an explanation for the positive association found for lung cancer rather than radiation exposure. They further note that the association with lung cancer should be interpreted with caution and point out the need for further investigation in studies with better ascertainment of smoking habits and other occupational exposures.

EFFECTS OF INTERNALLY DEPOSITED RADIONUCLIDES

Human Experience

A substantial body of information exists on the late-occurring effects of internally deposited radionuclides in human populations. Mettler and Upton (2008) have reviewed these studies and others. Some of the key populations studied are listed in Table 18.16.

The earliest evidence of the cancer-causing potential of exposure to internally deposited radionuclides, as already noted, came from the miners in Central Europe who developed respiratory maladies later to be identified as lung cancers. This experience recognized in the mid-nineteenth century was to be repeated in North America in the mid-twentieth century when uranium mining was initiated on a large scale and yet later in the late twentieth century in China. The development of quantitative estimates of the lung cancer–causing potential of exposure to radon and its daughter products from studies of the uranium miners has been challenging for multiple reasons. First, it has been difficult to assemble the population cohorts because workplace records were not always complete for the early years when the exposures to radon and its daughter products were likely highest and the lung cancer risk greatest. Second, it has been challenging to quantitatively estimate the radiation dose to the miners from radon and its daughter products throughout the work times of the miners. Third, the challenge of estimating

TABLE 18.16
Key Human Populations Studied Following Intake of Radioactivity

Population	Radiation Source	Target Organ	Key References
Uranium miners	Radon and daughters	Lung	BEIR VI (1999)
Radium dial painters	^{226}Ra and ^{228}Ra	Skeleton	Evans et al. (1972) Rowland (1994) Taylor (1989)
Thorotrast patients	ThO_2	Liver and spleen	Olsen et al. (1990) Taylor (1989)
Thyrotoxicosis patients	^{131}I	Thyroid	Dobyns et al. (1974) Ron et al. (1998) Holm et al. (1980)
Thyroid diagnosis patients	^{131}I	Thyroid	Holm et al. (1989)
Marshall islanders	^{131,132,133,135}I	Thyroid	Conrad et al. (1980)
Mayak workers	^{239}Pu	Lung, skeleton, liver	Sokolnikov et al. (2008)

the cigarette smoking history of the workers, especially that of deceased workers, has been difficult. Knowledge of the cigarette smoking history of the subjects in any epidemiological study of the association of any occupational or environmental factor with lung cancer is critical because of the clear and substantial role of cigarette smoking in causing lung cancer. In most studies of lung cancer, the individuals with a long history of cigarette smoking will have a tenfold or greater increase in lung cancer compared to nonsmokers. Indeed, the Smoking-Attributable Mortality, Morbidity, and Economic Cost (SAMMEC software; SAMMEC 1992) analysis as cited in BEIR VI (1999) stated the following: "For lung cancer, the relative risk values assumed, versus never-smokers, are 22.4 and 9.4 for male current and former smokers, respectively, and 11.9 and 4.7 for females."

BEIR Committee VI (BEIR 1999) reviewed the lung cancer risks of exposure to radon. The BEIR VI analysis included over 65,000 workers in underground miners from 11 different studies conducted in 6 countries. Although most of the miners had been employed in uranium mines, three of the populations had worked in other kinds of mines, including over 17,000 Chinese workers mining tin. Detailed smoking data (duration, rate, and cessation) were only available in 5 of the 11 studies. In considering these studies, it is important to recognize that for the uranium miners, the period of follow-up was from 1943 to 1990. By 1990, most countries had implemented regulations and related control strategies to limit the exposures of miners to radon and its daughters. The most effective control of radon and its daughter products in mines is enhanced ventilation.

The primary purpose of the BEIR VI Committee analyses and deliberations was to reexamine the risk of health effects on exposure to radon in homes. In placing reliance on the use of the miner data, the report noted the following: "Another critical issue in the extrapolation of risks to the general population is that exposure rates in homes are a thousand-fold to a hundred-fold less from those in mines." Other key considerations in extrapolating from the miners to the general population are that the miner population consisted primarily of men, and their smoking history was likely different than the general population. Concern for the general population would include concern for both genders and individuals with exposures starting at birth.

A key consideration in extrapolating from the miner populations to the general population is the nature of the exposure–response relationship extending to the lower exposures of the general population. The BEIR IV Committee chose to use a linear relationship between risk and low doses of radon progeny without a threshold. That choice was based primarily on mechanistic considerations, which were summarized as follows:

> Those considerations are related to the stochastic nature of energy deposition by alpha particles; at low doses, a decrease in dose simply results in a decrease in the number of cells subjected to the same insult. That observation, combined with the evidence that a single alpha particle can cause substantial permanent damage to a cell and that most cancers are of monoclonal origin, provides the mechanistic basis of the use of a linear model at low doses. In addition,— exposure-response relationships estimated from the observational studies in miners with the lowest exposures, and from the case-control studies of indoor radon, are consistent with linearity.

The RRs from the pooled analysis of underground miner studies are shown in Figure 18.17. Note that the data are plotted as RR relative to radon concentration in contrast to the earlier discussions of external radiation effects in which a dose metric was used. The analysis was restricted to exposures under 50 WLMs, the RRs from meta-analysis of indoor-radon studies that are also reviewed in BEIR VI, and an estimated linear RR based on an ecologic analysis by Cohen (1995).

In considering Figure 18.16, it is important to recognize that only 5.7% of homes in the United States are estimated to have radon levels above 148 Bq/m^3, the level approximating the U.S. EPA's guidance level (4 pCi/L) for remedial action to reduce residential radon levels. The committee estimated that eliminating exposures at concentrations above 148

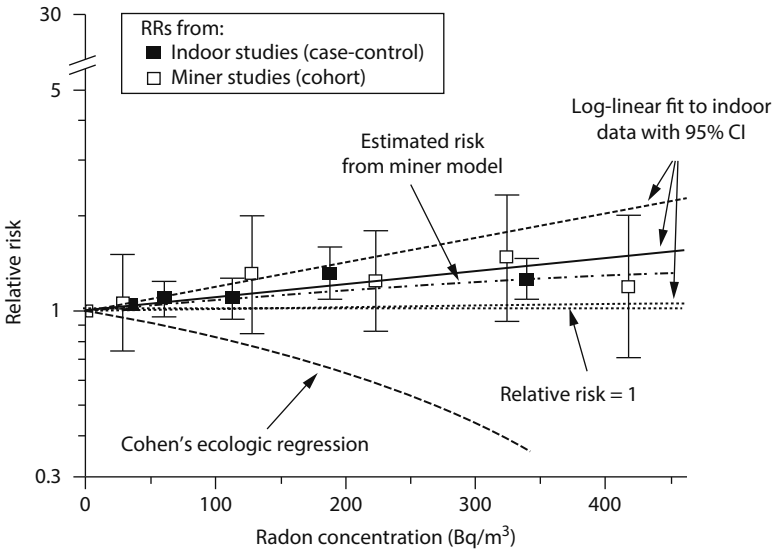

FIGURE 18.17 Summary RR from meta-analysis of indoor-radon studies and RRs from pooled analysis of underground miner studies, restricted to exposures under 0.175 Jhm–3 (50 WLM). Included are RR of 1, fitted exposure–response and its 95% CI from indoor-radon studies, and estimated linear RR based on ecologic analysis by Cohen (1990). (From NRC, *Health Effects of Exposure to Radon: Committee on Health Risks of Exposure to Radon*, BEIR VI, National Academy Press, Washington, DC, 1999.)

Bq/m^3 results in an effective attributable risk of about 4%. The committee then noted the following: "Thus, 10–15% of all lung cancers are attributable to indoor radon, and eliminating exposures in excess of 148 Bq/m^3 (4 pCi/L) would reduce the lung cancer burden from radon to 7–11% of all lung cancer cases." Of course, this conclusion is dependent on the assumption of a linear exposure–response relationship below 148 Bq/m^3, the exposure range where all data points are statistically indistinguishable from the *control* risk. I served on the BEIR VI Committee and suggested, with some other committee members, that it was important to recognize that the burden of excess lung cancer risk at all levels of radon exposure, including the low levels, was borne primarily by the cigarette smokers. The important message should not be lost if you are concerned about lung cancer; quit smoking, then check the radon level in your home, and if the level is high, take action to reduce it. This message is clear from a graphic rendering as prepared by Brooks (2013) (Figure 18.18). In my opinion, the Brooks' graphic illustrates the importance of focusing on the disease of concern, in this case, lung cancer, and not becoming excessively concerned with a specific etiological agent.

The next population exposed to internally deposited radionuclides that warrants discussion are the radium dial painters studied principally in the earliest days in the United States by physicians in New Jersey, later intensely by scientists at the Massachusetts Institute of Technology (MIT), and later yet by scientists at the Argonne Cancer Hospital (a part of the University of Chicago) and Argonne NL. The MIT team was a major contributor in developing a radon breath exhalation

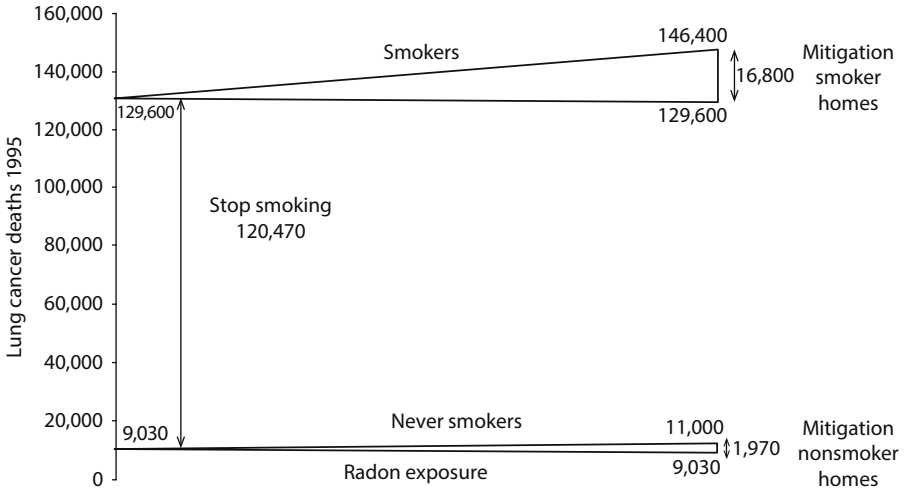

FIGURE 18.18 Relationship between exposure to radon and its daughter products and lung cancer and the impact of cigarette smoking and mitigation of radon and daughters. (From Brooks, A.L., *Health Phys.* 105, 407, 2013.)

test and, later yet, the use of a Na iodide crystal detector for estimating the bone burden of ^{226}Ra in subjects.

Information from the radium dial painter studies was key early in World War II to provide radiation protection guidance for the workers in the Manhattan Project who would be handling large quantities of the newly discovered ^{239}Pu. Very soon after that discovery when only minute quantities were available, Glenn Seaborg, who discovered ^{239}Pu, insisted that animal studies be conducted to determine its fate in the body. It was discovered that ^{239}Pu given intravenously to rats went primarily to liver and bone. Thus, based on the radium dial painters' experience, there was concern for ^{239}Pu-causing bone cancer. Soon studies were initiated in which patients, thought to be terminally ill, were administered trace quantities of ^{239}Pu to provide information on the ^{239}Pu excretion pattern as an aid in developing procedures for monitoring workers potentially exposed to ^{239}Pu by collecting and analyzing their urine for ^{239}Pu. The approach to assessing the potential ^{239}Pu hazard was straightforward as illustrated in the following:

$$\frac{^{239}\text{Pu (Estimated human hazard)}}{^{226}\text{Ra (Known human hazard)}} = \frac{^{239}\text{Pu (Animal experience)}}{^{226}\text{Ra (Animal experience)}}$$

The human experience with ^{226}Ra came from the dial painters. The initial animal experience came from studies in rats and was based on ^{226}Ra and ^{239}Pu tissue distribution data and calculated radiation dose to the skeleton. It is fortunate that ^{226}Ra and ^{239}Pu have one marked difference in their biological behavior. ^{226}Ra, like Ca, is absorbed rather readily from the gastrointestinal tract while ^{239}Pu is very poorly absorbed. As will be discussed later, a major program was initiated post–World War II at the University of Utah to conduct cancer bioassay studies of bone-seeking radionuclides using beagle dogs.

A major criticism of the several cohorts of radium dial painter patients was that the cohorts were assembled in an ad hoc manner not consistent with the expectations of many expert epidemiologists. It was true that the cohorts were initially heavily populated with individuals seeking attention because of their health concerns and there was no contemporary control population. Later, the investigators expended substantial effort to try to locate as many individuals employed as dial painters as could be found. On the positive side, a few epidemiology studies of occupational hazards have rigorous estimates of the body burden (and, thus, dose) of the agent of concern as were developed for the radium dial painters, and numerous publications document the saga of the studies. Excellent summaries can be found in the proceedings of a conference held October 6–9, 1975 (Jee 1976), a summary document on the U.S. radium dial painter studies (Rowland 1994) and several of the last papers from the MIT program (Evans et al. 1972; Evans 1974).

Figures 18.19 and 18.20 summarize the key findings from the radium dial painters studies showing a body burden–related increase in the incidence of bone cancers. Figure 18.19 shows the high incidence, over 20%, in individuals with burdens calculated to have a cumulative skeletal dose of 1000 cGy or greater and an absence of an effect below 500 cGy. It is important to recall some of these dial painters were ingesting radium every

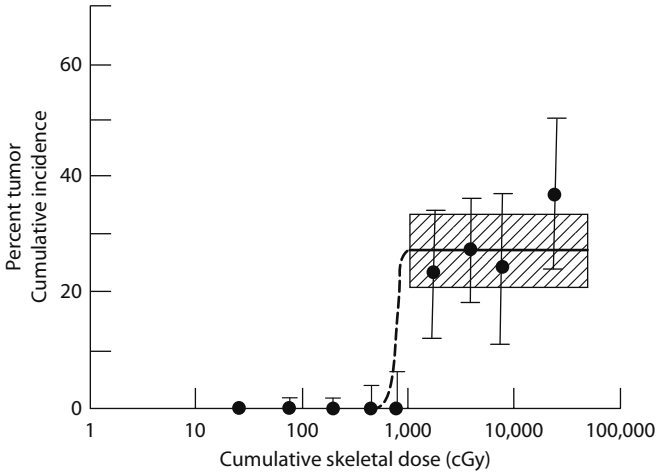

FIGURE 18.19 Cumulative bone sarcoma incidence in people exposed to ^{226}Ra as a function of cumulative dose to the skeleton.

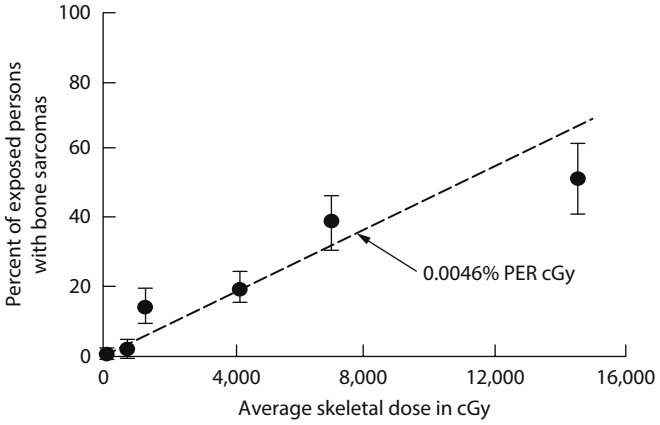

FIGURE 18.20 Hypothetical linear dose–response model fit to bone sarcoma rates in people exposed to ^{226}Ra as a function of lifetime skeletal dose.

working day for several years as they tipped the brushes with their lips. The acute effects were minimal, so they accumulated large body burdens of radioactivity, and the effects were not manifest until later. Robley Evans interpreted this dose–response relationship as reflecting a *practical threshold*, a potential for an increase in bone cancers at lower doses limited by the natural life span of the subjects. If cancers were potentially initiated by the radiation dose to the skeleton from ^{226}Ra, the individuals did not live long enough to manifest them. An alternative interpretation of the same data is shown in Figure 18.20 (Mays and Lloyd 1972). Note the dose scale is different than in Figure 18.19 yielding what appears to be a linear dose–bone cancer response for ^{226}Ra. The *practical threshold* advocated by Evans et al. (1972) and shown clearly in Figure 18.19 has apparently disappeared (Figure 18.20). It is apparent that how data are summarized and presented can profoundly influence how they are interpreted. Two additional presentations of the same basic data on the radium dial painters are shown in Figures 18.21 and 18.22 (Raabe 2010). Figure 18.21 is a 2D logarithmic plot of a fitted 3D model for ^{226}Ra-induced disease

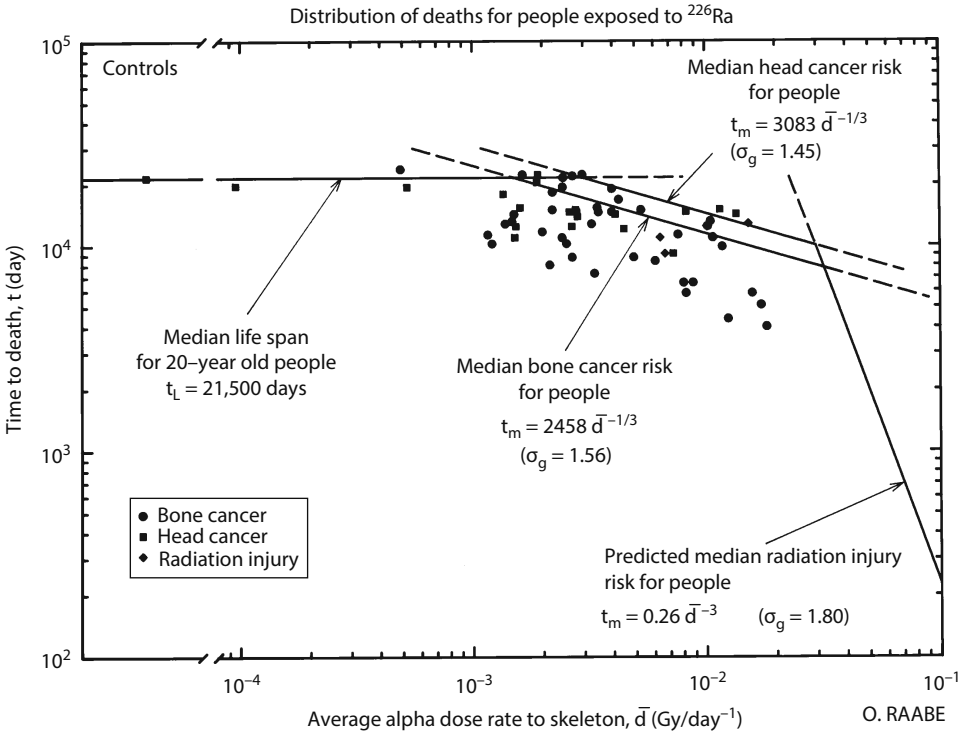

FIGURE 18.21 A 2D logarithmic representation of the fitted 3D model for radiation-induced bone sarcoma and radiation-induced head carcinoma deaths in people after intake of ^{226}Ra based on the U.S. radium studies (Rowland 1994). The cases appear displaced from the median lines because of the simultaneous combined risk of the two types of fatal radiation-induced cancer. (From Raabe, O.G., *Health Phys*., 98, 515, 2010.)

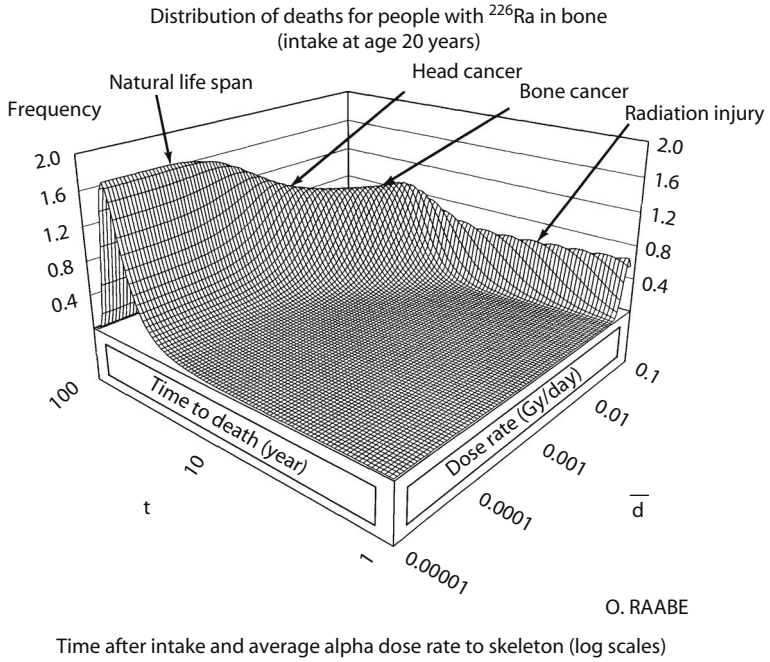

FIGURE 18.22 A 3D logarithmic representation of the skeletal alpha radiation average-dose-rate/time/response relationships for people after intake of ^{226}Ra shown as the probability density distribution frequency of the combined risk of dying from causes associated with natural life span, radiation-induced head carcinoma, radiation-induced bone sarcoma, and radiation injury, as a function of time-weighted average dose rate to skeleton and elapsed time after intake. (From Raabe, O.G., *Health Phys*., 98, 515, 2010.)

shown in Figure 18.22. This figure is a graphic depiction of average-dose-rate/time/response models with Weibull probability distribution fit by maximum likelihood methods (Raabe 1987). The *practical threshold* advanced by Evans et al. (1972) and Evans (1974) is apparent in both plots.

As soon as ^{131}I became available, it was used to treat various thyroid diseases. From this experience came suggestive evidence of its cancer-causing capabilities that complemented what was known from x-irradiation of the thyroid gland. The exposure of Marshall Island residents as a result of weapons testing in the Pacific Ocean also provided early insight into the cancer-causing capabilities of exposure to short-lived ^{132,133,135}I and the 8-day half-life of ^{131}I (Conrad et al. 1966, 1980). The excess of thyroid cancer observed in the Marshallese was likely a result of irradiation with the short-lived radionuclides as well as the ^{131}I. The short-lived radioiodines were more effective per gray than the ^{131}I. There is also some evidence of the role of ^{131}I in inducing thyroid cancer as a result of releases of ^{131}I from the Chernobyl reactor accident (Gavrilin et al. 2004).

A number of studies have been conducted on populations of individuals who received ^{131}I for medical purposes. In general, these studies have shown no excess of thyroid cancers related to the radiation dose from administered ^{131}I, an outcome very different than noted for exposure of the thyroid to external LET radiation (Ron et al. 1995). Dobyns et al. (1974) studied 16,000 patients with hyperthyroidism treated with ^{131}I and found no increase in thyroid cancer. The dose to the thyroid was a mean of over 85 Gy. Ron et al. (1998) reported on the follow-up of 35,593 adult patients that received therapeutic doses of ^{131}I, doses to the thyroid on the order of 100 Gy. A small increase in thyroid cancer incidence was observed, most within 4 years of treatment, suggesting the cancers may have been related to the underlying disease for which they were treated. There was no statistically significant increase in mortality related to either ^{131}I treatment. It is apparent that the protracted exposure of the thyroid from ^{131}I, recall its 8.05-day half-life, is much less effective than acute irradiation of the thyroid by x-irradiation. This same increased effectiveness of brief exposures to high-dose-rate x-rays compared to protracted irradiation of the thyroid from ^{131}I was observed in sheep (McClellan et al. 1963).

One of the most important sources of information on the human effects of plutonium came from studies of workers at the MPA facilities in Russia. These studies conducted jointly by Russian and U.S. scientists reflect a positive outcome of the end of the Cold War. The MPA in the Russian Federation was built to produce ^{239}Pu for the Soviet Nuclear Weapons Program. It included uranium-fueled reactors and fuel-reprocessing facilities remarkably similar to those built and operated at Hanford, Washington. The first reactor became operational in June 1948; irradiated fuel began to be processed in February 1949. Recall that the U.S. Air Force detected airborne ^{131}I from the MPA fuel reprocessing in early 1949. This, in turn, led to the *Green Run* experiment described earlier. Weapons-grade plutonium produced from the MPA was used in the first Soviet A-bomb detonated on August 29, 1949. It is now apparent that MPA workers received significant external radiation exposure as well as internal contamination, including substantial burdens of ^{239}Pu. As discussed earlier, substantial amounts of radionuclide contamination was also released to the local environment, especially the Techa River. The MPA nuclear workers (Sokolnikov et al. 2008), the Mayak residents, and the Techa River Cohort have all been the subject of extensive investigations (Schonfeld et al. 2013).

A cohort of MPA workers initially hired between 1948 and 1972 has been assembled. This population of 17,740 workers is of special interest because of their exposure to ^{239}Pu. By December 31, 2003, a total of 681 lung cancer deaths, 75 liver cancer deaths, and 30 bone cancer deaths had occurred. Of the 786 cancer deaths observed, 239 (30%) were attributed to plutonium exposures (Sokolnikov et al. 2008). Significant plutonium dose–response relationships were observed for the cancers in the three organs, with the lung and liver cancer risks reasonably described by linear functions (Figures 18.23 and 18.24). At attained age 60, the ERRs per gray for lung cancer were 7.1 for males and 15 for females, the averaged attained age ERRs for liver cancer were 2.6 for males and 29 for females, and the ERRs for bone cancer were 0.76 for males and 3.4 for females. Note that the dose metric used is in gray without a conversion using a quality factor (QF) or radiation-weighting factor (W_R) to convert to Sv. Although the occupationally exposed MPA cohort and the atomic bomb survivor cohort differ in many ways beyond the type of radiation exposure sustained, it is still of interest to cautiously compare them by developing RBEs. The ERR per gray for lung cancer in the MPA cohort was 7.1 for males and 15 for females compared with 0.63 for the atomic bomb survivors representing a crude RBE of 11 for males and 24 for women compared with the W_R factor of 20 for alpha particles assigned by the ICRP. The ERR per gray for liver cancer in

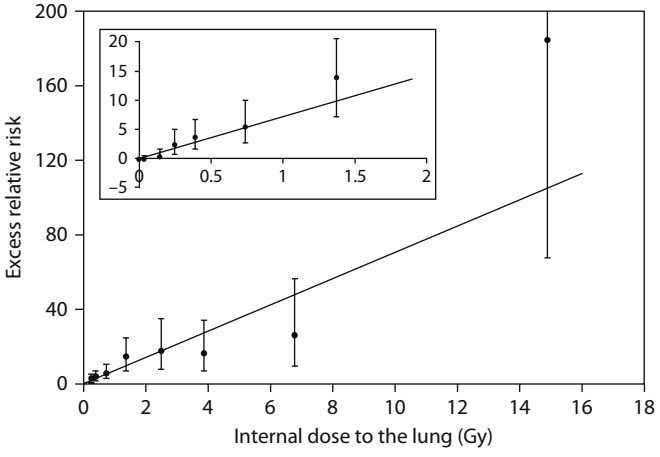

FIGURE 18.23 ERR (with 95% CI) of lung cancer by categories of plutonium dose to the lung for Mayak Production Association workers. Data shown for males at age 60 with an estimated linear function relating dose to ERR. (From Sokolnikov, M.E. et al., *Int. J. Cancer*, 123, 905, 2008.)

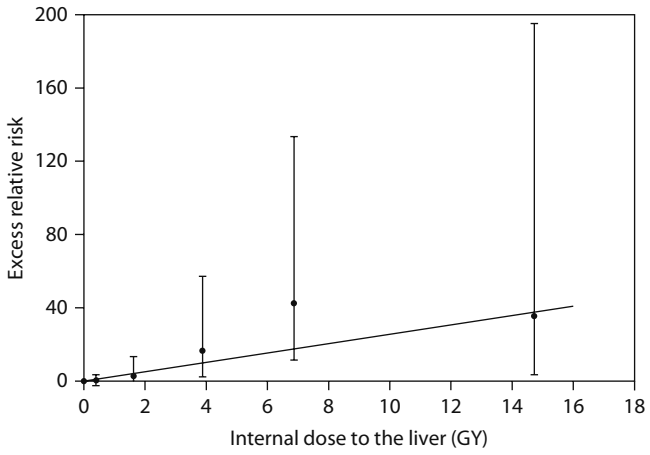

FIGURE 18.24 ERR (with 95% CI) of liver cancer by categories of liver dose for Mayak Production Association workers. Data shown for males of all ages with an estimated linear function relating dose to ERR. (From Sokolnikov, M.E. et al., *Int. J. Cancer*, 123, 905, 2008.)

the MPA cohort was 2.6 for males and 29 for females compared with 0.36 for the atomic bomb survivors representing a crude RBE of 7 for men and 80 for women. In my opinion, these crude comparisons give confidence that the W_R of 20 for alpha particles is reasonable. Tokarskaya et al. (2006) have noted that the liver cancer risk in the MPA works was likely an influence of both plutonium exposure and alcohol consumption. In considering the MPA findings, it is also of interest to recall the mutation data comparing alpha and low-LET gamma irradiation shown in (Figure 18.10). The comparative potency of high LET versus low LET in the short-term mutagenicity study was remarkably predictive of the long-term carcinogenic effects in humans.

An updated analysis of lung cancer data for the MPA workers including follow-up through 2008 has been published by Gilbert et al. (2013). The Gilbert et al. (2013) analysis is based on improved plutonium and external dose estimates allowing special attention to be given to the joint effect of plutonium exposure and smoking and the shape of the plutonium dose–response function. This results in improved models for assessing the risk of plutonium exposures for future nuclear operations. However, it is important to recognize that the U.S. regulatory policy has always had a goal of zero plutonium exposure. Thus, any time there was potential for exposure to plutonium, workers wore full-face respirators or full-body suits with supplied air. All workers with potential for exposure to plutonium participated in bioassay surveillance programs that involved regular collection of urine samples and radioanalysis.

The results of the Gilbert et al. (2013) analysis are presented graphically in Figure 18.25. The dose–response relationship for lung cancer is well described by a linear function, and an LQ function did not significantly improve the fit ($P > 0.5$). A statistically significant plutonium dose–response relationship was observed for workers exposed prior to 1964, but not for post-1964. The ERR per gray for males at age 60 was 7.4 (95% CI of 5.0–11), only slightly higher than the earlier analysis of Sokolnikov et al. (2008). For women over age 60, the ERR per gray was 24 (95% CI of 11–56) compared with 15 observed in the earlier analysis.

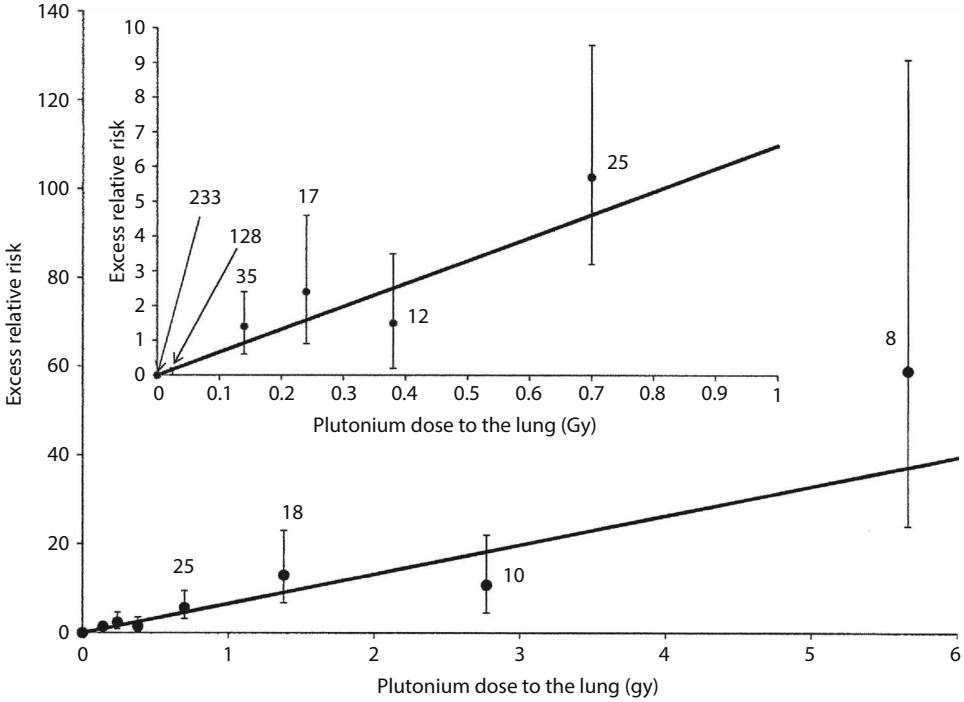

FIGURE 18.25 ERR (with 95% CI) of lung cancer and the number of lung cancer deaths by categories of plutonium dose to the lung. Shown for males at age 60. Estimated linear function also shown. (From Gilbert, E.S. et al., *Radiat. Res*., 179, 332, 2013.)

The authors noted that 98% of the plutonium person-years of exposure were for lung doses of less than 1 Gy and 91% had lung doses less than 0.2 Gy. Thus, no extrapolation is necessary in this low-dose region.

The authors (Gilbert et al. 2013) noted that while there was uncertainty in estimating risks below 0.2 Gy, confidence in the finding was enhanced by the findings with occupational exposure to radon (NRC 1999). Analysis of the 12,708 workers with information on smoking indicated that the relationship of plutonium exposure and smoking was likely submultiplicative ($P = 0.011$) and strongly indicated that it was superadditive ($P < 0.001$), a finding similar to that reported earlier by Tokarskaya et al. (2002). A study of smoking and plutonium exposure conducted in rats (Mauderly et al. 2010) gave results consistent with the findings in the Mayak workers.

The findings in the MPA workers with excess cancer risk associated with exposure to plutonium contrast sharply with the absence of such findings in the U.S. nuclear weapons complex in workers handling plutonium. The findings of lung, liver, and bone cancers in the MPA plutonium workers are consistent with the observations in dogs that inhaled plutonium, as will be discussed later.

The U.S. and British experience contrasts sharply with the Mayak experience. Lung cancer in U.S. and British plutonium workers was consistent with their cigarette smoking history. The maximum body burden of plutonium among U.S. and British workers was 3.2 kBq (Wiggs et al. 1994; Voelz et al. 1997; Omar et al. 1998; Brown et al. 2004; Wing et al. 2004). More than 400 MPA workers had plutonium burdens that exceeded this level.

The workers in the MPA inhaled high concentrations of ^{239}Pu released in the workplace over long periods of time. Follow-up studies of the MPA workers are still underway, including reconstruction of their exposures to ^{239}Pu and other radionuclides and external exposures. They are a unique population because the exposures to ^{239}Pu were quite high, much higher than encountered by ^{239}Pu workers in the United States or other countries. The data from the MPA cohort are likely to be the cornerstone of any future standards for ^{239}Pu and other actinide radionuclides.

The information on the Thorotrast patients with thorium dioxide administered in a colloidal form provides a unique source of information on alpha particle irradiation of the liver, spleen, and cancer induction. Thorotrast was used as a contrast agent in many countries before the risks of its use became known. The Japanese studies date to World War II and include soldiers treated for war wounds. Rate ratios for all-cause mortality and specific diseases began to increase 20 years after the administration of Thorotrast. Rate ratios for liver cancer, liver cirrhosis, leukemia, and lung cancer were 35.9, 6.9, 12.5, and 2.0 times, respectively, higher than controls (Mori et al. 1999). The radiation doses were not well characterized but undoubtedly were high. In addition, the potential exists for the chemical effects of the colloidal thorium dioxide contributing to the development of these chronic diseases.

Another population of special interest is the cohort of patients with ankylosing spondylitis treated in Germany from 1945 to 1955 with injections of Peteosthor, a mixture of short-lived ^{224}Ra (half-life of 3.66 days), red dye eosin, and colloidal platinum. As it turned out, the ^{224}Ra went to bone whether it was injected in a pure form or in Peteosthor. A total of 899 patients have been followed in a special cohort. The cohort is of particular interest because the ^{224}Ra decays by alpha particle emission with a half-life of 3.66 days compared with the 1600-year half-life of ^{226}Ra. The administered doses were quite large, 0.66 MBq/kg, and the mean bone surface doses were correspondingly high, 30 Gy. The most striking finding as of the 1998 follow-up was the occurrence of 56 malignant bone cancers that peaked about 8 years after the administration of ^{224}Ra compared with an expected less than one case.

Laboratory Animal Experience

Introduction to Laboratory Animal Studies

Studies of the toxicity of internally deposited radionuclides were conducted as part of the Manhattan Project at the University of Chicago, UC-Berkeley, and the University of Rochester. Post–World War II, the University of Chicago programs became the core of the Argonne NL. The Argonne NL toxicology research efforts on internally deposited radionuclides were substantially expanded to focus on ^{90}Sr-causing bone cancers in laboratory animals and the occurrence of bone cancers in the radium dial painters as just discussed.

The University of Rochester program during World War II focused on the inhalation toxicity of inhaled uranium and concluded that its primary effects were related to the chemical characteristics of uranium rather than its radiological characteristics (Stannard and Baalman 1988). This program continued post–World War II focusing on the inhalation toxicity of a number of radionuclides and basic radiobiological research. Perhaps the most substantial contributions from the University of Rochester were the development of practical and scientific inputs to the fields of aerosol science and inhalation toxicology and the training of many students. The first president of the Society of Toxicology, Harold Hodge, was a member of the University of Rochester team. The UC-Berkeley efforts post–World War II became a part of the Lawrence Berkeley Laboratory. Later, the Lawrence Livermore National Laboratory (LLNL) was developed as a competitor of the Los Alamos National Laboratory (LANL) for the design and development of nuclear weapons. Even later, LLNL would develop a radiation effects research program linked to the *Plowshare Program*, an AEC effort to use nuclear detonations for peaceful uses such as blasting harbors or canals or stimulating production of natural gas.

This section of the chapter will focus on a series of projects concerned with the effects of injected, ingested, or inhaled radionuclides, especially those concentrating in bone (Table 18.17). The IARC has reviewed the results of studies on internally deposited radionuclides with regard to cancer hazard (IARC 2001). As traditional in its reviews,

TABLE 18.17
Major Life Span Studies with Internally Deposited Radionuclides in Beagle Dogs with Multiple Exposure Levels and Life Span Observation

Laboratory	Radionuclides	Route of Administration
University of Utah	^{226}Ra, ^{228}Ra, ^{228}Th, ^{239}Pu, ^{241}Am, ^{90}Sr	Single intravenous injection
UC-Davis	^{226}Ra	Multiple intravenous injections
	^{90}Sr	Chronic ingestion
Hanford	$Pu^{238}PuO_2$, $^{239}RaO_2$, ^{239}Pu $(NO_2)_4$	Inhalation of polydisperse aerosols
Lovelace	^{90}Y, ^{91}Y, ^{144}Ce, and ^{90}Sr Chloride	Inhalation of polydisperse aerosol
	^{137}Cs Cl_2	Intravenous injection
	^{90}Y, ^{91}Y, ^{144}Ce, and ^{90}Sr in FAPs	Inhalation of polydisperse aerosol
	$^{238}PuO_2$ and $^{239}PuO_2$	Inhalation of monodisperse particles

IARC focuses only on the cancer hazard and did not rigorously address dose–response relationships. Thus, the IARC review essentially documents what is well known—internally deposited radionuclides were a carcinogenic hazard. At the conclusion of World War II, substantial uncertainty remained as to the toxic potential of ^{239}Pu in humans. It was anticipated that ^{239}Pu would continue to be a vital part of the continuing nuclear weapons program and would have a role in civilian nuclear programs. The very preliminary research conducted during World War II indicated the utility of the approach that leveraged off of the radium dial painters' experience. There were concerns that the rat might not be a good experimental model for studying ^{239}Pu because the ratio of bone to liver content of ^{239}Pu in the rat appeared to differ from that observed in humans and dogs. Moreover, the liver burden of ^{239}Pu in the rats appeared to clear relatively rapidly as contrasted to observations in humans and dogs. This finding would later be found to be generally applicable to how the rat metabolizes both lanthanide and actinide elements.

The decision was made that the issue of the comparative toxicity of ^{238}Pu, ^{226,228}Ra, and ^{228}Th, the latter three radionuclides linked back to the radium dial painters' experience, should be studied in a dedicated program using beagle dogs, a relatively long-lived species, as the experimental subject (Jee 1976). The University of Utah, which was starting a medical school, was selected to carry out what was anticipated to be a long-term program, perhaps 20 years, because the beagle dogs were expected to have a life span of about 15 years. To mimic the brief oral intake of radioactivity by the dial painters and minimize issues with radioactive contamination, it was decided to give young adult beagle dogs a single intravenous injection of the radionuclide. For each radionuclide, there were multiple dose level groups.

Soon after the University of Utah program was initiated, concern increased over ^{90}Sr as an important component of weapons fallout. As an alkaline earth element, it behaves in the body like Ca and deposits in the skeleton where it serves as a source of beta particle irradiation of bone, bone surfaces, and bone marrow. This gave rise to concern for its potential to cause bone cancer and leukemia. The decision was made to add ^{90}Sr to the University of Utah experimental matrix.

As the University of Utah program moved forward in the 1950s, even more concern developed for the potential effects of fallout of ^{90}Sr recognizing that all children ingesting milk were being exposed to low concentrations of fallout ^{90}Sr via the contaminated pasture/forage–cow–cows' milk–intake by children pathway. Concern also developed that the single intravenous injection approach used at the University of Utah did not adequately mimic the pattern of exposure of children ingesting ^{90}Sr in milk from dairy cows or their mother's milk. This led to the decision to create another dedicated laboratory program at the UC-Davis to study the effects of bone-seeking radionuclides.

Scientists at the UC-Davis were already conducting whole-body x-irradiation studies with beagle dogs for the U.S. Air Force, so it was considered reasonable to leverage on that experience by adding radionuclide toxicity studies to their research agenda. The UC-Davis program was envisioned as linking to the University of Utah studies. Young adult female beagle dogs were given a series of injections of ^{226}Ra (to better mimic the intake of the dial painters) or fed food containing ^{90}Sr. Thus, the offspring of these dams were exposed to the radionuclides in utero and during lactation by ingesting their mother's milk. After weaning, they were provided ^{90}Sr-contaminated feed. Multiple dose levels were used and the animals observed for life.

In 1959, a somewhat similar program with ^{90}Sr was initiated at the Hanford Laboratories (McClellan and Bustad 1964). For that study, it was decided to utilize miniature pigs weighing about 70 kg at maturity as the experimental subjects. Female pigs were given a dose of ^{90}Sr in their feed each day, bred, and gave birth to offspring that were entered into the experiment. Thus, this F_1 generation and a later F_2 generation were exposed to ^{90}Sr in utero and early in life by ingesting their mother's milk. At weaning, they began receiving an oral dose of ^{90}Sr each day and were observed for development of leukemia and bone cancers. As in the other radionuclide studies, multiple levels of ^{90}Sr were studied.

As noted earlier, the Hanford site was the earliest and primary U.S. production site for ^{239}Pu. Later, the AEC would develop the capability to produce plutonium and tritium at a complex in Savannah River, Georgia. Operation of the uranium-fueled reactors and reprocessing plants led to concern for potential exposure of workers to ^{239}Pu and various fission products. This led to the early development of a research program at Hanford on the inhalation toxicity of various radionuclides with a focus on inhaled ^{239}Pu. The physical half-life of ^{239}Pu is 24,110 years. Again, the beagle dog was used as the experimental animal. A series of studies were initiated in the late 1950s in which beagle dogs received single, brief

inhalation exposures to ^{239}Pu, the disposition of the radionuclide was evaluated, and the animals were observed for their lifetime. Studies were conducted with both ^{239}Pu oxide and ^{239}Pu nitrate, and multiple dose levels were used.

In the early 1960s, it became apparent that shorter-lived ^{238}Pu (radioactive half-life of 87.7 years) would be useful as a thermal electric power source in space, part of what was initially called the Space Nuclear Applications Program (SNAP). This raised concern for the potential toxicity of ^{238}Pu to workers and, with accidental release, exposure of the public. Concern for the toxicity of ^{238}Pu was further heightened when the launch of a unit, SNAP 9a, containing 17,000 C of ^{238}Pu metal was aborted over east Africa. The unit *burned up*, releasing the ^{238}Pu into the atmosphere at a high altitude. The form of the ^{238}Pu was changed in later SNAP units from metal to pellets of ^{238}Pu oxide, which in the event of an accident would be more resistant to burning. This led to the addition of ^{238}Pu to the experimental program at Hanford and Lovelace.

With continued use of uranium-fueled reactors to produce ^{239}Pu and the beginning of the civilian nuclear reactor program for generation of electricity, concern developed over the potential consequences of an accident involving a nuclear reactor or a fuel-reprocessing plant and release of mass quantities of fission product radionuclides. The staff at the Brookhaven NL prepared the landmark report that scoped the issue (USAEC 1957). The scoping study, sometimes referred to as the WASH-740 or Brookhaven National Laboratory study, estimated the possible effects of a *maximum credible accident* for a commercial nuclear reactor generating electrical power. The report postulated total release of the reactor fuel core of uranium and associated radionuclides with the plume of released radioactivity traveling over New York City. It projected 3400 deaths, 43,000 injuries, and property damage of $7 billion (1957 dollars). Needless to say, numerous assumptions were used in projecting mortality and morbidity and substantially impacted the calculated health risks.

An examination of key input parameters in the analysis based on current knowledge revealed that essentially no information was available on the health effects of the fission products that were postulated to be released and potentially available to be inhaled by *downwind* populations. To address this shortcoming, the AEC in 1960 initiated the Fission Product Inhalation Program at the Lovelace Foundation for Medical Education and Research in Albuquerque, New Mexico. Over the years, this program would evolve into what was called the Lovelace Inhalation Toxicology Research Institute. I had the pleasure of providing leadership for that program from 1966 to 1988. The program continues today as part of the Lovelace Respiratory Research Institute. Although the research program would use many different laboratory animal species, the major core inhalation studies used beagle dogs as in the programs at the University of Utah, UC-Davis, and Hanford. The AEC's Division of Biology and Medicine that funded the four programs viewed them as scientifically interrelated since all were concerned with the health effects of internally deposited radionuclides.

In 1967, the scope of the long-term program at Lovelace was finalized (McClellan et al. 1986). The approach was to study major beta-emitting radionuclides found in a reactor inventory: ^{90}Y, ^{91}Y, ^{144}Ce, and ^{90}Sr. Each of the radionuclides has different radiological characteristics; however, all of their beta particle emissions are the major source of their radiation dose to tissues. The radionuclides would be administered via inhalation in a relatively soluble form as a chloride and in a relatively insoluble form achieved by incorporating the radionuclides into fused aluminosilicate particles (FAPs). When inhaled in a soluble form, the radionuclides translocated from the lung to other tissues as predicted based on their chemical characteristics: Y and Ce to liver and skeleton and Sr to bone. Soluble ^{137}Cs was also studied. However, for the LSS, the dogs were administered with the ^{137}Cs intravenously since it was observed that soluble ^{137}Cs distributed throughout the body whether it was ingested, inhaled, or injected (Boecker 1969a,b). The use of intravenous injection rather than inhalation exposures to deliver the ^{137}Cs substantially reduced the potential for radiation exposure of personnel.

Multiple approaches were considered for delivering relatively insoluble radioactive particles to the respiratory tract, including creating oxide aerosols of each radionuclide of interest. Ultimately, the decision was made to use a common vector (FAPs) that would contain the various radionuclides. These particles were created by exchanging the cation radionuclides, such as ^{144}Ce, into montmorillonite clay. The clay suspension was washed to remove any unbound cation. The suspension of the radiolabeled clay particle was aerosolized and the aerosol passed through a high-temperature furnace to fuse the radiolabeled particles. The result was a suspension of radiolabeled glass particles that were used to expose the laboratory animals in a nose-only inhalation exposure system.

The deposition and retention of the FAPs in the respiratory tract and their dissolution were determined by the FAP matrix. However, the pattern of irradiation of the lung was determined by the physical half-life of the radionuclide ^{90}Y (64 h), ^{91}Y (58.5 days), ^{144}Ce (285 days), and ^{90}Sr (28.8 years). Thus, the pattern of chronic beta irradiation differed for each radionuclide: the dose from ^{90}Y-FAP was delivered at a decreasing dose rate over a few weeks, the lung dose from ^{90}Sr-FAP over several years, and intermediate time durations for ^{91}Y-FAP and ^{144}Ce-FAP. The study of each radionuclide and form involved multiple dose levels and detailed medical evaluation of the animals for their life span.

As noted earlier, concern developed in the 1960s over the potential hazards associated with an accidental release of ^{238}Pu from a SNAP thermal electric device. The SNAP accident with the release of ^{238}Pu over Africa at a high altitude raised questions as to whether the potential for inhaled ^{238}Pu causing lung cancer would be altered if it were inhaled as many, very small particles versus a few large particles. Contrasting views were presented by Bair et al. (1974) and Tamplin and Cochran (1974). During this era, increased interest also developed in the use of ^{239}Pu as a reactor fuel. Indeed, the U.S. Breeder Reactor Programs in the late 1970s included plans for constructing a prototype breeder reactor,

using a ^{239}Pu–uranium mixed oxide fuel, to generate electrical power at Oak Ridge. This intensified concern for the potential hazards of ^{239}Pu. At that time, it was not anticipated that any effects would be observed in U.S. plutonium workers, and most importantly, the experience at the MPA facility in Russia had not yet come to light. Thus, it was thought that studies in beagle dogs with inhaled plutonium were critical to estimating human hazards.

To address the uncertainties in the inhalation toxicity of ^{238}Pu and ^{239}Pu, Lovelace developed a technique for producing monodisperse aerosols of ^{238}Pu and $^{239}PuO_2$ (Kotrappa et al. 1972; Kotrappa and Moss 1971). The typical aerosol used in the inhalation exposure studies at Lovelace and Hanford was polydisperse in size distribution with an aerodynamic diameter of about 2 μm and a geometric standard deviation of about 2. These polydisperse aerosols are not remarkably similar to the aerosols used in most studies of the inhalation toxicity of chemicals. It is a challenge to produce monodisperse aerosols. A new system developed at Lovelace produced monodisperse Pu particles with an aerodynamic size ranging from 0.5 to 4.0 μm and, most importantly, a geometric standard deviation of less than 1.2. As a result, it was possible to select specific monodisperse sizes for study (^{239}Pu oxide, 0.75, 1.5, and 3.0 μm; ^{238}Pu oxide, 0.75 and 1.5 μm). As noted earlier, ^{239}Pu has a half-life of 24,110 years compared with 87.7 years for ^{238}Pu. Thus, the same amount of radioactivity was contained in a few particles of ^{238}Pu compared with many particles of ^{239}Pu, and for a given sized particle, the alpha emissions will be many times greater for a $^{238}PuO_2$ particle than a $^{239}PuO_2$ particle. For either ^{238}Pu or ^{239}Pu particles, the smaller the diameter, the lower the physical mass and the lower the alpha particle emission rate.

Thus, it was possible to design inhalation toxicity studies in which the amount of radioactivity inhaled and the number of particles deposited in the lung differed. With this approach, it was possible to test whether a few ^{238}Pu particles with very nonuniform irradiation of the lung were more, equally, or less effective in producing lung cancer than more uniform alpha irradiation of the lung from many ^{239}Pu particles. It turns out the more uniform the alpha irradiation from ^{238}Pu or ^{239}Pu, the greater the lung cancer hazard.

Linking Exposure to Dose for Radionuclide Toxicity Studies

Detailed studies were conducted to determine the pattern of retention of the specific radionuclide and chemical form in the body in all of the animal studies (recall Table 18.17) with injected, ingested, or inhaled radionuclides. Scientists studying the toxicity of internally deposited radionuclides recognized that exposure, whether measured as the quantity injected or ingested, was at best a poor surrogate for tissue dose (recall Figure 18.2). With inhaled radionuclides, the situation is even more complicated than with injection or ingestion. It is not sufficient to know the air concentration and duration of exposure or even the amount of radioactive material deposited in the respiratory tract. As a starting point, it is necessary to characterize the aerodynamic size distribution of the aerosol used for animal exposures since aerodynamic size is the primary determinant of the fraction of the aerosol that will be deposited and where it deposits in the respiratory tract. In addition, there is a need to know the concentration and duration of the exposure. In the studies conducted at Lovelace, a whole-body plethysmograph was used during the dog exposures to measure the respiration of the dog (Boecker et al. 1964). Knowing the volume of contaminated air inspired and the radionuclide concentration, it was possible to develop accurate estimates of the total amount of radioactivity inhaled. In some cases, the radiological characteristics of the radionuclide studied allowed the body and lung burden of radionuclides to be determined by external monitoring of radiation emissions detected externally in a whole-body counter, scanner, or imaging system. In other cases, the initial respiratory tract and lung burden may be accurately estimated based on the radioanalysis of tissues and excreta. Using this array of data, it was possible to accurately determine the fractional deposition of the inhaled radionuclide and follow the pattern of retention and translocation of each radionuclide and aerosol form for a period of time determined by the physical half-life of each radionuclide.

The information from the radionuclide retention and tissue distribution studies provided essential input for calculating the time course of internal irradiation of the various organs and tissues, taking account of the tissue content and the specific emissions of each radionuclide. The generic biokinetic model (Figure 18.26) was used for integrating and interpreting the data. The duration of the biokinetic studies varied dependent on the physical half-life of the radionuclide studied with some disposition studies for short-lived radionuclides being a week or so in duration, while those with long-lived radionuclide studies extended over years. In addition, for the long-lived radionuclides, the organ burden of radioactive material could be determined for dogs in the LSS when they died.

Several examples will be given to illustrate the kind of results that were obtained. The first example is the retention of ^{137}Cs in the beagle dog following inhalation of ^{137}Cs chloride (Figure 18.27) (Boecker 1969a,b). It is apparent that the concentration of ^{137}Cs found in the several specific tissues and averaged across the total body is very similar, reflecting the rapid translocation of ^{137}Cs from the lung to other tissues. This pattern was fully expected recognizing the Cs was behaving like K. The exception to the relatively uniform tissue distribution was the lower content of ^{137}Cs in the femur, reflecting the fact that it is largely a calcified bone with only the active red marrow accumulating Cs.

The contrast in the disposition of the radionuclide, ^{144}Ce, inhaled as a relatively soluble $CeCl_3$ or in a quite insoluble form, ^{144}Ce, within a fused aluminosilicate matrix, is apparent in Figures 18.28 and 18.29 (Boecker and Cuddihy 1974; Muggenburg et al. 2001). In both cases, radioactive particles deposited in the upper respiratory tract, trachea, and bronchi were rapidly cleared to the oropharynx, ingested, and excreted in the feces. This is the case since either form of

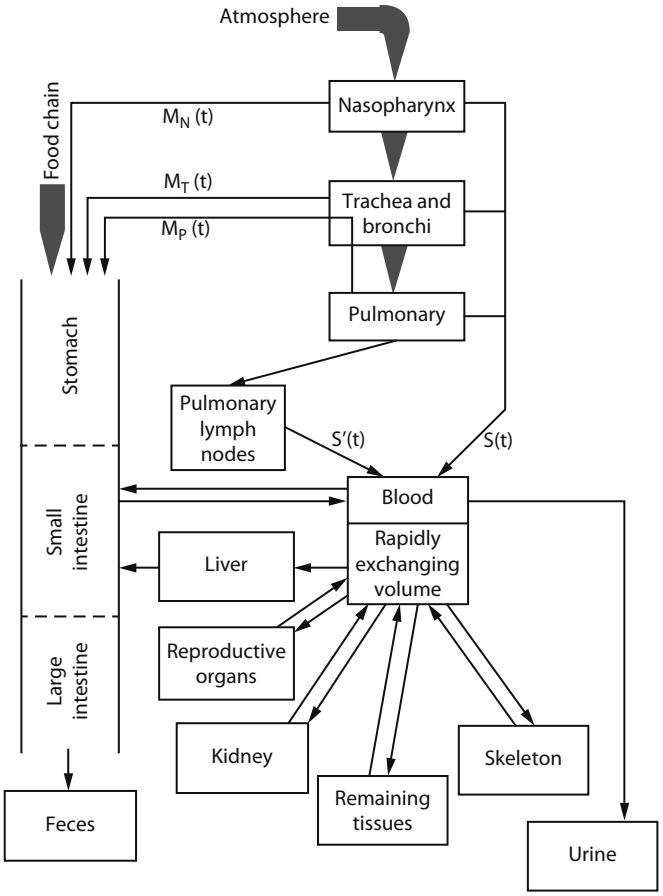

FIGURE 18.26 General model used to simulate organ distribution and retention patterns for inhalation, ingestion, or injection of radioactive elements. Clearance of material from the respiratory tract is considered to involve competing mechanical clearance to the gastrointestinal tract, M(t), or lymph nodes and dissolution with absorption into blood, S(t). Both rates may be time-varying functions depending upon the physical–chemical characteristics of the inhaled material. Other transfer rates are considered to be first-order unless better information is available for particular radionuclides. (From Cuddihy, R.G. et al., *Health Phys*., 30, 53, 1976.)

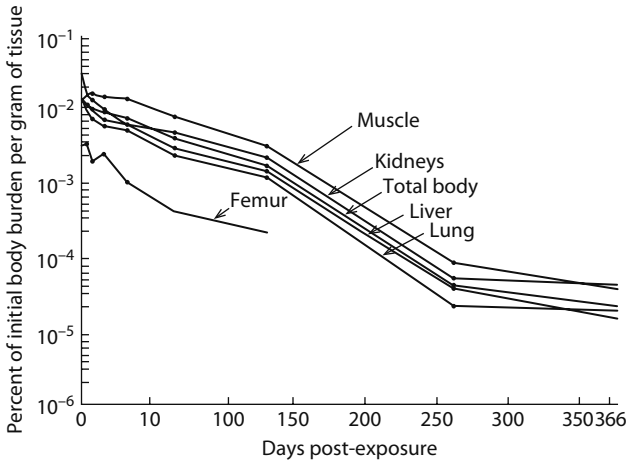

FIGURE 18.27 Distribution of ^{137}Cs in beagle dogs following a single brief exposure to $^{137}CsCl_2$ particles. (From Boecker, B.B., *Proc. Soc. Exp. Biol. Med.*, 130(3), 966, 1969.)

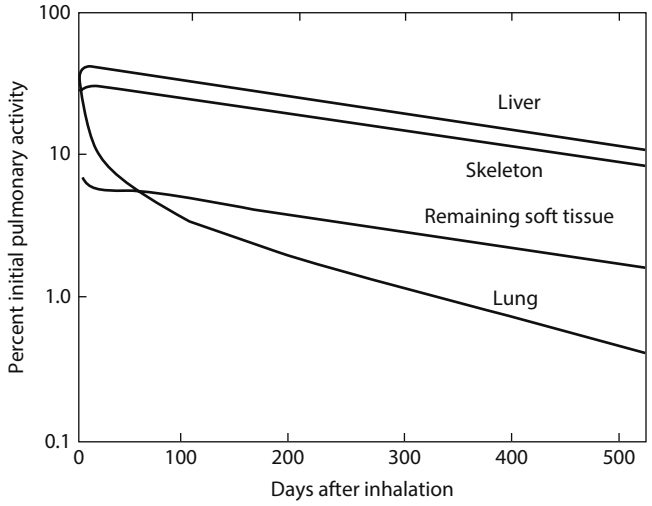

FIGURE 18.28 Distribution of ^{144}Ce in beagle dogs following a single brief exposure to $^{144}CeCl_3$ particles. (From Boecker, B.B. and Cuddihy, R.G., *Radiat. Res*., 60, 133, 1974.)

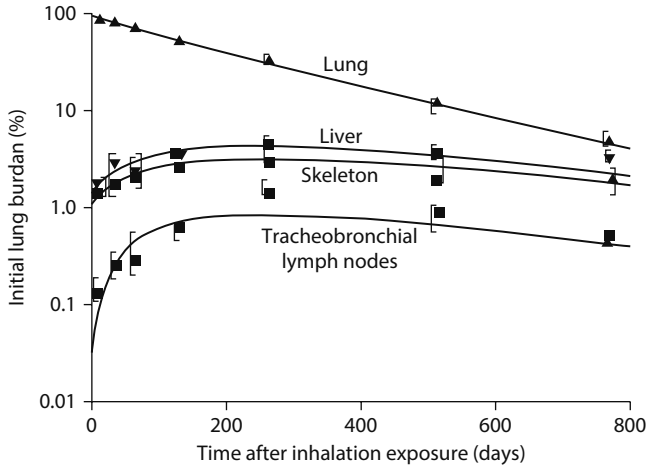

FIGURE 18.29 Distribution of ^{144}Ce in beagle dogs following a single brief inhalation exposure to ^{144}Ce incorporated into FAPs. The ^{144}Ce in the lung (●) clears over time and appears in liver, skeleton and tracheobronchial lymph nodes (●). (From Muggenburg, B.A. et al., *Radiat. Res*., 146, 171, 2001.)

Ce is poorly absorbed from the gastrointestinal tract. Data in both figures are plotted as percent retention of the ^{144}Ce initially deposited in the pulmonary region.

When ^{144}Ce is inhaled as ^{144}Ce chloride, the ^{144}Ce is rapidly translocated to the liver and skeleton as expected for a lanthanide rare earth element (Figure 18.28). The portion in liver and skeleton soon exceeds the lung burden of ^{144}Ce. The portion translocated to liver and skeleton is avidly retained in those organs. The situation is quite different when the ^{144}Ce is inhaled in a relatively insoluble matrix (Figure 18.29) (Cuddihy et al. 1976). In this case, the ^{144}Ce in the FAP is avidly retained in the lung. The ^{144}Ce slowly leaves the lung as FAPs dissolve, and the ^{144}Ce is translocated to the liver and skeleton. Note that the ratio of ^{144}Ce in liver to skeleton is quite similar to that observed for the translocated ^{144}Ce when

it was inhaled as ^{144}Ce chloride and rapidly translocated to these organs. As typically observed with a material inhaled as relatively insoluble particles, translocation to the tracheobronchial lymph nodes is observed. Note that data in Figures 18.28 and 18.29 are given a percent initial lung burden in each organ or tissue. Because the tracheobronchial lymph nodes are quite small, the concentration of radioactivity is quite high as is the corresponding radiation dose.

When inhaled or injected in the same chemical form, different radionuclides of the same element, for example, ^{85}Sr and ^{90}Sr or ^{90}Y and ^{91}Y, inhaled in the same chemical form, have similar disposition in the body when corrections are made for the differences in physical half-life. However, the situation can be different when the radionuclides have markedly different half-lives and specific activities. This is the case for ^{238}Pu (half-life of 87.7 years) versus ^{239}Pu (half-life of 24,110 years). In this case, the ^{239}Pu oxide particles are much more avidly retained in the lung than are the ^{238}Pu oxide particles. It is thought that the intense alpha decay within the ^{238}Pu oxide particles results in the particle fragmenting and having an increased rate of dissolution with the dissolved ^{238}Pu translocating from the lung to other tissues, principally the skeleton and liver. By about 500 days after inhalation of ^{238}Pu oxide particles, approximately equal amounts of the initial lung burden are found in lung, liver, and skeleton. The translocation from lung to liver and skeleton continues with avid retention of the ^{238}Pu in liver and skeleton. There is also some accumulation in the tracheobronchial lymph nodes and kidney. It is noteworthy that the general pattern of disposition of the actinide Pu is generally similar to that of the lanthanide element Ce.

At Hanford, dogs were also exposed to ^{238}Pu $(NO_3)_4$, which was retained much less avidly in the lung than $^{239}PuO_2$ and somewhat less avidly than $^{238}PuO_2$. Both ^{238}Pu and ^{239}Pu distributed primarily to the skeleton and liver after leaving the lung. Wilson et al. (2009) compared the findings from the Hanford and Lovelace studies with inhaled ^{238}Pu (NO_3), $^{238}PuO_2$, and $^{239}PuO_2$ in dogs with the findings in the workers at the MPA. As an initial step in the comparison, Wilson et al. (2009) estimated the absorbed dose from alpha irradiation of the lung, liver, and skeleton (Figure 18.30). The influence of both the radionuclides (^{238}Pu versus ^{239}Pu) and form (oxide versus nitrate) normalized to a 10 KBq deposited lung burden is apparent. It is noteworthy that in the Hanford studies with $^{239}PuO_2$ dogs that died at long time periods, postinhalation exposure had a substantial portion of the retained body burden in the lung-associated lymph nodes (Park et al. 2012).

In closing this discussion of the fate of plutonium, it is appropriate to note that a meeting on *plutonium contamination in man* was convened in 1966 to discuss approaches to treatment of accidental worker exposures at the AEC's Rocky Flats facility in Colorado, where large quantities of plutonium and uranium metal were machined. From this meeting came the idea of creating a registry of plutonium and uranium workers to receive donated tissues and bodies from workers who had been exposed to plutonium and uranium. The intention was to have exposure records, in-life bioassay results, and tissue analysis results maintained in one location for detailed study and evaluation. The activity continues today as the U.S. Transuranium and Uranium Registries (USTUR) operated by Washington State University at Richland for the U.S. DOE (http://www.ustur.wsu.edu). It is not appropriate to review here all of the USTUR findings. It can be noted that observations made on the fate of Pu and U in workers exposed during the course of their work are in general agreement with the findings in the dog studies. The unique value of the registry is apparent from the work of Nielsen et al. (2012). They used tissues from the registry to compare the microdistribution of

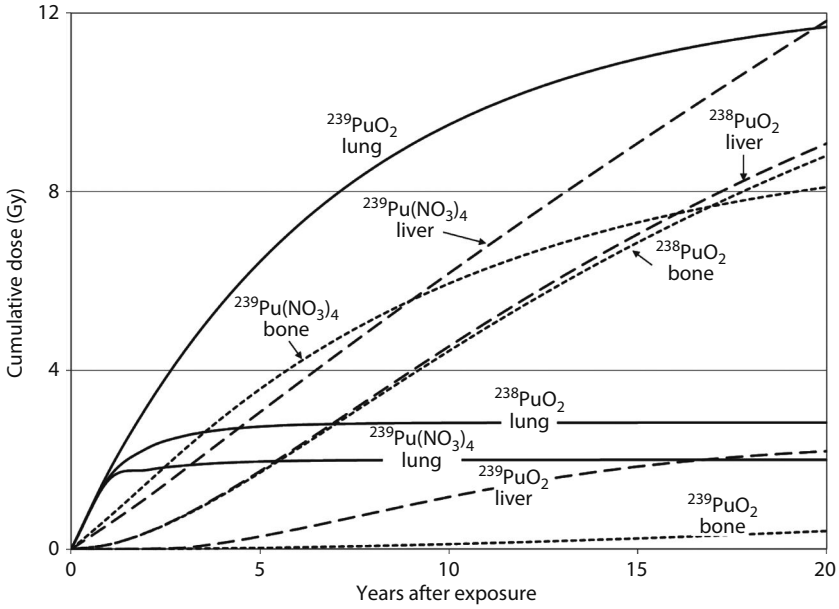

FIGURE 18.30 Cumulative absorbed dose to the lung, bone, and liver over time for a 10 kg dog exposed to 10 kBq of $^{238}PuO_2$, $^{239}PuO_2$, or ^{239}Pu $(NO_3)_4$. (From Wilson, D.A. et al., *Health Phys*., 96(2), 175, 2009.)

^{239}Pu in the respiratory tract of a worker accidentally exposed to ^{239}Pu $(NO_3)_4$ with the tissues of dogs exposed to different forms of ^{239}Pu. It is noteworthy that most of the tissue samples from workers contain such small amounts of radioactivity that it has been necessary for the USTUR scientists to develop new and innovative techniques for quantifying the retained radioactivity (Hare et al. 2010). It is apparent that U.S. workers in the facilities at Hanford, Los Alamos, and Rocky Flats did not receive exposures to plutonium similar to the high-level exposures of the Russian workers at the MPA discussed earlier. Most of the specimens from U.S. plutonium workers in the USTUR have radioactivity content substantially below that of specimens from MPA workers.

Effects of Internally Deposited Radionuclides in Laboratory Animals

The studies conducted at the University of Utah with injected radionuclides, at the UC-Davis with injected and ingested radionuclides, at the Hanford Laboratories (designated later as the Pacific Northwest Laboratories), and at the Lovelace Laboratory all had a primary focus of understanding the potential for induction of cancer (Thompson 1989). Hence, in all four laboratories, the studies involved detailed clinical observations on each subject until they died or were euthanized because of clinical illness and a desire to avoid pain associated with what was thought to be a life-threatening clinical condition. It is important to emphasize again that the tissue burden and radiation dose received were estimated for each subject allowing for detailed analyses as dose–response relationships for populations of the subjects. The original investigators and others have reported the results of the LSS in a number of publications. It was possible for other investigators to conduct secondary analyses since the laboratories conducting the studies frequently published detailed information on the various studies, including information on each animal. Some examples of key results from the LSS will be presented here.

It was generally thought at the initiation of the *internal emitter* programs that the primary late effects would be cancer and that would be the focus of the research with observations made over the life span of the experimental subjects. In the case of the Lovelace Inhalation Toxicology Program, there was an interest in evaluating the acute health effects of exposure to fission products that workers or other individuals close to the location of an accidental release might encounter.

The results of the controlled exposure studies with dogs exposed to radioactive aerosols with sufficient body burdens of radioactivity to cause early effects played a key role in the development of the health effects models developed for the conduct of nuclear power plant consequence analyses (Scott 1979; Scott and Hahn 1980; Scott et al. 1984; Evans et al. 1993). Moreover, it was recognized that evaluating the health effects of a brief exposure to a high-concentration radionuclide aerosol would establish an anchor point analogous to the maximum, for these radiation toxicity studies, tolerated dose used to establish the highest exposure levels in chronic bioassays of chemicals. When the inhalation studies with radionuclides were initiated, it was not known the degree to which protracted delivery of the radiation dose from inhaled radionuclides would be less effective than brief external radiation exposures.

The results of the Lovelace studies with beta-emitting radionuclides (^{90}Y, ^{91}Y, ^{144}Ce and ^{90}Sr) have been reported in a number of peer-reviewed publications. Summary papers on ^{137}CsCl are Boecker (1972) and Redman et al. (1972); on $^{91}YCl_2$ are Muggenburg et al. (1998); on $^{144}CeCl_3$ are Boecker and Cuddihy (1974a), Hahn et al. (1996), and Hahn et al. (1997); on $^{90}SrCl_2$ are Gillett et al. (1987, 1992); on ^{90}Y-FAP are Hobbs et al. (1972) and Mauderly et al. (1973); and on ^{144}Ce-FAP are Hahn et al. (1973a,b), Hahn and Boecker (1980), and Mauderly et al. (1980a). A paper by McClellan et al. (1986) provides an overview of the experimental approach and results.

The results will be briefly summarized here. In the studies with soluble forms of these radionuclides, the predominant deterministic effects related ARS-bone marrow damage from beta irradiation of the marrow by the radionuclides deposited in the skeleton. The primary stochastic effects were induction of cancers in the lungs, skeleton, and liver. The site of occurrence and incidence were organ dose dependent.

With the radionuclides inhaled in a fused aluminosilicate vector, the principal deterministic effects were fibrosis and associated cardiopulmonary functional effects. The principal stochastic effects were induction of cancers in the lungs, nasal cavity, and tracheobronchial lymph nodes. The sites of occurrence and incidence were organ dose dependent.

The answer to the important question of dose protraction effects is shown in Figure 18.31 where the risk of developing ARS related to bone marrow damage from either brief exposure to external 1 MeV x-rays or gamma rays is compared to that of dogs injected with ^{137}Cs (Scott et al. 1988). As discussed earlier, ^{137}Cs distributes throughout the body like K. Thus, the internally distributed ^{137}Cs is a source of protracted whole-body exposure to beta particles and gamma rays from decay of the ^{137}Cs. The internal dose decreases with an effective half-life of about 30 days. It was found that the internal radiation dose from the ^{137}Cs was about one-fourth as effective as a very brief exposure to the external x-radiation or gamma radiation.

Similar results for acute hematopoietic effects were observed in the beagle dogs that inhaled ^{91}Y, ^{144}Ce, or ^{90}Sr as a chloride. The relatively soluble radionuclides were rapidly absorbed from the lungs with translocation based on their elemental characteristics. All three elements were translocated to bone. The lanthanide elements Y and Ce were also translocated to the liver. With ^{90}Sr, ^{91}Y, ^{144}Ce, and ^{137}Cs, the beta emissions were the primary source of irradiation of the tissues, including the bone marrow. As may be noted in Figure 18.31, a dose of about 1000 rad delivered to whole body and bone marrow (^{137}Cs) or primarily to bone marrow (^{91}Y, ^{144}Ce and ^{90}Sr) over 30 days is required to produce deaths from hematopoietic injury as compared with the much smaller total dose from acute x-irradiation delivered essentially instantaneously (recall Figure 18.2) (Figure 18.32).

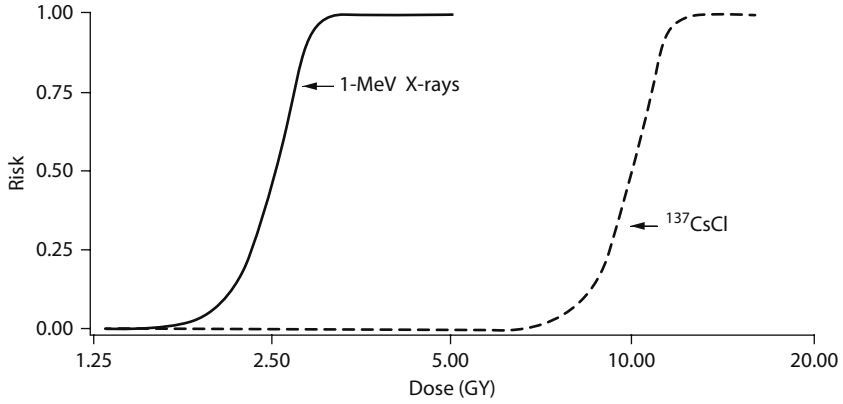

FIGURE 18.31 Comparative effect of acute x-irradiation (1-Mev x-rays) versus protracted irradiation (^{137}CsCl) in producing bone marrow syndrome in dogs. (From Scott, B.R. et al., *Risk Anal.*, 8(3), 393, 1988.)

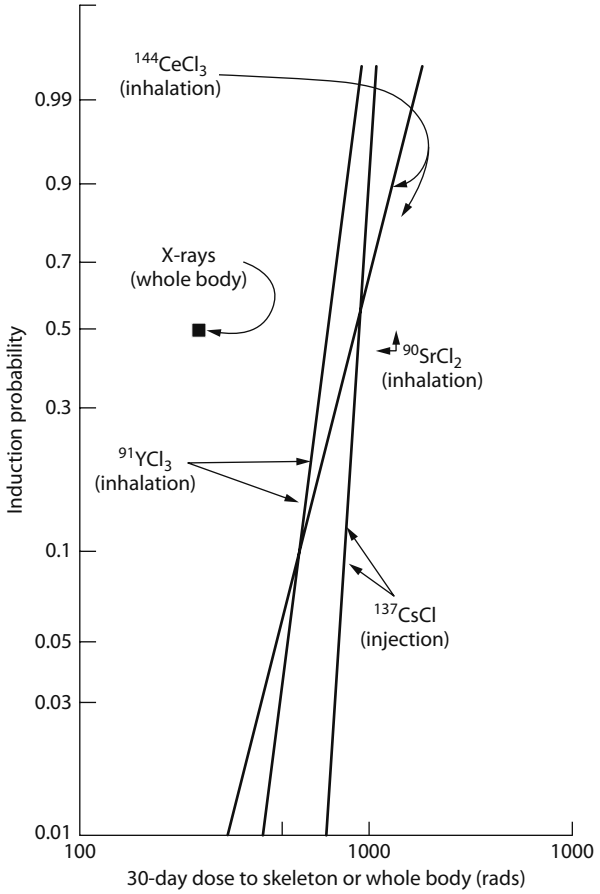

FIGURE 18.32 Decreased effectiveness of protracted internal beta irradiation from soluble forms of ^{91}Y, ^{144}Ce, or ^{90}Sr inhaled or injected ^{137}Cs in producing deaths due to hematopoietic injury. For comparison a value (■) is shown for x-rays (whole body) irradiation is shown. (From Scott, B.R., *Health Phys*., 36, 323, 1979.)

For inhalation exposure to relatively insoluble radionuclides that result in substantial radiation doses to the lungs, a primary early deterministic effect was development of pneumonitis. Summary radiological information on the four beta-emitting radionuclides incorporated into FAPs is given in Table 18.18 (Scott 1980; Scott et al. 1990). In these studies, the

TABLE 18.18
Physical and Effective Half-Lives and the Length of Time Required for Deposition of 90% of the Total Dose

Radionuclide-Infused Aluminosilicate Particles	Physical Half-Life	Effective Half-Life in Lung (9 days)	Time to Deliver 90% of Total Dose
^{90}Sr	29 years	600	5.5 years
^{144}Ce	285 days	175	1.6 years
^{91}Y	59 days	50	0.5 years
^{90}Y	2.6 days	2.5	8 days

FAPs were cleared in a similar manner with long-term retention in the lungs. The difference in effective half-life and the resulting differences in the pattern of irradiation of the lungs were a reflection of the different physical half-lives for the four radionuclides. The survival curves for radiation-induced pneumonitis producing deaths within 500 days of inhalation exposure for the four radionuclides are shown in Figure 18.33 and compared to a similar clinical outcome resulting from brief x-irradiation of the lungs (Van Dyk et al. 1981;

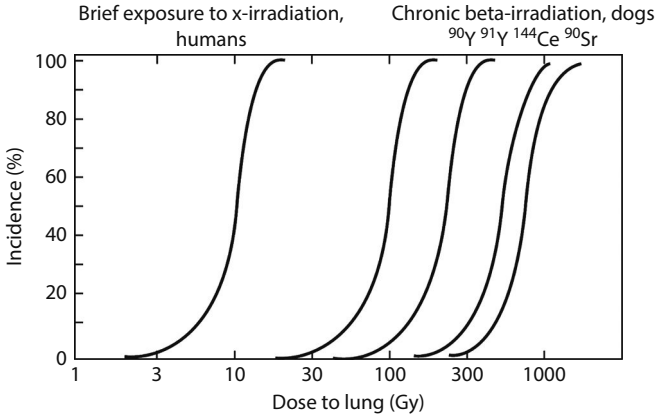

FIGURE 18.33 Decreased effectiveness of protracted internal beta irradiation compared with very brief external x-irradiation in producing pneumonitis. (From Scott, B.R. et al., *Risk Anal.*, 8(3), 393, 1988.)

Scott et al. 1988). The sparing effect of dose protraction is clear. Compared with brief x-irradiation exposures protracting the delivery of 90% of the dose over about 8 days, ^{90}Y requires a radiation dose nearly 10 times greater than from brief x-irradiation to produce equivalent clinical effects. With ^{91}Y, 90% of the dose is delivered in about 6 months and about 20 times more radiation dose is required. With ^{144}Ce and ^{90}Sr, 90% of the radiation dose is delivered over 1.6 years or longer, so it takes a dose 30 times greater to produce equivalent clinical effects as a brief exposure to x-irradiation. It is clear that dose protraction substantially reduces the effectiveness of each gray of dose. The threshold dose for brief x-irradiation causing pneumonitis was about 2 Gy delivered to the lungs. The threshold radiation dose for ^{90}Y-FAP was increased to about 15 Gy, for ^{91}Y-FAP to about 50 Gy, for ^{144}Ce-FAP to about 100 Gy, and for ^{90}Sr-FAP to about 300 Gy related to protraction of the low-LET beta dose to the lungs.

The results of the studies at both Lovelace and Pacific Northwest Laboratories (PNL) with dogs inhaling ^{238}Pu and ^{239}Pu have been published in numerous open literature publications. Some key references on the effects of $^{239}PuO_2$, $^{238}PuO_2$, and $^{238}Pu(NO_3)_2$ are Muggenburg et al. (1983, 1986, 1996, 1999, 2008), Park et al. (1997, 2008, 2012), and Mauderly et al. (1980b).

The primary deterministic effects observed were lymphopenia and pulmonary pneumonitis and fibrosis, although, as discussed later, the issue of whether the pulmonary noncancer effects were deterministic or stochastic is a matter of definition. The primary stochastic effects were principally lung cancer and, at lower incidence, liver and bone cancer. The sites of occurrence of the plutonium-induced cancers and incidence were organ dose dependent.

Pneumonitis was seen in dogs that inhaled ^{238}Pu or ^{239}Pu oxide, which resulted in higher-LET alpha irradiation of the lungs. Both radionuclides have very long half-lives and when inhaled in the oxide form have a long retention time in the lungs. This results in protracted alpha irradiation. Muggenburg et al. (1999, 2008) and Park et al. (1997, 2008, 2012) have noted that this results in some cases of pneumonitis developing several years after the single brief inhalation exposure to the Pu oxide aerosols. Wilson et al. (2009) examined the time course of a diagnosis of lung fibrosis or lung cancer in 359 dogs exposed to either $^{238}PuO_2$ or $^{239}PuO_2$ in the Lovelace experiments (Muggenburg et al. 1999, 2008). The time course of the two diseases is shown in Figure 18.33. Note that both lung fibrosis and lung cancer were observed in control dogs with the earliest cases at about 12 years after the initiation of the studies when the dogs were just over a year old. In contrast, the first cases of lung fibrosis (in the dogs with the highest lung burdens and largest alpha radiation doses) were observed about 1 year after exposure to $^{239}PuO_2$ and about 2 years after exposure to $^{238}PuO_2$. Additional cases of lung fibrosis were observed in dogs with lower lung burdens of ^{239}Pu or $^{238}PuO_2$ at later times. A model with a linear function of dose (Figure 18.34) best describes the hazard function for developing lung fibrosis. A threshold is apparent for $^{238}PuO_2$-induced lung fibrosis at about 0.7 Gy and for

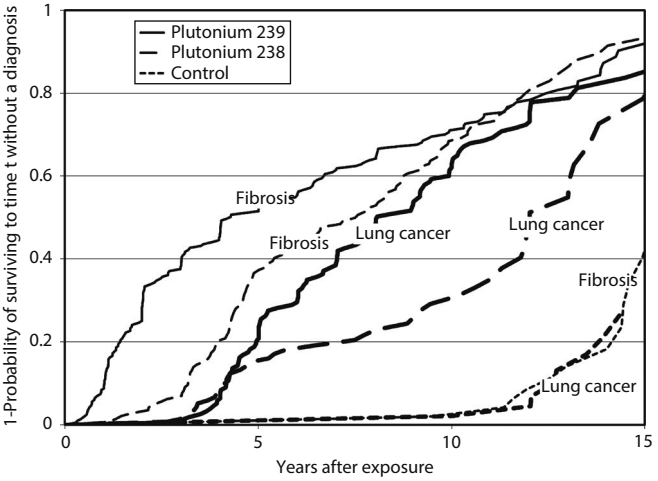

FIGURE 18.34 The unadjusted probability of a diagnosis of lung fibrosis or lung cancer by years after exposure for dogs exposed to $^{239}PuO_2$ or $^{238}PuO_2$. (From Wilson, D.A. et al., *Health Phys*., 96(2), 175, 2009.)

$^{239}PuO_2$ at about 1.5 Gy. The $^{238}PuO_2$ particles were more effective than the $^{239}PuO_2$ particles. This may have been influenced in part by the alpha decay–related fragmentation of the $^{238}PuO_2$ particles (Diel and Mewhinney 1983). Note that the doses have been expressed as gray units without any consideration of RBE or W_R and a conversion to Sv.

In the lifespan studies conducted in the four laboratories (Table 18.19 and Figure 18.35), an excess of cancers was observed compared with the occurrence of cancers in the control dogs. The site of the cancers was closely related to the organs and tissues irradiated. Hence, these cancers were primarily observed in the lungs, skeleton, and liver. Numerous papers have been published on the results of the studies; in this chapter, key results will be briefly summarized. In considering the results, the reader should take special care to note that in the figures, different scales have been used in accordance with the published material. In some cases, results are presented as the number of observed cases, while in others, the results are expressed as RR. The dose metric may be average dose to an organ/tissue, cumulative dose to death or with a specified lag. The original authors used

TABLE 18.19
Summary of Relative Carcinogenic and Dosimetric Ratios from Four Beta-Emitting Radionuclides Inhaled in FAPs and Inducing Lung Cancer

Nuclide	Emission Type	Organ	Laboratory	Potency Ratio
^{90}Y	β	Lung	Lovelace ITRI	1.0
^{91}Y	β	Lung	Lovelace ITRI	0.51
^{144}Ce	β	Lung	Lovelace ITRI	0.27
^{90}Sr	β	Lung	Lovelace ITRI	0.22

Source: Raabe, O.G., *Health Phys*., 98, 515, 2010.

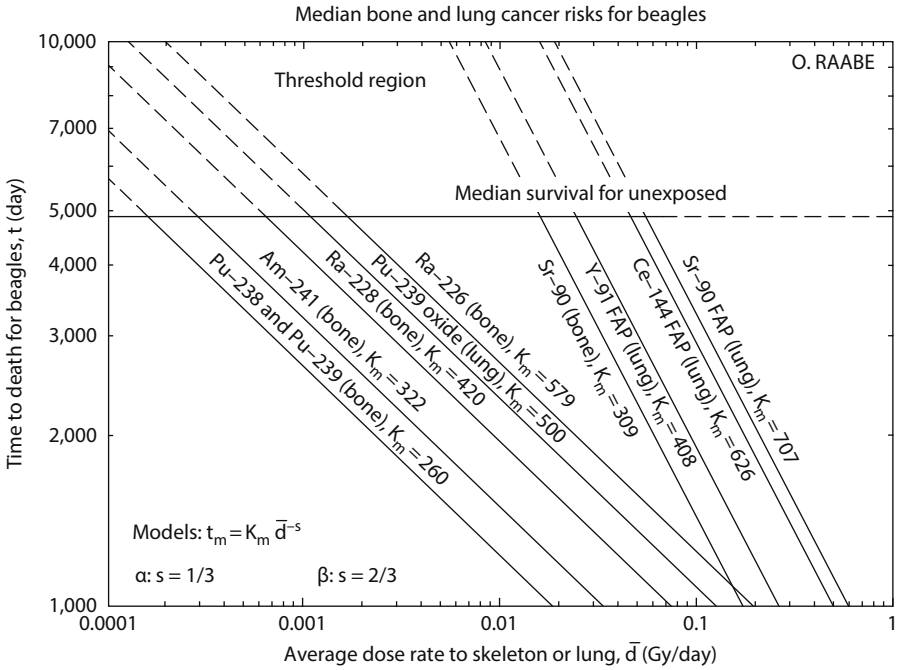

FIGURE 18.35 Illustration of bone sarcoma and lung carcinoma risk functions for beagles demonstrating similar target organ average-dose-rate/time/response patterns with life span virtual thresholds at low dose rates. The positions of the lines vary because of inherent differences in irradiation of the target cells by the different radionuclides and forms. Data were selected from lifetime laboratory studies of internally deposited radionuclides in beagles, including skeletal deposits of ^{90}Sr after exposure by ingestion at Davis and by injection at Utah; lung deposits of inhaled ^{144}Ce, ^{91}Y, and ^{90}Sr in FAP at ITRI; lung deposits of inhaled $^{239}PuO_2$ a PNL; skeletal deposits of injected ^{226}Ra at Davis and Utah; inhaled $^{238}PuO_2$ at ITRI; and skeletal deposits of injected ^{226}RA and ^{241}Am at Utah. (From Raabe, O.G., *Health Phys*., 98, 515, 2010.)

both arithmetic and logarithmic scales and, in some cases, logarithmic–logarithmic scales and even 3D slots.

The study results with inhaled radionuclides and induction of lung cancer will be considered in detail. In considering these results, it is important to note that when these studies were initiated, the only significant data available on induction of lung cancer by inhaled radionuclides were the emerging quantitative data from the studies of exposure to radon and its daughter products that produced lung cancer in miners. It is now apparent that exposure to radon and its daughter products results primarily in alpha particle irradiation of the cells lining the conducting airways. This pattern of irradiation of the respiratory tract cannot be readily extrapolated to other inhaled radionuclides where the radiation dose is primarily delivered to the more distal airways and pulmonary region as occurs with inhalation of respirable-sized particles.

To understand late-occurring effects of the internally deposited radionuclides, Raabe (2010) developed summary comparative potency estimates by modeling data published by the original investigators at the University of Utah, UC-Davis, PNL, and Lovelace. These effects were best described by 3D average-dose-rate/time/response surfaces that compete with other causes of death during an individual's lifetime. Recall the 3D plot in Figure 18.22. A 2D summary plot of Raabe's (2010) results for nine different exposure scenarios is shown in Figure 18.35. In four scenarios, the target organ is the lung, and in five scenarios, it is the skeleton. Four of the scenarios involve low-LET beta irradiation of lung or skeleton, and five involve high-LET alpha irradiation of lung or skeleton. Raabe (2010, 2011) makes a strong case for using average radiation dose rather than cumulative dose in modeling the effects of the internally deposited radionuclides. In this chapter, the results are presented as published by the original authors. Both approaches have pros and cons that deserve careful consideration and are beyond the scope of this chapter. Using maximum likelihood survival regression methods, the characteristic logarithmic slope for cancer induction for each dose pattern and organ was estimated. Across this range of dose patterns for low-LET beta irradiation, with dose protraction, there was a sparing effect of more than a factor of 4.

The beta-emitting radionuclides all yielded Weibull cancer risk power function distributions with negative slopes of about two-thirds, as will be noted later. In contrast, Raabe (2010) found that for alpha irradiation, the negative slopes were about one-third. He interpreted these results as indicating two beta particles were needed to cause the same cellular transformation as that produced by one alpha particle.

Wilson et al. (2009) and Raabe (2010) modeled the data from studies of the alpha-emitting ^{239}Pu and ^{238}Pu conducted at Lovelace and Battelle. Shown in Figure 18.36 is a traditional hazard function plot of the cumulative hazard function for lung cancer induction by lung irradiation from inhaled ^{239}Pu and ^{238}Pu developed by Wilson et al. (2009).

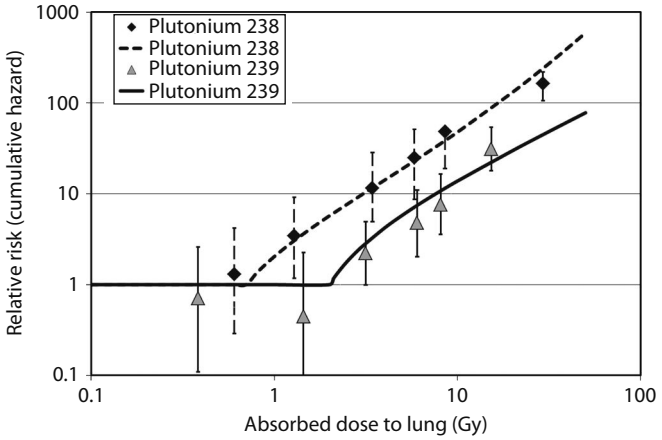

FIGURE 18.36 Estimated cumulative hazard of lung fibrosis in dogs for various levels of initial lung burden of $^{238}PuO_2$ or $^{239}PuO_2$, with 95% CI, and the fitted hazard functions of cumulative dose to the lung. (From Wilson, D.A. et al., *Health Phys*., 96(2), 175, 2009.)

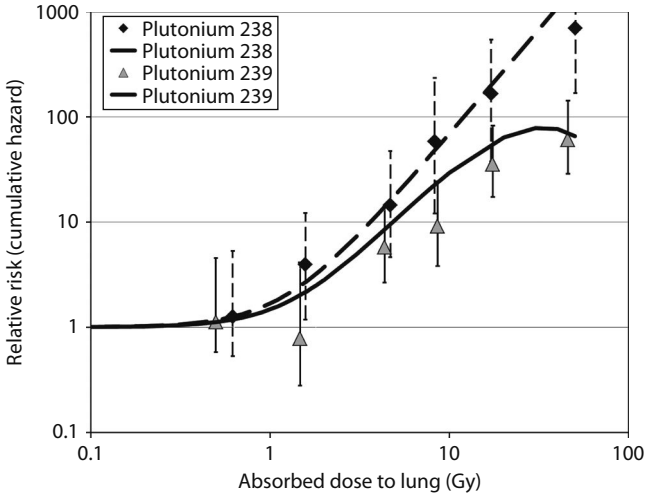

FIGURE 18.37 Estimated cumulative hazards of lung cancer for each level of initial lung body burden, with 95% CIs, and fitted hazard functions of cumulative dose to the lung from inhaled $^{238}PuO_2$ and $^{239}PuO_2$. (From Wilson, D.A. et al., *Health Phys*., 96(2), 175, 2009.)

They explored a range of models of dose–response relationships, including use of various lag periods. They found that

> Models of the risk of lung cancer using doses adjusted for thresholds from 0 to 1 Gy were not significantly different; thresholds over 1 Gy were significantly worse. The models were significantly improved by adjusting the cumulative dose estimates to account for lags of at least 1 year. In models with the dose-response function as the predictor variable, the hazard of lung cancer was consistent with a linear-no threshold model in the combined data and in dogs exposed to $^{239}PuO_2$. However, when covariates were added, the linear-no threshold model was not the best choice; the lung cancer hazard was better described with a model incorporating a quadratic dose-response function.

A comparison of Figures 18.36 and 18.37 illustrates that over the dose range of 1–10 Gy, lung fibrosis and lung cancer are competing risks.

Raabe (2010) modeled the lung cancer data from both the Lovelace and Pacific Northwest Laboratory studies of inhaled ^{239}Pu and ^{238}Pu. His analyses are of special interest because they provide a basis for considering the comparative potency of the several different alpha radiation dose patterns (Figure 18.36 and Table 18.20). He found that the $^{239}Pu(NO_3)_4$ aerosol exposure was most potent at inducing lung cancer. As noted by Wilson et al. (2009), a $^{239}Pu(NO_3)_4$ particle will contain less ^{239}Pu than a $^{239}PuO_2$ particle of the same physical size as a $^{239}PuO_2$ particle because of the substantial contribution of the nitrate to the total mass. Thus, it will take more $^{239}Pu(NO_3)_4$ particles to deliver the same total radioactivity and initial radiation dose with the dose delivered over a greater portion of the lung. Another phenomenon to consider as an influencing factor is the radiation-induced fragmentation reported by Diel and Mewhinney (1983). The $^{238}PuO_2$ aerosols studied at the Pacific Northwest Laboratory was about a factor of 4, less effective than the $^{239}Pu(NO_3)_4$. This difference is relatively modest in view of the range of particle sizes (both polydisperse and monodisperse) and the two different chemical forms studied.

TABLE 18.20
Summary of Relative Carcinogenic and Dosimetric Potency Ratios as a Function of Radiation Type for Inhaled Alpha-Emitting Radionuclides Producing Lung Cancer

Nuclide/Form	Type	Organ	Laboratory	Potency Ratio
$^{239}Pu(NO_3)_4$	α	Lung	Pacific Northwest Lab	1
$^{238}PuO_2$	α	Lung	Lovelace ITRI	0.54
$^{239}PuO_2$ 0.75 μm	α	Lung	Lovelace ITRI	0.48
$^{239}PuO_2$ 1.5 μm	α	Lung	Lovelace ITRI	0.42
$^{239}PuO_2$	α	Lung	Pacific Northwest Lab	0.37
$^{239}PuO_2$ 3 μm	α	Lung	Lovelace ITRI	0.31
$^{238}PuO_2$	α	Lung	Pacific Northwest Lab	0.26

Source: Raabe, O.G., *Health Phys*., 98, 515, 2010.

Fourteen different exposure scenarios (Table 18.21) with seven different elements and ten different radionuclides were studied that resulted in an excess of bone cancer. Figure 18.38 is a classical dose–response plot for the incidence of fatal leukemia, bone sarcoma, oral/nasal carcinoma, and periodontal carcinoma in dogs fed ^{90}Sr, from exposure started in utero and continued to adulthood at the UC-Davis. Raabe (2010) interpreted the data as demonstrating a life span virtual threshold for all radiation-induced cancers occurring at calculated cumulative skeletal beta radiation doses above 10 G (10 Sv). He further noted that the absence of bone sarcoma cases in the three lowest dosage groups was significantly less than those found in controls (<0.047).

TABLE 18.21
Summary of Relative Carcinogenic and Dosimetric Potency Ratios for Bone-Seeking Radionuclides Producing Bone Cancer

Nuclide/Form	Type	Organ	Laboratory	Potency Ratio
^{228}Th	α	Bone	University of Utah	12.4
^{239}Pu	α	Bone	University of Utah	12.0
^{238}Pu	α	Bone	Pacific Northwest Lab	11.8
^{238}Pu	α	Bone	Lovelace ITRI	10.6
^{240}Cf	α	Bone	University of Utah	7.5
^{224}Ra	α	Bone	University of Utah	7.0
^{241}Am	α	Bone	University of Utah	5.9
^{239}Pu	α	Bone	Pacific Northwest Lab	4.5
^{252}Cf	α	Bone	University of Utah	4.4
^{228}Ra	α	Bone	University of Utah	2.7
^{226}Ra	α	Bone	University of Utah	1.1
^{226}Ra	α	Bone	UC-Davis	1
^{90}Sr (injection)	β	Bone	University of Utah	1.2
^{90}Sr (ingestion)	β	Bone	UC-Davis	1

Source: Raabe, O.G., *Health Phys*., 98, 515, 2010.

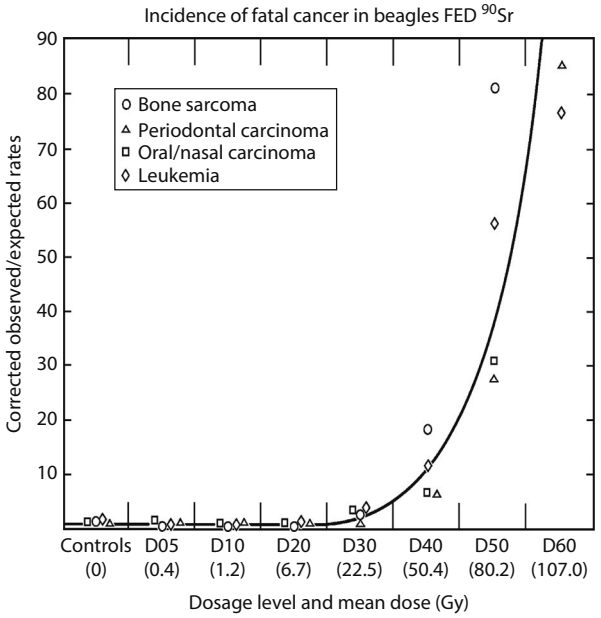

FIGURE 18.38 Survival analysis using the method of Peto et al. (1980) for the incidence of fatal leukemia, bone sarcoma, oral/nasal carcinoma, and periodontal carcinoma in beagle dogs exposed to ^{90}Sr in utero and fed ^{90}Sr to young adulthood at the UC-Davis as a function of dose group (with mean cumulative beta radiation dose to the skeleton). (From Raabe, O.G., *Health Phys*., 98, 515, 2010.)

The bone cancer results from studies conducted at the University of Utah and UC-Davis with injected ^{226}Ra, at UC-Davis with ingested ^{90}Sr, at the University of Utah with injected ^{90}Sr, and at Lovelace ITRI with inhaled ^{90}Sr are shown in Figure 18.39 (Raabe 2010). Note that this plot includes only the dogs dying with a bone cancer and does not include dogs that died of other causes. The median life span for the control dogs for the several studies is shown. The data for the two studies with ^{226}Ra and the other three studies are quite closely grouped; the range of data points on the dose metric and the average dose rates to skeleton are a reflection of the calculation of dose for each subject as contrasted to presentation of the data shown in Figure 18.38 by dose group.

The difference in the dose–response relationships for groups of dogs administered ^{226}Ra (alpha irradiation) and ^{90}Sr (beta irradiation) is striking. As noted earlier, Raabe (2010) interpreted the difference as related to a different mode of action for the alpha versus beta irradiation. It is noteworthy that with a high dose rate, approximately 0.1 Gy/day, the effects of the two types of irradiation in bone sarcoma induction are about equally as effective per gray in causing bone cancer. At dose rates an order of magnitude lower, the alpha irradiation is about four times as effective per gray as the beta irradiation. At even lower dose rates of greater relevance for occupational exposure and, certainly, for environmental exposure of people, the difference in relative effectiveness per gray is even more pronounced with the high-LET alpha irradiation being about 10 times as effective as the low-LET beta irradiation.

Using a similar analytical approach for all the studies, Raabe (2010) calculated the relative carcinogenic and dosimetric potency estimates for each of the 14 types of exposure (Table 18.21). The ^{228}Th, ^{239}Pu, and ^{238}Pu, which deposit preferentially on bone surfaces, are high-LET alpha emitting as the most potent. The low-LET beta emitter ^{90}Sr and high-LET alpha emitter ^{226}Ra were least potent. The ^{90}Sr deposits throughout the skeleton accompanying Ca. However, the relatively energetic beta particles from ^{90}Sr and its short-lived daughter ^{90}Y provide relatively uniform irradiation of the skeleton. In contrast, although ^{226}Ra deposits throughout the skeleton like Ca, much of the alpha emissions are *wasted* radiation because of the short track length of the alpha particles in dense bone. Only a portion of the alpha particle energy is dissipated in cells lining the bone surface, the cells that are presumed to be the origin of the bone cancers.

An excess of liver cancer was observed in the beagle dogs that inhaled $^{238}PuO_2$ or $^{239}PuO_2$ $(NO_3)_4$. Both types of exposure resulted in the translocation of plutonium from the lungs to liver (Figure 18.30). Liver cancer mortality increased significantly in the dogs receiving $^{239}PuO_2$ with cumulative liver doses above 0.2 Gy and in the dogs receiving $^{238}PuO_2$ with cumulative liver doses above 1.0 Gy. With both types of exposures, the cumulative liver doses were less than 3.0 Gy. The difference in the apparent threshold doses for $^{238}PuO_2$ may relate to its more gradual release from the lung than in the case of ^{239}Pu $(NO_3)_4$, which left the lungs more rapidly. A portion of the alpha dose from $^{238}PuO_2$ may have been *wasted*.

In considering the very substantial amount of data from the dogs administered internally deposited radionuclides, a key question is its relevance to predicting human hazard and risk. Recall that when the studies with beagle dogs were initiated, no human data were available on these radionuclides

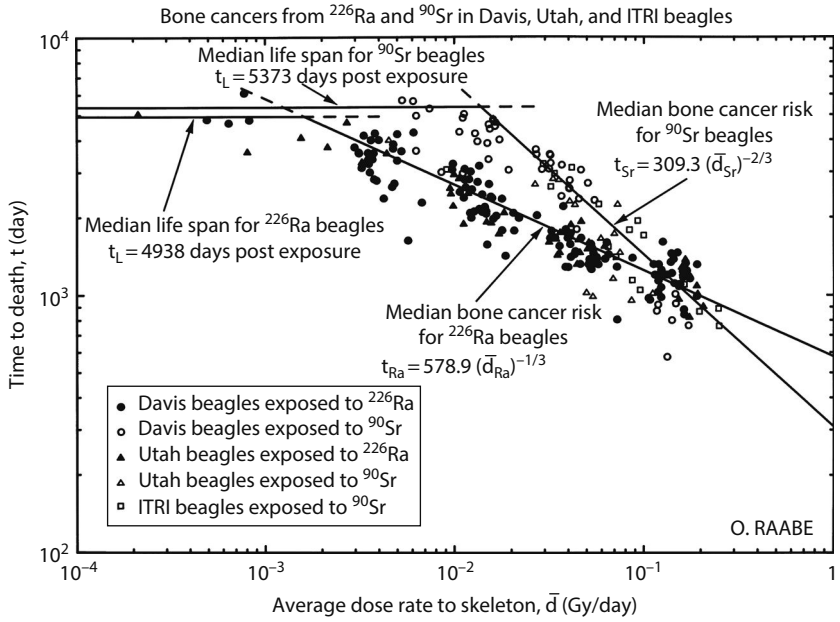

FIGURE 18.39 Comparison of bone sarcoma cases in beagle dogs at UC-Davis, the University of Utah, from injected ^{226}Ra, at UC-Davis with ingested ^{90}Sr, at the University of Utah with injected ^{90}Sr, and from Lovelace ITRI with inhaled ^{90}Sr as a function of the average alpha or beta radiation dose to the skeleton. (From Raabe, O.G., *Health Phys*., 98, 515, 2010.)

with the exception of ^{226}Ra and, to a lesser extent, ^{228}Th, information gained from the radium dial painter studies. The University of Utah dog studies would later predict, using the ratio approach discussed earlier, that ^{239}Pu reaching the skeleton would be about 10 times as potent as ^{226}Ra in producing bone cancer.

An alternative approach to assessing the predictive nature of the dog study results is to directly compare the observed effects of $^{239}Pu(NO_3)_4$ and $^{239}PuO_2$ in the dogs purposefully exposed and workers occupationally exposed at the MPA complex starting in 1948. That is exactly the approach taken by Wilson et al. (2010). The MPA worker data are from Sokolnikov et al. (2008) who analyzed data on 17,740 workers (25% females) observed until death, lost to follow-up, or through December 31, 2003. The workers typically had multiple exposures to $^{239}Pu(NO_3)_4$ and $^{239}PuO_2$ during their employment, which in some cases dated to 1948. Mortality included 354 lung cancer deaths, 40 liver cancer deaths, and 11 bone cancer deaths as discussed earlier. It is obvious that some of the workers smoked cigarettes and consumed alcohol (Tokarskaya et al. 2002, 2006). The beagle dog population consisted of 837 dogs exposed to $^{239}PuO_2$, $^{238}PuO_2$, or $^{239}Pu(NO_3)_4$ at either Lovelace ITRI or Pacific Northwest Laboratory and followed for their life span. In this population, 231 lung cancer deaths, 45 liver cancers, and 158 bone cancers were observed. It is obvious that none of the dogs smoked or consumed alcohol. Half of the dogs were females and half were males. None of the female dogs was allowed to reproduce.

The lung cancer mortality rates for the MPA workers (354 cases) and the dogs (231 cases) are shown in Figure 18.40. The overall agreement is striking with the greatest deviation at the highest lung doses for the MPA workers. In my opinion, the similarity in dose–response relationship for the workers occupationally exposed and dogs purposefully exposed is especially remarkable considering the much greater uncertainty in estimating lung doses for workers than for the dogs and the always challenging issue of accounting for cigarette smoking as a major confounder.

The liver cancer mortality rate for the MPA workers (14 controls, 26 exposed) and the beagle dogs (7 controls, 39 exposed) is shown in Figure 18.41. Again, the similarity in the dose–response relationships for workers and dogs is remarkable, especially for liver cases below 3.0 Gy. The lower dose groups included 34 of the 35 cases observed in exposed dogs and 14 of the 26 cases observed in workers. Twelve of the liver cancers were observed in workers with liver doses of over 3.0 Gy. An additional critical factor in considering liver cancer mortality is the role of alcohol consumption as a confounder in the worker population (Tokarskaya et al. 2006).

Recalling that concern for ^{239}Pu-producing bone cancer was a major driver of the U.S. research program starting in World War II, it is ironic that bone cancer proved to be a less significant outcome than the development of lung and liver cancer. The MPA workers cohort only experienced 11 cases of bone cancer, 5 in controls, 3 in the 0.3–0.5 Gy subgroup, and 3 in the subgroup with estimated bone doses of over 10 Gy. The low number of bone cancers in the MPA workers (5 in controls and 6 in exposed workers) contrasts with the beagle dogs (1 case in a control and 157 cases in exposed dogs). This contrast deserves further investigation and suggests the possibility that the dog is more sensitive to induction of bone cancer by alpha irradiation than people.

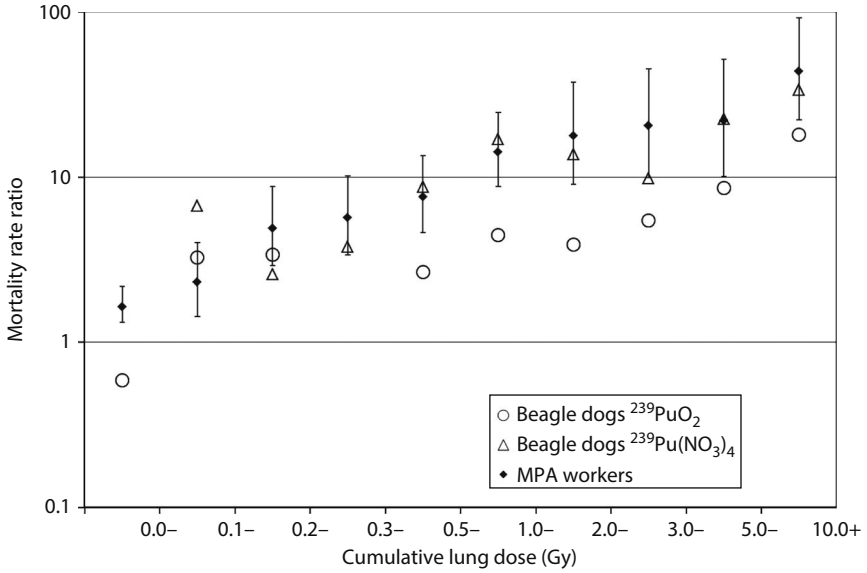

FIGURE 18.40 Lung cancer mortality rate ratio (with 95% CIs) for Mayak Production Association workers with known plutonium exposure plotted with rate ratios in beagle dogs exposed by inhalation to plutonium by level of cumulative lung dose. (Adapted from Wilson, D.A. et al., *Health Phys*., 98(1), 42, 2010.)

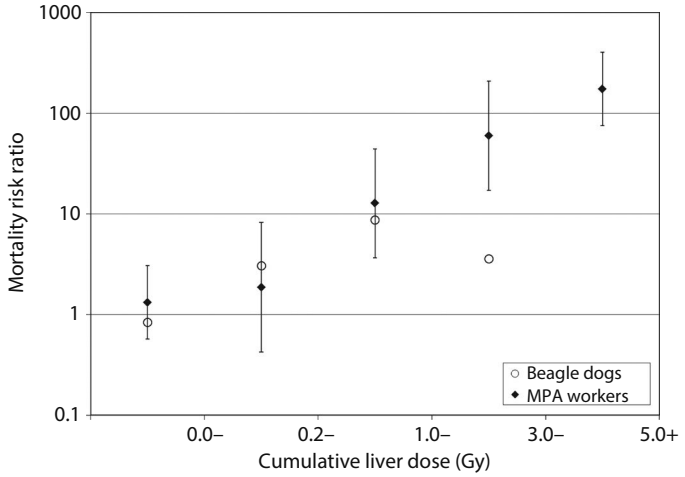

FIGURE 18.41 Liver cancer mortality rate ratios (with 95% CIs) for Mayak Production Association workers with known plutonium exposure plotted against rate ratios in beagle dogs exposed to plutonium by level of cumulative liver dose. (From Wilson, D.A. et al., *Health Phys*., 98(1), 42, 2010.)

Wilson et al. (2010) modeled the data in several ways. In general, the linear dose–response models fit the data reasonably well. They noted that linear function of dose given in Sokolnikov et al. (2008) did not fit the data as well as a quadratic function with inclusion of a cell-killing response function. It is likely that additional attention will be given to these valuable data sets in the future to gain further insight into the biological mechanisms involved in the two species. Indeed, Gilbert et al. (2013) have already updated the MPS worker data providing an additional 5 years of follow-up.

It would be very useful to conduct a critical analysis of extensive data available from rats exposed to plutonium aerosols and assess how well the dose–response relationships for plutonium-exposed rats relate to the observations on beagle dogs and humans. Such an analysis could be especially useful since the results of inhalation studies with rats have been used to assess human hazards of a number of chemical toxicants.

In my opinion, the Wilson et al. (2009) reanalysis offers reassurance that risk estimates for intake of radionuclides and associated alpha irradiation of lung, liver, and skeleton developed from the MPA workers and the beagle dog studies are not likely to underestimate the true risk of radiation-induced disease. It is useful to recall that Cantril and Parker (1945) noted that human misfortune was the cornerstone of radiation protection guidance at that time drawing heavily on the radium dial painter experiences for radionuclides deposited in the skeleton. Now more than a half century later, rigorous data analysis from another unfortunate experience,

that of the MPA workers, validates the importance of the dog studies with internally deposited radionuclides as predictors of radiation-induced effects in humans.

RADIATION COUNTERMEASURES

Pharmaceutical Approaches

Since the late 1940s, there has been interest in the development of agents that could be used to protect against radiation risks. Recent acts of terrorism and concern for the potential use of devices that might involve radiation exposure have renewed interest in the development of such agents (Weiss and Landauer 2009; DuMont et al. 2010; Williams et al. 2010; Mettler et al. 2011; Koukourakis 2012). There are three major classes of radiation countermeasure agents: radioprotectants, radiation mitigators, and therapeutic agents. Radioprotective agents are intended for use before irradiation. Radiation mitigators are given after irradiation but before expression of tissue injury. Therapeutic agents are intended for use after irradiation as palliative or supportive measures as indicated by clinical signs and symptoms.

Thousands of agents have been tested for radioprotective effects (Weiss and Landauer 2009). In view of the role of free radicals in producing tissue injury from irradiation, much attention has been directed toward evaluating compounds with antioxidant properties. This has led to substantial research on sulfhydryl compounds. Unfortunately, at doses of these compounds required to provide some protection against acute radiation injury, the sulfhydryl compounds produce nausea and vomiting (Grdina et al. 2002). In his comprehensive review, Koukourakis (2012) identifies more than 60 compounds with radioprotective activity linking them to specific mechanisms of radiation injury. One of these compounds, aminothiol, amifostine (Ethyol, Medimmune), is the only agent approved by the U.S. Food and Drug Administration for clinical use (Wasserman and Brizel 2001). It is limited to use for some radiotherapeutic situations to reduce xerostomia or dry mouth. Unfortunately, it has serious side effects requiring careful medical monitoring of the patient. Looking to the future, Williams et al. (2010), based on a literature review, have recommended animal models that may prove useful in developing medical countermeasures to radiation exposure.

Reducing Radiation Dose

The only radioprotective agent generally accepted for use in treating large, accidentally exposed populations is potassium iodide to block unbound ^{131}I from accumulating in the thyroid. However, in considering its use, it is important to recognize that some individuals are sensitive to potassium iodide. Thus, special care should be exercised in using it in situations involving accidental release of ^{131}I. It is obvious that the preferred approach is to limit ^{131}I intake, which can be accomplished in many situations by avoiding ingestion of ^{131}I-contaminated milk.

The U.S. Food and Drug Administration in 2003 approved the use of Prussian blue (PB; Radiogardase) for reducing body burdens of radiocesium (Thompson and Church 2001). Melo et al. (1996), in studies at Lovelace, demonstrated the effectiveness of PB in enhancing the elimination of ^{137}Cs from beagle dogs. The reductions in the average total whole-body doses by PB were age-related: 51% in immature dogs (4.7 months), 31% in young adults (2.4 years), and 38% in aged dogs (13.5 years). The ratio of fecal to urinary ^{137}Cs was enhanced by PB administration from 0.8 in untreated dogs to 2.2 in treated dogs. A key question with PB is what level of ^{137}Cs intake would warrant treatment by this agent.

Chelation Therapy

Since the beginning of nuclear operations in the mid-1940s, there has been interest in approaches to removing radionuclides that have been inhaled or entered via puncture rounds. ^{239}Pu handled in a variety of forms as $^{239}PuO_2$, $^{239}Pu(NO_3)_4$, and as a metal has been of special concern. Early research demonstrated that chelating agents administered intravenously were effective in removing soluble plutonium (Taylor et al. 2007; Durbin 2008; Kazzi et al. 2013). Zinc diethylenetriamine pentaacetate (DTPA) has been shown to be effective in removing plutonium and americium when administered intravenously or by inhaling a nebulized aerosol. In the case of ^{239}Pu or ^{241}Am in wounds, the usual approach has been to surgically remove as much of the radioactive material as possible and then administer DTPA intravenously (NCRP 2008). The U.S. Food and Drug Administration has given limited approval for the use of Ca- and ZN-DTPA to treat workers accidentally contaminated with plutonium, americium, and other actinides. It has encouraged pharmaceutical companies to seek approval for wider usage in the event of population exposures to actinide radionuclides. As an aside, based on my experience in aerosol science and in studying the health effects of inhalation exposure to actinides, I cannot envision situations in which the air concentrations of actinides and the associated projected radiation doses would warrant mass treatment of populations with DTPA.

Bronchopulmonary Lavage

In the late 1950s and 1960s, widespread interest developed in using lung fluid and cells recovered by lung lavage as a diagnostic tool in human clinical medicine. Interest in the technique was further stimulated when fiber-optic bronchoscopes became available and could be used to recover fluid and tissue from specific areas of the airways and lung. A pioneer in the field, Ramirez-Rivera et al. (1963), described their use of whole-lung lavage to remove material from the lungs of patients with pulmonary alveolar proteinosis. This work served as a stimulus to Lovelace ITRI scientists to explore the use of bronchopulmonary lavage in order to obtain samples of lung surfactant for evaluation, for biochemical and cytological evaluation as a diagnostic tool, and most importantly, for evaluation of the use of bronchoalveolar lavage (BAL) as a therapeutic procedure for removing inhaled radioactive particles (Pfleger et al. 1969; Henderson et al. 1974, 1975).

The bronchopulmonary lavage procedure can be carried out in several ways in dogs, primates, and human subjects.

The basic procedure is conducted with the individual under general anesthesia and intubated with either a single or double lumen catheter; the latter allows separation of the two lungs. The lavage procedure involves introducing saline fluid and removal of the fluid from the lungs by gravity. Typically, the procedure is repeated several times. In a well-oxygenated patient, the entire lung can be washed several times.

Multiple studies were conducted at Lovelace ITRI with dogs exposed to radioactive aerosols, including $^{239}PuO_2$ (Pfleger et al. 1969; Boecker et al. 1974; Felicetti et al. 1975; Muggenburg et al. 1976, 1977, 1980, 1990). The lavage procedure was found to be effective in removing inhaled, relatively insoluble radioactive particles from the pulmonary region. The largest amount of radioactivity was removed if the lavage procedure was carried out soon after the radioactive particles were inhaled but decreased over time. However, particles could be removed by bronchopulmonary lavage as long as 8 months after a single brief exposure to radioactive particles. It is doubtful that the particles remained in the alveolar region for that long. Rather, their presence in the lung and lung lavage fluid many months after a brief inhalation exposure probably reflected continued movement of macrophages carrying particles between the alveoli and interstitial lung tissue. The Lovelace ITRI studies demonstrated that typically 40%–60% of the initial lung burden of radioactive particles could be removed by lavage if the procedure was used soon after the inhalation exposure. Moreover, the use of chelation therapy increased the removal of the radionuclides that had become solubilized. The risk of radiation-induced disease from the inhaled radionuclides was reduced proportionally.

A worker from the Rocky Flats facility was exposed to a ^{239}Pu aerosol in an accident when a container exploded and dispersed the ^{239}Pu into the laboratory. The worker was aware of the research conducted at Lovelace with the BAL procedure. He and his physician requested that he be treated in Albuquerque, New Mexico, with BAL and intravenous Zn-DTPA. It was estimated that his lungs contained about 0.5 μCi of ^{239}Pu. To complement the Lovelace team of scientists and physicians, a physician and an anesthesiologist from Duke University Medical Center, who routinely performed BAL on children to treat cystic fibrosis and alveolar proteinosis, were added to the team. One lung was subjected to BAL followed 2 days later by BAL of the other lung and then on the fourth day BAL of the lung initially washed. In addition, DTPA was given intravenously. The BAL fluid that was recovered was straw colored and contained many macrophages with engulfed ^{239}Pu. Many macrophages found in lung fluid also contained debris characteristically recovered from the lungs of smokers or recent former smokers. This patient indicated he had been a smoker. A total of about 30% of the initial lung burden of ^{239}Pu was recovered in the BAL fluid and urine. The use of BAL in treating humans accidentally exposed to plutonium has been reviewed (Dean 1997; Morgan et al. 2010). Any use of the BAL procedure and Zn-DTPA, as with any medical procedure, requires an evaluation of the potential risks and benefits to the patient of reduced risk of radiation-induced diseases. The greatest risks associated with the use of the procedure are the risks associated with general anesthesia, a common medical procedure.

RADIATION HORMESIS

Thus far in this chapter, primary consideration has been given to two fundamentally different dose–response relationships for radiation: the sigmoid curve with a threshold usually associated with deterministic health outcomes and the linear no-threshold (LNT) model used to describe stochastic effects such as cancer and hereditable effects. The LNT model has been accepted as the model of choice for radiation protection purposes by the BEIR Committee, the NCRP, and the ICRP.

An alternative model that has received substantial attention invokes the concept of hormesis, derived from the Greek word *hormaein*, to excite. The concept of hormesis had its roots in the cellular pathology studies of Virchow in the nineteenth century. The hormesis hypothesis, which is not restricted to radiation, states that low-level stress from different agents or environments stimulates a system of protective biological processes at the cellular, molecular, and organismic levels, decreasing the incidence of cancer and other deleterious health effects below the spontaneous level. In short, adaptive responses such as antioxidant defenses, enzymatic repair of DNA, removal of DNA lesions, apoptosis, and immunologic stimulation are triggered by low doses of various agents, while the harmful stochastic effects, such as cancer, are observed at higher doses.

The potential for radiation having hormetic effects was advanced soon after the discovery of x-rays (Atkinson 1898) and was at least partially the basis for the public flocking to radon spas for their alleged health benefits in the early 1900s and, indeed still today, in some parts of the world. As already noted, the Manhattan Project stimulated the conduct of many studies of radiation effects on many health endpoints, including LSS. One of the earliest was by Lorenz and colleagues at the National Cancer Institute with mice, which suggested that protracted exposure to low doses of radiation increased life span compared with the nonirradiated controls (Lorenz 1950; Lorenz et al. 1955). Other investigators soon reported similar results in mice and rats (Carlson et al. 1957; Brown et al. 1963). In other cases, attempts to reproduce the hormetic effects were unsuccessful (Bustad et al. 1965a). Indeed, it is noteworthy that the life span of the Japanese atomic bomb survivors with doses of less than 149 mGy was reported to be prolonged (Mine et al. 1990). Colonge and Preston (2000) provide more recent evaluation of the longevity of atomic bomb survivors. There has been an increased interest in radiation hormesis in recent decades (Luckey 1980, 1991, 2007; Calabrese 1992, 2011; Calabrese et al. 1999; Calabrese and Baldwin 2000, 2003; Upton 2001; Scott 2005, 2007, 2008; Sanders 2010). Readers interested in exploring this topic in detail will find the 217-page book by Sanders especially useful. Much of the debate has centered on the estimation of risks for low dose rate and low dose in setting radiation protection standards and criteria for cleanup of radioactively

contaminated sites (Little et al. 2009; Tubiana et al. 2009; Feinendegen et al. 2011). This issue was the basis of a conference I coorganized with sponsorship by the Council of Scientific Society Presidents (CSSP) (1998) to create a strategy for developing a science-based policy approach to addressing conflicting views on assessing the health risks of low-level exposure to ionizing radiation. The CSSP conference, in turn, had major influence on the creation of the U.S. DOE's Low-Dose Radiation Research Program that continued for about a decade (1998–2008) exploring doses below about 100 mSv. The program emphasized a multiparametric approach extending from molecular-level observations to those at the level of the mammalian organism (Brooks 2005; Brooks and Couch 2007; Dauer et al. 2010). Additional information on the DOE Low-Dose Program can be found at http://www.lowdose.energy.gov.

In recent years, the debate over the use of the LNT model in radiation risk assessment and standard setting has been extended into the chemical toxicology risk assessment and standard setting arenas. The argument for hormesis is based on the premise that the LNT routinely overestimates health risks for cancers induced by low-LET radiation exposure, especially for low dose rate and low total doses. It further acknowledges that at high doses, ionizing radiation and certain chemicals (or their metabolites) that damage DNA may share common mechanisms in producing cancer. The hormesis argument is that these mechanisms not be operative with low-level chronic exposure to chemicals and ignore repair mechanisms; thus, extrapolation of the LNT model based on radiation experience to assess the risks of chemical exposures is not appropriate. Abelson (1994), a longtime editor of *Science*, addressed this issue in one of his classic editorials:

> To calculate effects of small doses, a linear extrapolation from large doses to zero is employed. The routine use of this procedure implies that the pathways of metabolism of large doses and small doses are identical. It implies that mammals have no defense against effects that injure DNA. It implies that no dose, however small, is safe. Examples of instances in which these assumptions are invalid become numerous …. The use of linear extrapolation from huge doses to zero implies that one molecular event can cause cancer. This assertion disregards the fact of natural large-scale repair of damaged DNA.

Sanders (2010) reviews substantial data to support Abelson's argument (1994). Sanders' arguments use data extending from the molecular level to intact organisms to populations of laboratory animals and people. He notes the adult human body contains more than 100 trillion cells of about 100 cell types. With 10^{11} cell divisions a day, about 10^{16} new cells are created over a 70-year life span. All of these cells are attacked by reactive oxygen species a million times a day; however, the chance of one of those cells ultimately undergoing genetic changes such that its cell progeny leads to a lethal cancer during a lifetime is about 25%. Table 18.22 provides a comparison of DNA damage from reactive oxygen species and natural background of about 2 mSv/year.

TABLE 18.22
Comparison of DNA Damage from Reactive Oxygen Species and a Background Level of 2 mSv/year

Type of Damage	Spontaneous ROS	2 mSv/year
DNA oxidative adducts/cell/day (Pollycove 2002)	10^6	1×10^{-3}
DNA damage/cell/year (Billen 1990)	70×10^6	4
Double-stranded DNA breaks/cell/year (Stewart 1999)	40	~0.1

Source: Reprinted with permission from Sanders, C.L., *Radiation Hormesis and the Linear-No-Threshold Assumption*, Springer-Verlag, Berlin/Heidelberg, Germany, 2010, Table 2.1.

The author of this chapter has been an ardent proponent of the concept of hormesis beginning with admonishment from two grandmothers: "Everything in moderation, avoid excesses." That common sense view was reinforced by my experience as a student in veterinary medicine studying nutritional diseases and learning that every vitamin and trace element has an optimum intake with both deficiencies and excesses causing disease. Later, as a scientist studying radiation and chemicals, I was struck by variable responses to exposure to low-level radiation, especially on life span. However, in my view, the variable hormetic responses for different endpoints to various agents and among varied populations create a dilemma for devising a scheme for developing protective guidance for radiation and chemicals that takes account of hormesis. Indeed, to date despite much-heated discussion over hermetic responses for both radiation and chemicals, no one has proposed a specific strategy for incorporating the consideration of hormesis in a regulatory system to protect against the risks of either radiation or chemical exposure.

MEDICAL CONSEQUENCES OF NUCLEAR ACCIDENTS

Mettler and Upton (2008) in their book, *Medical Effects of Ionizing Radiation*, provide an excellent review of the medical consequences of a number of accidents involving radiation exposure, including the Three Mile Island and Chernobyl accidents in the context of the knowledge about the acute and chronic effects of radiation. The insights of Fred A. Mettler are especially valuable since he participated in the medical care and follow-up of the Chernobyl first responders. Included in their book is a chapter on "Perceptions of radiation and psychological effects." Fear of radiation, that is, radiation phobia, is a real phenomenon that was clearly evident in all the accidents, irrespective of the magnitude of the radioactive releases. Moreover, it has been recognized as a real phenomenon for years as the public reacts to announcements of potential sitings of nuclear facilities and radioactive waste sites (Slovic 1987). Bromet (2013) has recently reviewed the emotional consequences of nuclear power plant disasters.

The Three Mile Island accident, as noted earlier, involved a minimal release (Table 18.4). As noted by Mettler and Upton (2008), the image of an accident was fueled by media coverage. "The maximum radiation dose at the plant boundary for the course of the entire 'accident' was less than 1 mGy, substantially less than the dose from an abdominal x-ray." Baum et al. (1983) have chronicled the emotional and psychological response of the incident of the Three Mile Island incident. Talbott et al. (2003) reported no untoward health responses in 20 years of follow-up of residents who lived near Three Mile Island.

A comprehensive review of the medical consequences of the Chernobyl accident has been published (WHO 2006) and summarized by Mettler and Upton (2008). They noted, "The mental health impact of Chernobyl is the largest public health problem caused by the accident to date," while also noting it was, by far, the worst industrial disaster on record. The extraordinary quantities of radioactive material released in the Chernobyl accident were noted earlier. When the author visited Kiev, near Chernobyl, in the fall of 1986, the enhanced level of radioactivity in the environment was clearly evident. However, to provide context, the readily measurable increase in background radiation levels was equivalent to that experienced by a resident of Chicago, Illinois, moving to Denver, Colorado. Cardis et al. (2005) have reported an excess risk of thyroid cancers in a case–control study with 276 cases of childhood (less than 15 years of age at the time of the accident) thyroid cancers compared with 1300 control cases. A few cases received over 2 Gy with most of the cases receiving less than 0.5 Gy. The ERR per gray was on the order of 5–10. Using information on increased background radiation levels across Europe attributed to the Chernobyl accident, Cardis et al. (2006) estimate that the accident may have resulted in 2400 excess leukemia cases and 1650 excess leukemia deaths, an attributable fraction of 0.04%. As Mettler and Upton (2008) noted it would not be possible to detect this small of a hypothetical increase.

The Fukushima accident in Japan beginning on March 11, 2011, was an extraordinarily complex event triggered by an earthquake and a tsunami that caused huge loss of life (over 20,500 killed), property damage, and relocation of large populations (over 320,000 people) whose homes, schools, and places of work were destroyed. Ohnishi (2012), Yamashita (2013), and WHO (2013) have described the disaster. Laid on top of this was the release of a large quantity of radioactivity from the severely damaged nuclear station (Table 18.4). The actions of Japanese officials and the local populations were exemplary, resulting in a substantial reduction of potential radiation exposure from the passing cloud of radioactive material, ingestion of radioactively contaminated foodstuffs, and external radiation exposure originating from deposited radioactivity. The response to the accident was a testimonial to the disciplined character of the Japanese people.

The WHO (2013) has developed preliminary dose estimates for populations exposed as a result of the Fukushima nuclear accident associated with the 2011 Great East Japan earthquake and tsunami. The results are reassuring. The estimated maximum thyroid effective dose was 35 mSv, 26 individuals received 16–35 mSv, and 1054 received less than 15 mSv. In contrast, the mean thyroid dose for 1576 cases following the Chernobyl accident was 490 mSv (Cardis et al. 2005). The estimated external exposure doses for Fukushima are also low, with very few in the range of 5–15 mSv and most less than 1 mSv. Based on these dose estimates, it is very unlikely that an associated increase in cancer will be observed. It is especially noteworthy that a comprehensive medical follow-up plan has already been initiated. As anticipated, whenever a population is given detailed medical examinations, an increase will be observed in the number of medical conditions observed irrespective of their origin. It is quite possible that a positive outcome of the program will be increased diagnosis and treatment of health conditions that would have otherwise not been observed until later and possibly gone untreated.

Boice (2012), a very experienced epidemiologist who has conducted many studies on populations exposed to radiation and currently serves as the president of the NCRP, has offered a useful perspective on the Fukushima accident. He noted that

> Studies of the Fukushima population should be and are being considered for reassurance and health care reasons. Apart from the extreme psychological stress caused by the horrific loss of life following the tsunami and the large-scale evacuation from homes and villages, such studies have limited to no chance of providing information on possible health risks following low dose exposures received gradually over time – the estimated doses (to date) are just too small.

RADIATION PROTECTION STANDARDS

Development of Radiation Protection Standards

Relatively soon after the discovery of radiation and radioactivity and recognition that radiation could produce harmful effects, action was taken to develop guidance to limit adverse health effects in the people using radiation and radioactivity. The first actions were voluntary and involved defining levels of exposure to x-rays that would or would not harm the skin: in short, the identification of a threshold dose. In 1902, Rollins proposed a tolerance dose: if a photographic plate is not fogged in 7 min, the radiation is not of harmful intensity. This represented the first association of an exposure or dose metric with a health outcome. Guidelines evolved most rapidly in Great Britain, which quickly embraced the use of radiation in the practice of medicine. It developed relatively comprehensive radiation protection guidelines as early as 1915.

Radiologists, medical doctors who specialized in using radiation in their practice of medicine, took a leadership role in creating international radiation protection criteria. This was also an era in which the disciplines of medical physics and radiological physics were developing with individuals trained in physics applying their expertise to medical issues. Different methods for measuring the intensity of x-rays were being advanced, and most importantly, concern was

increasing for the harmful effects of radiation. The British Institute of Radiology was one of the first professional societies to focus on these issues. In 1925, it invited delegates from a number of countries to the *First International Congress of Radiology*, which was convened in London. At the *Second International Congress of Radiology* in Stockholm in 1928, representatives from a number of countries met to prepare x-ray protection guidelines. This meeting resulted in the creation of the International X-Ray and Radium Protection Committee. After World War II, this committee evolved into the ICRP and the International Commission on Radiation Units and Measurements that continue today.

Lauriston Taylor, an employee of what was the U.S. National Bureau of Standards (NBS), attended the 1928 meeting in Stockholm. He brought the radiation protection criteria that had been agreed to at Stockholm back to the United States. Under Taylor's leadership, the advisory committee on X-Ray and Radium Protection was created under the auspices of the NBS. In 1946, the advisory committee was renamed the NCRP. In 1964, the NCRP received a U.S. Congressional charter to serve as an independent body to provide advice and recommendations on radiation protection matters in the United States. The NCRP continues today as an independent body governed by a self-elected council of 60 members. The author served as a member of the NCRP Council from 1971 to 2001 and has been designated as a distinguished emeritus member. The various reports prepared and issued by the NCRP have significantly impacted radiation protection in the United States. As of late 2012, the NCRP had issued 173 authoritative reports offering advice and guidance on a wide range of radiation protection topics germane to its mission. However, it is important to recognize that various laws have delegated legal responsibility for radioactive protection matters in the United States to the Nuclear Regulatory Commission, the Environmental Protection Agency, the Food and Drug Administration, the DOE, and state and local agencies.

It is noteworthy that various advisory committees of the NRC, most notably a continuing series of committees, have had a major role in the United States in interpreting literature on the health effects of ionizing radiation. These reports have had substantial influence on U.S. government agencies, especially on the Environmental Protection Agency after it was created. The first NRC report (NRC 1999) was a critique of the first UNSCEAR report (UNSCEAR 1958), which was soon followed by the Biological Effects of Atomic Radiation (BEAR) report (NRC 1960). The BEIR Committee has issued four reports focused on the effects of exposure to low levels of ionizing radiation (NRC 1972, 1980, 1990, 2006). In addition, the BEIR Committee issued a report on radon and other internally deposited alpha-emitting radionuclides (NRC 1988) and a seminal report later that was exclusively concerned with estimating the risks of residential exposure to radon (NRC 1999).

Evolution of a Radiation Protection System

As noted earlier, both U.S. national and international systems to guide radiation protection activities evolved rapidly in the 1920s and 1930s and was in place at the beginning of World War II. Taylor (1981) reviewed these activities in detail for the period from 1928 to 1974. Hendee (1993) and Jones (2005) have provided useful reviews of the history of radiation protection standards. As noted, a cornerstone of early radiation protection guidance was the concept of a tolerance dose. Cantril and Parker (1945) prepared a document dated January 5, 1945, intended to describe for workers at the Hanford site and other Manhattan Project sites the health risks of exposure to radiation and radionuclides. The document was intended for release before the start-up of the Hanford reactors in September 1944 and the beginning of fuel reprocessing in December 1944; however, release was delayed related to security classification concerns. In any event, the document was released coincidently with the start of major fuel reprocessing at Hanford and the associated handling of large quantities of fission product radionuclides and plutonium. As an aside, the radiation protection activities at Hanford, Oak Ridge, and Los Alamos proved effective as judged by the lack of observed radiation effects in workers. This stands in sharp contrast to the Mayak experience discussed earlier.

Herbert M. Parker, a radiological physicist trained in England, came to the United States in the late 1930s to work with Seymour T. Cantril, a radiation oncologist at Swedish Hospital in Seattle, Washington. Parker would later assume responsibility for developing and managing the radiation protection program at Hanford where I had the opportunity to work under him. Cantril and Parker provided remarkable insight into key issues concerning radiation risks that remain today and, most importantly, extend to nonradiation materials. A quote from the Cantril and Parker document is germane:

> In reviewing the subject of tolerance dose, it is most striking that animal experimental evidence has played practically no part in arriving at present day levels. In summary, there are only three tolerance levels, which have been established and accepted as a working basis for occupational exposure.
>
> 0.1 r per day for external X and gamma radiation
> 1×10^{-14} curie/cc for radon in the air of working rooms
> 0.1 μg of radium as the maximum allowable amount deposited in the body of a radium dial painter
>
> Each of these levels has been established by adding a safety factor to the amount, which has been known to produce lasting injury to persons so exposed. It is of interest also to note that in each case the factor does not exceed 10, and is more likely considerably less than 10. Human misfortune rather than animal experimentation were the basis for these levels.

In the same document, Cantril and Parker (1945) discuss LNT and threshold dose–response relationships (recall Figure 18.3), drawing heavily on Henshaw's earlier discussion of this topic (Henshaw 1941). Discussion and debate over the use of these two different dose–response relationships for exposure to ionizing radiation and chemicals continue today.

As discussed earlier, the Manhattan Project included initiation of a substantial research program on the health and

environmental effects of external radiation exposure and internally deposited radionuclides. This program was substantially expanded in the United States by the AEC. Similar, but more modest, research programs were initiated in the United Kingdom, France, the Soviet Union, and yet later in Japan. Substantial advances were made in understanding heritable and somatic effects of radiation in humans and the movement of radionuclides in the environment during the late 1940s and 1950s.

The NCRP in 1953 published Handbook 52, "Maximum permissible amounts of radioisotopes in the human body and maximum permissible concentrations in air and water" (NCRP 1953). These standards were intended primarily for nuclear workers; however, the report stated that the permissible concentrations were *for use beyond the control area*. NCRP Handbook 59 released in 1954 recommended that "in the course of their normal activities, protective measures be taken to make sure that no minor actually receives a weekly rate higher than one-tenth the respective permissible weekly doses for the critical organs" (NCRP 1954). In 1957, the NCRP issued an addendum to Handbook 59. It specified an occupational dose rate exposure limit of 15 rem per year, but the maximum accumulated dose was limited to five times the number of years beyond age 18 (NCRP 1957). This was adapted out of concern for what were then viewed as genetic effects, now more appropriately viewed as heritable effects. With regard to internally deposited radionuclides, the report recommended: "For individuals outside of the controlled area, the maximum permissible concentrations should be one-tenth of those for occupational exposure" (NCRP 1957). It also identified a per capita dose limit of 0.5 rem outside the control area for radiation from a controlled area. Out of concern for heritable effects, it noted that radiation exposure should be limited to a small portion of the total population.

NCRP Handbook 69 (NCRP 1959) further noted that radiation or radioactivity outside of a control area, attributable to control area operations, shall be such that it is improbable that any individual will receive a dose of more than 0.5 rem in any year from external radiation. It also noted that the maximum permissible body burden of radionuclides in persons outside the control area and attributable to the operations within the control area should not exceed one-tenth that for radiation workers. This included for internally deposited radionuclides the identification of critical organs based on the organs receiving the highest radiation dose from a particular radionuclide and chemical form.

In 1960, the NCRP (1960a,b) published a report of an ad hoc committee that stated, "We recommend the population permissible dose for man-made radiation be based on natural background level." They went on to note, "Although it is not our responsibility to determine the exact level, we believe that the population permissible somatic dose for man-made radiation, excluding medical and dental sources, should not be larger than that due to natural background radiation, without a careful examination of the reasons for, and the expected benefits to, society for a larger dose." The report assumed a background dose rate of 100 mrem per year.

These interrelated recommendations explicitly rejected the concept of a tolerance dose and advanced the idea of acceptable risk in contrast with no risk at some low level of radiation exposure. The move to adoption of an LNT predictive model of radiation injury for exposed populations had begun.

In 1958, the UNSCEAR (UNSCEAR 1958) issued its first report on the effects of radiation exposures on humans. It estimated the adverse effects of low-level radiation exposure using both the threshold and no-threshold models of radiation-induced risk. The report noted the difficulty of estimating with any precision the possible consequences of radiation exposure at low levels. It specifically noted the delayed effects of exposure and that the effects were not distinguishable from those produced by radiation occurring spontaneously or produced by other agents. Those who drafted the report were aware of the appearance of radiation-induced leukemia in the Japanese atomic bomb survivors who had received substantial radiation doses. UNSCEAR (1958) concluded that both the threshold and nonthreshold values had equal validity. The NRC Committee on Pathologic Effects of Atomic Radiation offered a commentary taking exception to the UNSCEAR conclusions and urged that greater attention be given to nonlinear relationships between dose and effect (NRC 1959). Later, UNSCEAR reports would emphasize that extrapolation of the LNT curve to low levels provided an upper limit to the risk of low-level exposures (UNSCEAR 1962, 1964). The ICRP endorsed this position (ICRP 1966) and reemphasized that viewpoint in its most recent report (ICRP 2007). The controversy over estimating radiation risks at low dose levels sparked substantial public interactions during a period in which concern was increasing about the effects of radioactive fallout from nuclear weapons tests. This was also a time period when tensions increased over permissible levels of radiation exposure for medical applications, with clear benefits to the individual, and radiation exposure from fallout and other nuclear operations without the exposed population receiving any benefits.

H.M. Parker (1960), in his congressional testimony, offered some useful insights on some aspects of the controversy: "In the period between 1945 and 1950, the information available on the genetic effects and the increasing suspicion that not all somatic effects of radiation were of a threshold type (i.e., a type that requires a minimum dose before manifestation) led to the change in terminology from 'tolerance dose' to the present 'maximum permissible dose.'" It was at this point that the concept of acceptable risk appeared.

I am convinced that the terminology was changed from one form to the other specifically to underline the acceptance of a no-threshold-dose concept for the production of gene mutations by radiation (p. 26 et seq. of handbook 59) (NBS 1959). From a language point of view, one could equally well have changed from a *maximum permissible dose* to *tolerable dose* or *tolerance dose*. The intent to change to an *acceptable risk* line of reasoning was partially obscured by an arbitrary choice of words.

The fundamental change in approach at this point has not always been sufficiently emphasized. The earlier clinical observations clearly pointed to the existence of threshold effects and perhaps conditioned observers to expect all deleterious effects to have a threshold. It was, for example, probably impossible to produce reddening of the skin with less than some prescribed dose. If such a threshold applied to every radiation effect, an intelligent, wholly technical, search could be made to define such thresholds with precision, and persons exposed to lesser amounts would be protected in the fullest sense.

If there is no threshold dose, there is no absolutely safe dose in the same sense. The determination of permissible limits then involves value judgments outside the areas of scientific and technical competence. It is essential that qualified observers recognize this limitation and reexamine their own role. I believe that it is equally essential that these same observers continue to play a basic role in recommending standards. This follows from the principle of better acceptance of standards having the authority of knowledge and from one further much-overlooked factor, that is, that although present-day standards appear to be established in detail by the considerations of physicists, biologists, and other scientists, they are basically validated by clinical experience. This experience does go beyond reproducible science and does include value judgments in accordance with the Hippocratic oath. Because some aspects of the genetic effects are beyond clinical observation, there is a needed separate voice of authority from geneticists. Then, because the decisions are necessarily based on cultural reactions to human welfare, many generations hence, the whole structure of ethical opinion must be included in some fashion.

The firm resolution of the evidence concerning the response at a very low dose rate and low total dose as to whether relevant radiation effects are threshold, nonthreshold and linear, or nonthreshold and nonlinear is the most important factor in establishing the authority of knowledge for radiation standards.

There is yet no complete definitive, scientific answer to these questions. The assumption of a nonthreshold type of response for somatic as well as genetic effects in setting limits, while plausible, is an assumption, and numerical estimates of effects calculated on this basis must be treated with reservation.

It is frequently indicated that the assumption of a nonthreshold response is a *safe* assumption. This can be followed by a stipulation that permissible exposures are zero. However, there are other consequences to the nation as a whole from eliminating radiation or radiation exposures. These consequences can be expressed in terms of a limitation in our ability to attain the many benefits that radiation can bring. In essence, then, the risk principle states that radiation exposure and the potential damage from radiation should be balanced against the benefits of radiation and the limits set at some level where the optimal benefits are attained, as compared with the losses. "To establish such limits for radiation and equally carefully for any other agent or influence on the national life, projected over an indefinite number of generations, is a Herculean task" (Parker 1960, pp. 4–6).

The testimony that Parker gave in 1960 is equally relevant today, more than one-half century later. The only difference is that if it were given today, it would have more emphasis on other agents, that is, chemicals, and a growing list of health endpoints in addition to cancer.

Out of the controversy of overestimating or underestimating radiation risks at low levels, the NRC Committee on BEAR emerged (NRC 1959). That committee would soon be renamed the Committee on Biological Effects of Ionizing Radiation (BEIR) and issue its first report in 1972 (NRC 1972). That report included estimates of cancer risk at low doses based on linear extrapolation from cancer mortality at high doses from the studies of the Japanese atomic bomb survivors and other groups. In this same time period, the AEC introduced the concept of *as low as reasonably achievable*, an approach advocated by Edward Lewis in the 1960 Congressional hearing. The concept implies that no threshold exists for radiation-induced disease and that exposures should be held as low as reasonably achievable to minimize any radiation-induced harm.

Parker (1976) later addressed the use of radiation as a prototypical toxic agent for considering the health risks of other agents:

> First, the case could be solved and safety extrapolated to low exposures if a generalized theory of the deleterious actions of the agent could be developed. This must be attempted for any new agent. Then a broader umbrella should be developed for classes of agents, if it exists technically. Short of these unifications, the problems of each new agent might be looked at as follows: Make an early attempt to determine whether the relevant risks have a threshold or not. In doing this, develop measurement systems that are unambiguous and acceptable to all. If animal data are going to be discarded because the only relevant subject is man himself, review early whether animal research is needed at all. If comprehensive theory for the particular agent is compatible with a hazard threshold or even a practical threshold for low exposure (due to latency period inversely related to exposure), there is an acceptable technological standard to be found. If not, the eventual standard involves value judgments. The efforts of the scientific fraternity will never be sufficient in themselves. The key element is to identify the plausible effects as early as possible and to promote informed public discussions as early as possible. (p. 40)

Much of the early radiation health effects research was concerned with developing a scientific basis for setting exposure limits. As Parker noted, the approach shifted over time from *tolerance dose* to *permissible dose* to *acceptable risk*. He appropriately noted that this introduced *value judgments* that go beyond scientific and technical competence. At this juncture, it is important to emphasize that the ICRP and NCRP are voluntary, nongovernmental bodies comprised principally of scientists, including medically trained personnel. The same can be noted for the NRC BEIR Committees. Their competence in evaluating scientific data that informs public policy decisions is above reproach. To be explicit, they can estimate the risks at differing radiation doses; however,

a legitimate question remains as to whether these groups are appropriately constituted to decide for society at large an acceptable level of risk that is associated with a particular radiation dose from a given source or all sources.

In 1977, the ICRP announced its clear shift to a risk-based approach to the establishment of standards for radiation protection (ICRP 1977). The ICRP's approach was to define the concept of acceptable risk from radiation exposure of workers by relating it to the fatal accident rate in so-called safe industries. Later, the NCRP (1987) would adopt a similar approach.

One stimulus to moving to an *acceptable risk* orientation was the need to become quantitative in estimating health risks as noted by Albert (1994). Another stimulus for the development of quantitative estimates of risk was the increasing attention given in the 1960s to the proactive conduct of *consequence studies*. Some of the earliest *consequence studies* were concerned with nuclear war scenarios and nuclear power reactor accidents. Others were a spin-off of studies related to the space program and included the consequences of failure of space systems that utilized nuclear reactors or radionuclide power sources fueled with plutonium or other radionuclides. One prominent consequence study dealt with nuclear power reactors and was conducted under the leadership of Norman Rasmussen of MIT (NRC 1975). Another major consequence study during the 1970s was concerned with the development of a breeder or plutonium recycle reactor (Cuddihy et al. 1977).

While the discussion here focuses on radiation protection guidelines, it is important to recognize that the developments concerning radiation were taking place on a larger stage. The 1950s and 1960s were a time of increasing concern for environmental quality and related impacts on human health. In many ways, Rachael Carson's book, *Silent Spring* (1962), served as a catalyst for public demands for change and regulation. The National Environmental Policy Act (NEPA) of 1969 signed into law by President Richard Nixon on January 1, 1970, in many ways, provided a foundation for a broad range of additional actions, including Nixon's creation of the U.S. Environmental Protection Agency by executive order and passage of the Clean Air and Clean Water Acts. It is noteworthy that the U.S. Environmental Protection Agency charged with administering the Clean Air Act found it useful to draw on the radiation experience in modeling dose–response relationships as it dealt with assessing the cancer risk of exposures to hazardous air pollutants (Albert 1994).

A key element of NEPA was a requirement that all federal agencies prepare environmental assessments and environmental impact statements for proposed major activity, including those involving the use of federal funds and permits issued by federal agencies. As a result, the application of NEPA and the development of environmental assessments and environmental impact statements extended to many state and local projects.

From the foregoing, it is apparent that the need for quantitative estimates of the potency of radiation for causing a range of health effects, especially cancer, was coming from two interrelated activities. The first need was to provide a scientific basis for setting radiation protection standards. The second was the need related to conducting consequence studies such as those required to meet NEPA requirements.

The consequence studies involving nuclear facilities from the very beginning were comprehensive in extending from sources of radiation and radioactivity to the transport and fate of radionuclides in the environment to the exposure of individuals, the internal dose to various tissues, and finally the estimated health consequences. This comprehensive structure is apparent in the schematic rendering presented earlier in Figures 18.1 and 18.3. With radioactive material, the distinction between external exposure and internal dose is obvious, a distinction that has also become apparent for chemical agents. It is fortunate when dealing with radiation and radionuclides that metrics for ionization exist that can be used to describe exposure and dose. Unfortunately, common dose metrics do not exist for describing dose across a diverse array of chemicals. All of the nuclear facility *consequence studies* required as input some estimate of the potency of radiation for producing deleterious effects and especially solid cancers and leukemia. The textbook edited by Till and Grogan (2008) provides a number of examples of *consequence studies* conducted to guide remediation activities at various nuclear sites. Till (2013), in his Lauriston S. Taylor lecture, elaborated on the key elements of the large number of dose reconstruction activities he and his colleagues have participated in as risk assessment evolved over the past six decades.

The NRC BEIR III Committee (1980) issued a report endorsing the use of an LQ model of radiation-induced cancer. An LQ model predicts that the cancer risks of low-level radiation exposures are less than predicted by a strictly linear model extrapolating from high doses. The report noted that developmental effects, a deterministic effect, from irradiation in utero may exhibit a threshold. It indicated that an LNT model provided the best estimate of genetic risk from radiation exposure. The report did note that it was unknown and probably not determinable whether dose rates on the order of 1 mSv per year, on the order of dose rates from background radiation, were detrimental to people. More recently, the NRC BEIR VII Committee (BEIR VII 2006) endorsed the use of a linear, no-threshold model to describe the relationship between radiation dose and excessive cancer risk for brief exposures to whole-body irradiation.

Current Radiation Protection System

Very early in the development of information on the effects of externally delivered radiation exposure, internally deposited radionuclides, and a related system of radiation protection, it became apparent that it would be very difficult to consider separately each type of radiation and radionuclide. An attractive alternative was at hand, taking advantage of the fact that all kinds of radiation and radionuclides produce biological effects by ionizing tissue. The approach that has evolved is to cover exposures, both external irradiation and from internally deposited radionuclides, from all sources in a holistic manner with absorbed tissue dose as the common metric.

Preston (2004) has provided a concise review of the radiobiological concepts that undergive radiation protection. Key elements of the system developed by the ICRP are presented in its reports (ICRP 1966, 1977, 1999, 2007).

A central element of the dose-based radiation protection scheme is the use of W_R, tissue-weighting factor (W_T), and a dose and dose rate effectiveness factor (DDREF) to allow calculation of effective dose expressed as Sv as a critical intermediate step to estimating total detriment from cancer and heritable effects.

The 2007 ICRP recommendations for W_R are shown in Table 18.23. The W_R factors are based on the RBE of the radiation of interest, to the absorbed dose in an organ or tissue in order to derive the equivalent dose. In simple terms, the system assumes that with the same ionizing tissue dose, alpha particles are 20 times as effective as gamma photons. The system uses W_T factors for the principal tissues and organs to calculate effective dose. The ICRP-recommended W_T factors are shown in Table 18.24. The W_T factors represent the relative contribution of each tissue or organ to the total detriment resulting from uniform irradiation of the whole body.

The DDREF is defined as the factor by which cancer risks observed after large acute doses should be reduced when the radiation is delivered at a low dose rate or in a series of small fractions. For stochastic effects, the ICRP recommends a DDREF of 2 for deriving nominal risk coefficients for low doses and dose rates of low-LET radiation. The BEIR VII (BEIR VII 2006) Committee recommended a DDREF of 1.5. Note that the DDREF combines both dose and dose rate. In my opinion, consideration should be given to separately identifying a dose effectiveness factor (DEF) and dose rate effectiveness factor (DREF). Based on the evidence reviewed earlier on the occurrence of cancer associated with exposure to low-LET beta irradiation, a DREF as high as 4 would be appropriate. Raabe (2011) has also emphasized the need for change in considering the effectiveness of low-dose-rate exposures. Brooks et al. (2009) have also commented on the need to separate the DREF from the DDREF for internally deposited radionuclides for which the dose rate is typically low and the dose protracted.

The ICRP system distinguishes between deterministic effects and stochastic effects (recall Figure 18.4). Deterministic effects such as the development of a radiation-induced cataract of the eye are assumed to have a threshold dose below which they are not manifest. Above that dose, the severity of the effect is assumed to increase with increasing dose. Stochastic effects are radiation-induced effects such as cancer and heritable effects that increase in proportion to increasing dose without any change in the severity of the effect. The ICRP retains its fundamental hypothesis that the induction of stochastic effects is a linear function of absorbed dose without a threshold. The current ICRP (ICRP 2007; Wrixon 2008) recommendations for detriment-adjusted nominal risk coefficients for stochastic effects after exposure to radiation at low dose rate (10^{-2} Sv^{-1}) are shown in Table 18.25. The 2007 values are based on cancer incidence weighted for lethality and life impairment, whereas the 1990 values were based upon fatal cancer risk weighted for nonfatal cancer, relative life years lost for fatal cancers, and life impairment for nonfatal cancers. Note that while the cancer risk coefficients changed very little from 1990 to 2007, the risk coefficients for heritable effects were substantially reduced. Wrixon (2008) noted, "The decimal places in the table are not intended to indicate a high level of precision; they are simply the outcome of ICRP calculations." For comparison purposes, the NCRP (1993)-recommended dose limits are also shown in Table 18.25.

The ICRP (2007; Wrixon 2008)-recommended dose limits for planned exposure situations are also shown in Table 18.25. Note that the effective dose, driven by concern for stochastic effects, principally radiation-induced cancer, is different by a factor of 20 for occupational exposures versus population exposures. In contrast, the deterministic effects limits differ by a factor of 10 between occupational exposures versus population exposures. In considering these dose limits, it should be noted that they are provided

TABLE 18.23
Recommended Radiation-Weighting Factors (W_R)[a]

Radiation Type	Radiation-Weighting Factor (WR)
Photons	1
Electrons and muons	1
Protons and charged pions	2
Alpha particles, fission fragments, heavy ions	20
Neutrons	A continuous function of energy

Source: ICRP (International Commission on Radiological Protection), The 2007 Recommendations of the International Commission on Radiological Protection, ICRP Publication 103, 2007.

[a] W_R are used to adjust the absorbed dose in an organ or tissue to derive the equivalent dose.

TABLE 18.24
Recommended Tissue-Weighting Factors (W_T)[a]

Tissue	W_T	Sum of W_T Values
Bone marrow (red), colon, lung, stomach, breast, remainder tissues[b]	0.12	0.72
Gonads	0.08	0.08
Bladder, esophagus, liver, thyroid	0.04	0.16
Bone surface, brain, salivary glands, skin	0.01	0.04
Total		1.00

Source: ICRP (International Commission on Radiological Protection), The 2007 Recommendations of the International Commission on Radiological Protection, ICRP Publication 103, 2007.

[a] Used to derive the effective dose.

[b] Remainder tissues: adrenals, extrathoracic (ET) region, gall bladder, heart, kidneys, lymphatic nodes, muscle, oral mucosa, pancreas, prostate (♂), small intestine, spleen, thymus, uterus/cervix (♀).

TABLE 18.25
Recommended Dose Limits (mSv in a Year)

Type of Limit	ICRP	NCRP
A. *Occupational exposure*		
Stochastic effects		
Effective dose limit (cumulative)	20 mSv/year averaged over 5 years, not to exceed 50 mSv in any one year	10 mSv × age
Annual	50 mSv/year	50 mSv/year
Deterministic effects		
Dose equivalent limits for tissues and organs (annual)		
Lens of eye	150 mSv/year	150 mSv/year
Skin, hands, and feet	500 mSv/year	500 mSv/year
B. *Embryo/fetus exposure*		
Effective dose limit after pregnancy is declared	0.5 mSv/month	Total of 1 mSv to abdomen surface
C. *Public exposure (annual)*		
Effective dose limit, continuous or frequent exposure	No distinction between frequent and infrequent—1 mSv/year	1 mSv/year 5 mSv/year
Effective dose limits, infrequent exposure		
Dose equivalent limits	15 mSv/year	15 mSv/year
Lens of eye	50 mSv/year	50 mSv/year
Skin and extremities		
D. *Negligible individual dose (annual)*	No statement	0.01 mSv/year

Sources: ICRP (International Commission on Radiological Protection), The 2007 Recommendations of the International Commission on Radiological Protection, ICRP Publication 103, 2007; NCRP (National Council on Radiation Protection and Measurements), Recommendations for limits for exposure to ionizing radiation, NCRP Report No. 116, NCRP, Bethesda, MD, 1993.

within an overarching framework in which the goal is to have doses *as low as reasonably achievable*. The ICRP (2007; Wrixon 2008) has also provided a framework for dose constraints and reference levels (Tables 18.26 and 18.27).

The most significant sources for developing the estimates of radiation potency have been the results periodically reported from follow-up studies of the survivors of the atomic bombings of Hiroshima and Nagasaki, Japan, such as the paper of Ozasa et al. (2012). The data relating cancer or leukemia deaths to absorbed dose have been fit with a number of different mathematical functions, usually including a linear or LQ model of the relationship between low doses of radiation and cancer (Lewis 1957; NRC 1990, 2006; Ozasa et al. 2012). Models with a linear component at the lowest doses have been used, even though at the lowest doses of concern, the presence or absence of a linear relationship between dose and cancer response and the absence of a cancer response above the background cannot be proven

TABLE 18.26
Framework for Dose Constraints and Reference Levels

Bands of Effective Dose, mSv (Acute or Annual)	Characteristics	Requirements	Examples
20–100	Controlled by action on exposure pathway	Consider reducing doses	Reference level for radiological emergency
1–20	Controlled by action or source or exposure pathway	For planned exposure situations, individual dose assessment and training	Constraints for occupational exposure. Constraints for comforters and carers of patients treated with radiopharmaceuticals. Reference level for radon in dwellings
<1	Controlled by action on source	Periodic cheques on exposure pathways	Constraints for public exposures in planned situations

Source: ICRP (International Commission on Radiological Protection), The 2007 Recommendations of the International Commission on Radiological Protection, ICRP Publication 103, 2007.

TABLE 18.27
Detriment-Adjusted Nominal Risk Coefficients for Stochastic Effects after Exposure to Radiation at Low Dose Rate (10^{-2} Sv^{-1})

	Cancer		Heritable Effects		Cancer Total Detriment	
Exposed Population	**1990[a]**	**2007[b]**	**1990**	**2007**	**1990**	**2007**
Whole	6.0	5.5	1.3	0.2	7.3	5.7
Adult	4.8	4.1	0.8	0.1	5.6	4.2

[a] 1990 cancer values based upon fatal cancer risk weighted for nonfatal cancer, relative life years lost for fatal cancers, and life impairment for nonfatal cancer.

[b] 2007 cancer values based upon the data on cancer incidence weighted for lethality and life impairment.

as discussed by multiple groups and scientists (NRC 1988, 1990; Hoel and Li 1998; Clarke 2008).

Clarke (2008), who has served as chair of the ICRP, has addressed the uncertainties of estimating effects at low doses using Figure 18.42 to frame the discussion. He has stated that

> Studies on DNA repair and the cellular/molecular processes of radiation tumorigenesis provide no good reason to assume that there will be a low-dose threshold for induction of tumors in general. However, curvilinearity of the dose response in the low-dose region – perhaps associated with biochemical stress responses or changing DNA repair characteristics – cannot be excluded as a general feature. The mechanistic modeling of tumorigenesis is at a relatively early stage of development, but the data available tend to argue against a dose threshold for most tumor types.

In discussing Figure 18.42, he noted, "It is clear that the risk figures apply to the slope of the dose–response curve at the level of background, some few millisieverts per year. However, a strictly linear dose response should not be expected in all circumstances." "It must be recognized that ongoing and future studies in epidemiology and animal science, while remaining of great importance for quantitative risk assessment, will not resolve the uncertainties surrounding the effects in humans of low-dose radiation. Accordingly, there will be an increased need for weight-of-evidence judgments based on largely qualitative data from cellular and molecular studies of the biological mechanisms that underlies health effects; the provision of such judgment demands strong support from biologically validated computational models of risk." He goes on to note, "The development of mechanistic models of radiation risk demands more than a simple improvement in the understanding of cellular or molecular processes."

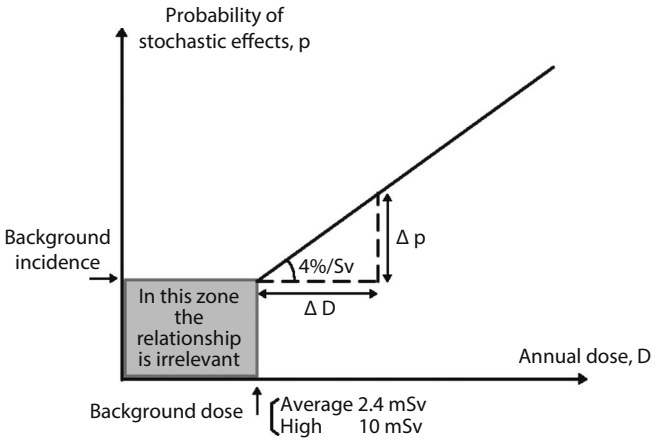

FIGURE 18.42 Region of the dose–response curve when risk factors apply. (From Clarke, R.H., The risks from exposure to ionizing radiation, in: *Radiological Risk Assessment and Environmental Analysis*, eds. J.E. Till and H.A. Grogan, Oxford University Press, New York, pp. 531–550, 2008.)

The concept of population dose and population detriment (NCRP 1960a,b) emerged during the 1940s and 1950s and was reinforced by the consequence studies. This was a natural outgrowth of concern for genetic effects and the use of population dose and detriment as a criterion of integrated injury to the gene pool. However, its use quickly moved beyond concern for genetic effects to cancer risks. It is obvious that the use of linear models of dose–response relationships and population dose and detriment go hand in hand. Using a linear model, it is easy to integrate dose over a population of any size over any time period. Then using a standard coefficient of excess cancer risk per unit dose, one can calculate the excess cancer risk for the population. Some individuals have noted cynically that if the calculated value seems low, then the population size or the duration of exposure can be increased or both population size and duration of exposure can be increased and the population risk recalculated. Unfortunately, many times only the excess cancer risk is presented in the absence of the underlying cancer risk that would have been observed if the added radiation exposure had not occurred. The result is a lack of context for considering the calculated excess radiation-induced risk.

FUTURE RESEARCH

This chapter is grounded in a view that ionizing radiation from external sources and internally deposited radionuclides has been studied more extensively than any other toxic agent. A wide range of adverse health effects have been identified and placed into two broad categories: deterministic effects and stochastic effects. The radiation dose–response relationships for both deterministic and stochastic effects are now very well known. Is it appropriate to ask if major issues can be addressed through further research to enhance our understanding of the effects of external radiation and internally deposited radionuclides? A separate, but important question follows: Is it likely that further scientific advances in understanding ionizing radiation can enhance its use as a diagnostic and therapeutic modality? The answer to that question

is a resounding yes! The issue of research needs related to better understanding the health impacts of exposure to external radiation and internally deposited radionuclides and the use of the knowledge acquired in setting radiation protection standards is a difficult and more complex, multifaceted issue. In the remainder of this section, some of the key issues will be discussed.

Advances in the management of individuals accidentally exposed to high doses of radiation will come from practical experience treating such individuals, as in the past and as is true of other acute toxicants. Let us hope that such situations occur very infrequently. The treatment approach will focus on the individual patient and be driven by symptoms and estimates of absorbed dose. Specific anticipatory research in this area is likely to have limited impact. Positive impacts will come from continuing advances in medical practice.

In my opinion, the prospects for developing pharmaceuticals as radiation-specific countermeasures is quite low based on more than a half century of experience attempting to identify agents that are effective in mitigating radiation-induced effects. The complexity of the radiation injury process defies a *silver bullet* approach. It is obvious that physicians that face the need to treat victims of radiation exposures producing clinical effects would certainly welcome pharmaceuticals developed with a view to a broader application in treating the myriad of pathways involved in ARS. In the absence of specific pharmaceutical agents for treating radiation-induced injury, physicians will use the time-honored approach at treating the clinical signs and symptoms attempting to limit morbidity and mortality.

The most persistent issue concerning the health effects of ionizing radiation relates to concern for the stochastic effects, principally induction of cancer from low-dose-rate, low-dose exposures. Henshaw (1941) and Cantril and Parker (1945) scoped the issue in the World War II era, and in recent reviews, Morgan and Bair (2013) and Coleman (2013) identified some of the issues that underpin the controversy over the extent of radiation-induced effects, if any, associated with low-dose and low-dose-rate radiation exposures. In my opinion, it is unfortunate that neither recent review framed their discussion using the source–exposure–dose–response paradigm (Figure 18.3) strongly advocated in this chapter for integrating the knowledge of radiation-induced effects. I submit that the use of that paradigm has guided the most fruitful research to resolve uncertainties in our understanding of dose–response relationships for radiation effects. Morgan and Bair (2013), as they noted, arbitrarily define a low dose of ionizing radiation as an acute exposure of <100 mGy (mSv). To provide perspective on this dose, it is about 30 times the average annual natural background dose and 30 times the average annual dose from anthropogenic sources.

Another perspective on the issue of understanding potential radiation-induced effects at low doses comes from the analysis of Mettler and Upton (2008). They call attention to the statistical methods discussed earlier by Land (1980) and BEIR VII Committee (2006) and proceed to describe the issue:

> The sample size needed to find or estimate an increased heath risk in a population depends on the size of the risk already present in the population. Thus, to find an excess of an effect that occurs rarely in a population, only a small number of cases may be needed; however, to find a small increase in the incidence of cancer (which occurs spontaneously in 40% of people), a large exposed population is needed. As the additional risk due to any noxious agent becomes smaller, the sample size must increase as the inverse square of the risk to maintain statistical precision and power. For example, if excess risk is truly proportional to dose and if a sample size of 100,000 is necessary to determine the effects of a 0.1 Gy exposure, a sample of approximately 10 million persons will be needed statistically to find the same effect for 0.01 Gy.

Mettler and Upton (2008) noted that a sample size of 1000 is needed to detect a response from 1 Gy. These calculations are fully consistent with the findings to date in the Japanese A-bomb survivors followed very carefully for 58 years (1945–2003) after the bombings. As of 2003, in that population of 86,611, with observations beginning in 1950, there were 10,929 cancer deaths with 527 attributed to radiation exposure and 35,685 noncancer deaths with 353 attributed to radiation exposure (Table 18.15).

It is apparent that most of the deaths attributable to radiation in the Japanese atomic bomb survivors were associated with quite high doses, over 0.1 Gy, that is, the dose Morgan and Bair (2013) arbitrarily suggested as being a low dose and then proceeded to advocate more research at lower doses. In the aggregate, only about 5% of the cancers and 1% of the noncancer deaths in the A-bomb survivors were attributable to radiation with the vast majority of the deaths at doses greater than 0.1 Gy. These statistics should be sobering to epidemiologists, experimental toxicologists, and all those engaged in assessing hazards and risks and those planning experiments on mechanisms of radiation-induced diseases at low doses. As noted by Mettler and Upton (2008), this analysis is relevant to all noxious agents. For the radiobiologists and experimental toxicologists who note that they work with molecular and cellular systems in which more sensitive responses can be characterized, I remind them of the need to place their typical deterministic experimental outcome findings in the context of the statistical framework for stochastic effects discussed earlier. It is clear that cancer occurs as a stochastic disease, and hence, the mechanisms and modes of action that are critical to the occurrence of cancer are of a stochastic nature.

It is obvious that the door should always be open to the conduct of research driven by intellectual curiosity, simply to better understand how humans respond to radiation exposure. However, from a pragmatic viewpoint, it is important to ask if there are key uncertainties in our knowledge of radiation-induced effects, either of a deterministic or stochastic

nature (including hereditable effects or cancer) that limit policy makers in making societal decisions on radiation protection standards, establishing cleanup criteria for radionuclide-contaminated sites or making decisions on the siting of nuclear facilities, including reactors, fuel-reprocessing plants, or nuclear waste storage facilities. In my opinion, the answer is a resounding NO! How the current knowledge is used is a matter of policy, precision estimates of radiation hazards at doses below 0.1 Gy are not the stumbling block to policy decisions, or is it causing potential radiation exposure populations to be inadequately protected.

EPILOGUE: IMPLICATIONS FOR CHEMICAL TOXICITY AND RISK

This textbook focuses on evaluating chemical toxicity, hazards, and risks; thus, it is appropriate to ask what has been learned from the study of radiation and radionuclides that is applicable to chemicals. In my opinion, internally deposited radionuclides and internally deposited chemicals are directly analogous with external radiation exposure being relatively unique. The source–exposure–internal dose–health response paradigm presented in Figure 18.3 is equally relevant to the study of chemicals and internally deposited radionuclides. A clear lesson from the study of internally deposited radionuclides is the importance of absorbed dose in understanding the mechanisms of action and dose–response relationships and comparing the radiation of different qualities delivered at different dose rates.

Absorbed dose is the critical link between exposure and mechanisms of action that lead to alterations in the occurrence of disease in addition to that which would have been observed in the absence of exposure to radiation. While absorbed tissue dose of ionizing radiation is well accepted as a common dose metric for internally deposited radionuclides, a similar unifying metric does not exist at this time for the diverse array of chemicals that are of interest. Perhaps such a metric for chemicals will be devised sometime in the future. In the field of chemical toxicology, there is a rich history of studying the toxicokinetics, the disposition in the body over time, of individual chemicals. However, these studies have not always been well integrated with studies of toxicodynamics and rarely have been linked to studies of late-occurring health effects of either brief or protracted exposures to chemicals within an exposure–dose–response paradigm. Indeed, some scientists and agencies have taken a view that they will first conduct a long-term bioassay using only an exposure metric (i.e., mg of agent/m^3), and if the bioassay is positive, then they will conduct a toxicokinetic study to try to understand the exposure–response outcome. The experience with internally deposited radionuclides suggests that future studies of the toxicity and human health risks of chemicals should be anchored in the exposure–dose–response paradigm to be most fruitful.

Experience with internally deposited radionuclides has given clear evidence that the radiation quality and dose rate at which ionizing radiation is delivered to specific organs and tissues have a major impact on the effectiveness of the absorbed dose for producing either acute or chronic adverse effects over and above the background incidence of disease. Considering first radiation quality, is it possible that a broad class of chemicals operate via mechanisms analogous to alpha-emitting radionuclides, while other classes of chemicals are more analogous to the beta-emitting radionuclides? Recall the alpha-emitting radionuclides were about 10–20 times more potent than the beta-emitting radionuclides in causing cancer. Considering dose rate, is it possible that the influence of exposure rate for chemicals might be better understood if more attention was given to the absorbed dose rate in specific tissues and organs for chemicals? Recall that beta-emitting radionuclides had a factor of 4 differences in cancer-inducing potency related to the rate at which the radiation dose was delivered.

As clearly demonstrated by the data reviewed in this chapter, the health effects of internally deposited radionuclides can be conveniently grouped into deterministic effects with clear threshold dose–response relationships and stochastic effects, such as cancer, that have dose–response relationships best described with linear or LQ models of dose-ERR and an inability to ascertain, because of statistical limitations, if a threshold exists at low doses and low dose rates. The studies of internally deposited radionuclides that have yielded the most useful data for analyzing dose–response relationships have had dose data on individual subjects, irrespective of whether they were human subjects in an epidemiological study or dogs in a controlled exposure study. Some of the most fruitful analyses have used RR models; absolute risk models have not been as informative. Unfortunately, RR models have rarely been used in chronic chemical toxicity studies, especially experimental studies. No doubt this relates to the past focus on using chronic bioassays to identify health hazards including cancer induction rather than to understand exposure–dose–response relationships. Perhaps greater use should be made of chronic studies with chemicals that are specifically designed to understand exposure–dose–response (relative to background incidence) relationships for at least a few purposefully selected specific chemicals. The obvious candidates based on the radiation experience would be to study chemicals for which there is a strong base of exposure–dose–response data for human populations.

As noted repeatedly in this chapter, ionizing radiation is a relatively weak carcinogen. I would argue that most chemicals with the exception of a few purposefully active pharmaceuticals and perhaps a few *legacy* chemicals are likewise not very potent in causing cancer or other effects, and moreover, effects are only observed under conditions of high exposure rates and high cumulative exposures. *Blockbuster* exposures used in many animal and cellular studies are many times higher than likely to be experienced by most potentially exposed human populations. I question whether the mechanisms observed in these kinds of studies designed within the context of deterministic outcomes are relevant to understanding potential stochastic effects in which the ERRs are very small and occur randomly. Perhaps the time has arrived, with new molecular techniques emerging at a rapid pace, to rethink our experimental approach to studying both radiation and chemicals as

to their stochastic effects, such as cancer, which appear as a small excess late in life. Recall within the context of attributable risk the results of the 58-year follow-up of the Japanese A-bomb survivors. Irrespective of how exquisite the methods might have been used to evaluate each and every cancer, only about 5% of the cancers on a statistical basis have been attributed to radiation. The experimentalist studying tissues from deceased individuals is not able to determine which of the cases are radiation induced; they are only known in a statistical sense. I suggest it may be productive to take time out to explore some new approaches to studying both radiation- and chemical-induced excess disease. Indeed, a possible outcome is to conclude that enthusiasm for an agent-oriented focus on attributable disease may be misplaced and that greater benefits to society will be achieved if the focus is redirected to the multifactorial etiology of disease recognizing the complex family of disease in multiple sites and organs (Vogelstein et al. 2013). It is clear that science has not revealed the etiology of most cancers. It has revealed that radiation exposures are a minor etiological factor. In my opinion, the same can be said with confidence for exposure to chemicals.

It is true that life exists in a sea of radiation, radioactivity, and chemicals for most populations. Moreover, all living things consist of chemicals constantly undergoing complex interactions microsecond by microsecond in an elegant and well-controlled manner consistent with population having healthy lives that extend on average over 75 years in most industrialized countries. Fortunately, those well-controlled mechanisms function appropriately most of the time in complex mammalian organisms with only the infrequent occurrence of life-threatening diseases as a result of low-level exposures to radiation, radioactivity, and exogenous chemicals. Thus, these agents make small contributions to the burden of disease in advanced countries. Unfortunately, that reality is absent from the planning of most research, and the subsequent introduction or conclusions of the papers describing the research, the papers usually start by noting concern for exposure to radiation or chemicals. Perhaps the scientific community, when broadly championing the need for more research on these agents, inadvertently contributes to radiation phobia and chemical phobia.

SUMMARY

The goal of this chapter has been to provide the reader with an understanding of the health effects of ionizing radiation within the context of the multiple sources and uses of radiation and radioactivity. The distinction has been made repeatedly between radiation and radioactivity and between irradiation from external sources and internally deposited radionuclides. The chapter began with a review of the remarkable history of ionizing radiation noting key landmark events linked to creating sources of exposure to external radiation and internally deposited radionuclides and elucidating associated health hazards and risks. Roentgen's discovery of x-rays led to the rapid application of radiation in key diagnostic and therapeutic procedures that soon became central to the practice of medicine. Somewhat later, the first major nonmedical usage of radioactive materials began when instrument dials were painted with radium to make them luminous. Unfortunately, the discovery of the injurious effects of external radiation exposure and internally deposited radionuclides, especially their cancer-causing potential, accompanied these beneficial applications of radiation.

The discovery of nuclear fission in 1937 served as a benchmark for another radiation era. This soon led to controlled nuclear fission, the production of ^{239}Pu and ^{235}U as fissile materials, the development of the A-bomb, and its use in bombing two Japanese cities. Out of this tragedy came the remarkable epidemiological data on the A-bomb survivors, data that today serve as a cornerstone of our knowledge of the late-occurring effects, both cancer and noncancer, of brief exposure to external radiation. This data set, its analyses, and interpretations are the foundation of radiation protection standards used around the world. Data on the cancer-causing effects of exposure to radon and its daughter products provide complementary data on a unique radiation risk. Quantitative data on exposure–radiation dose to tissues–health effects acquired principally from LSS conducted in beagle dogs and epidemiological observations on a few key radionuclides are central to today's understanding of the health risks of internally deposited radionuclides.

Without question, ionizing radiation has been studied far more extensively than any other toxic agent. Despite the extraordinary amount of information acquired on radiation-induced disease, especially cancer, uncertainties remain and most of all clamorous debate continues on the precise nature of the dose–response relationship for ionizing radiation at low radiation doses to various organs and especially radiation doses given at very low radiation dose rates similar to background levels. Despite the uncertainties, it is apparent that ionizing radiation is a relatively weak carcinogen and even weaker toxicant in producing noncancer effects at low dose rates and total radiation doses. It is my professional opinion that there is a very low probability that the health risks of low radiation dose rates and total radiation doses have been underestimated using an LNT model, including its use for extrapolation to exposures below 0.1G and above background levels. Unfortunately, this viewpoint is frequently lost in the scientific and policy debates over the setting of radiation protection standards, the conduct of risk assessments, and the frequent call for more research.

The major questions concerning radiation and health risks and the use of nuclear technology relate to policy decisions that should be informed by the already abundant science on radiation-induced disease. Scientific information alone, no matter how precise or elegant, is insufficient for making those policy decisions that are concerned with very low levels of extrapolated risk relative to the inherent everyday risks of life and the central policy question: "What is an acceptable level of risks for a particular agent, action, or technology?" Scientific knowledge can inform the policy decisions at the core of that question, and science *cannot* answer that question!

QUESTIONS

18.1 What are the differences between deterministic and stochastic effects of radiation exposure and the dose–response relationships used to model the two kinds of effects?

18.2 Describe the fundamental differences between dose rate and pattern of total dose accumulation for the thyroid from a brief exposure to x-irradiation versus ^{131}I when the total delivered dose is 100 mSv? For a population of 10-year-old children, discuss the likelihood of either exposure producing an excess of thyroid cancer.

18.3 Which radionuclides, when ingested or inhaled in a soluble form, behave like potassium, calcium, and iodine?

18.4 Describe the evidence supporting the statement that radiation is a weak carcinogen.

18.5 Discuss the concept of hormesis. If you believe that radiation hormesis should be used in setting regulatory standards for radiation exposure, describe your proposed regulatory scheme.

18.6 Brief whole-body exposure to x-irradiation at sufficiently high doses has the potential for causing an increase in the incidence of cancer and heritable effects. Discuss the evidence for both health endpoints being observed in large populations whose total dose was 1 Sv.

18.7 Discuss the agricultural ecosystem pathways for human exposure to accidental releases of ^{90}Sr, ^{131}I, and $^{134,137}Cs$, including potential steps that should be taken to reduce exposures and dose to critical tissues.

18.8 Describe the evidence supporting the statement that the results of studies with beagle dogs exposed briefly to ^{239}Pu are reliable predictors of the effects of human inhalation exposures to ^{239}Pu aerosols.

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19 Plant and Animal Toxins

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CONTENTS

TOXINS IN NATURE

Nature is beautiful, but is it always safe? Nature and things natural can be hazardous and risky if individuals are not informed and constantly aware of the dangers lurking in the green foliage, the crystal clear waters, and the bushes and rocks!

Naturally occurring toxins may be grouped into those originating in plants, algae, fungi, bacteria, and various members of the animal kingdom. The natural hazards of bacterial toxins and those from fungal toxins in the category of mycotoxins will be discussed in Chapter 19. This contribution will present the toxic hazards from plants, algae, mushrooms, and toadstools and hazards from animal-origin toxins.

The toxins from such widely diverse naturally occurring organisms can be found throughout our environment. Foliage may have a variety of toxic plants, waters can carry algae, and where moisture is, various hazardous fungi may grow. The dry land may harbor reptiles, amphibians, spiders and insects, and even mammals capable of inflicting injury. Animals found in waters include various invertebrates and vertebrates found in oceans, shellfish, and fish carrying toxins from other sources and such unsuspected bearers of poisons such as sea turtles, polar bears, seals, and walruses.

In short, naturally occurring toxins are everywhere and have continuing potential for intoxication.

Natural toxicants (toxins) are unique in that they exist in the organism's structure, yet are not harmful to that host. They are also special in that individuals potentially affected by these natural toxins may become exposed by consuming the toxin-bearing material, such as when consuming poisonous plants or toxic marine animals, by receiving skin contact and producing durable irritation or traumatic injury, such as occurs from poison ivy or blistering agents in plants or from cactus spines penetrating body surfaces, or most commonly such harmful effects result from animals carrying venoms and producing envenomations (injections) through stings, bites, or other delivery systems. In many cases, these delivery systems are specialized to cause reactions on contact, embed materials that are then released by the victim's biological tissues, or result in direct administration into the victim's body tissues and fluids. As in all areas of toxicology, the amount of exposure or dose delivered to the victim largely determines the resulting clinical effect.

Exposure to toxins present in nature is in many cases accidental, as persons collecting green plants mistake a toxic variety for a similarly appearing green food plant. Individuals may elect to chew and swallow plants because of ignorance. In some cases, the use of native herbal medications produce hazards due to selecting inappropriate natural materials and/or poor approximation of amounts to include in various preparations. In other instances, malicious use of natural toxins may be involved in homicidal or retaliatory actions. One way or another, these natural materials when introduced into the human body are capable of producing mild, moderate, or lethal effects.

Naturally occurring toxins are complex. Unlike synthetic compounds used for therapeutic or specific chemical needs, toxins from plants or animals are mixtures of organic, proteinacious, enzymatic, and even mineral materials that are present in various proportions that fluctuate with the individual plant's or animal's maturity, location, species, and the various life changes that growing and maturing organisms go through. The relative proportion of the numerous components in these toxic *packages* varies sufficiently so that their impact upon biological systems is not only affected by the quantity administered but also by the relative proportion of each of the components in the mixture. At any one time, the dose received from a specific animal envenomation or plant weight will fluctuate significantly—so that an exposure to the same amount of a specific plant will not always produce the same biological effect. This creates significant problems when attempting to identify the toxic components of naturally occurring materials. Fractionation of the *mixed bag* of components in any toxic mixture thus becomes problematic when trying to work specifically with the active component responsible for a specific harmful effect. It also impacts the resulting toxicity since the dynamics of the clinical impact fluctuate with these various factors and often require that evaluation of toxicity be based on potential effects or the observation of the direct impact made by the exposure. A single bite from a known poisonous snake does not always induce toxicity or an expected amount of damage. The ingestion of a known amount of a recognized poisonous plant may not induce toxicity or may indeed cause more life-threatening damage than previously documented.

So why are these toxic and often lethal components present in these organisms? One could make the point that they are there *just because* and are part of the organism's normal body component of compounds needed for appropriate functioning. Their introduction into human systems and their adverse effect is just an unfortunate coincidence! Others might argue that the compounds' present is a defensive mechanism to avoid consumption or destruction by other organisms or predators; essentially providing a *distasteful* menu. Others see the types of chemicals present as weapons to be used by the organism, for example, a rattlesnake, to defend itself against predators or other individuals. *Defensive* then is to discourage attack by or consumption from other individuals. Certainly plant toxins have that coincidental effect by keeping browsing animals from consuming plants that produce illness in that species. Finally, the use of venoms or poisons to immobilize animals or fight off other animals is a view into the real world of nature; securing an appropriate diet or nutritional force involves capturing a victim and consuming it as needed. Interestingly, animals consuming prey that their bites have made available for consumption have little impact upon the consuming animal that has just envenomated the victim. Being able to consume one's own poison seems the ultimate path of self-preservation!

The vast amount of information to be gained from the study of these naturally occurring poisons has evolved into a highly specialized and unique aspect of science. The variety of fractions found in venoms and toxic plant materials has required elaborate instrumentation, knowledge, and expertise to deal with the understanding of how and why these toxic materials exist in nature. This study is *Toxinology* and has a broad scope of interest and impact. The science varies geographically and as widely as the range of toxic plant and animal species. The science has a unique and widely respected journal, *Toxicon* [1], and has wide applicability to not only understanding our own environment but also the components of nature and their potential harmful and more recently important beneficial contributions to the human race.

Toxinology seeks to understand the mechanism by which these widely diverse and selective natural chemicals produce their effect. Isolation of various components and demonstrating their structure and biological effects are exciting activities that keep individual scientists busy with one species of organism over a lifetime. The information is used to apply the individual venom or toxin components to potential treatments and use in biomedical research or treatment of human or animal diseases. The Southeastern pygmy rattlesnake possesses a venom containing eptifibatide that reduces the blood clotting process allowing blood to flow more freely by reducing the number of platelets. The drug (Integrilin) is used to reduce the risk of acute cardiac ischemic events in patients with unstable angina or non-ST-segment-elevation

myocardial infarction both in patients who are to receive nonsurgery medical treatment and those undergoing percutaneous coronary intervention. Understanding venoms and their chemical structure and function allows the production of antivenins through manipulation of animal and biological systems. A presence of such materials can be lifesaving in treatment of human animal bites and serious illnesses. The explosion in the use of *botox* for cosmetic surgery and treatment of various neurological disorders is a special example of how such applicability of natural toxins will evolve in future years. As these biological processes are better understood and the individual venom components become identified, their application and use in human medicine and in therapy for natural intoxications will be exciting and of significant health benefit.

Understanding the toxins from plants, algae, fungi, and animals is the substance of the remainder of this chapter.

TOXINS FROM PLANTS

Ever since the frequency of exposures of humans to toxins has been tallied, plants have ranked among the most frequent causes of exposures to toxic substances. In 2011, plants were the eleventh most common cause of human exposures with 47,561 instances—2.2% of the total exposures—reported in the American Association of Poison Control Centers, Toxic Exposure Surveillance System, Annual Report [2]. At the same time, plants and their chemical composition have been investigated for the potential of providing new medical agents and derivatives to treat illnesses and have been an ever common component of herbal drugs and folk medicines. From previous times and cultures, the herbalist-healer has gathered leaves, roots, blossoms and bark to brew remedies, potions, and elixirs with mixed results. Now, alert clinicians have observed that while some of these herbals seem to work well, and others fail, many at best exert a positive placebo effect… while others induce illness! Plants are a true example of balm or poison. A balance is needed to receive beneficial effects versus life-threatening doses from these tricky poisons.

Only very small amounts of some plant parts (flowers, fruits or berries, leaves, stems, bark, or roots) may be toxic and produce illness or even rather dramatic effects. Some plants are poisonous if they are chewed or swallowed. Others cause poisoning by initiating allergies, dermatitis, or mechanical injury due to spines or needles. Some plants are harmful only if eaten or chewed at certain stages of their growth; others are toxic at all stages of development. Thus both the species of the plant and its stage of growth or the season of the year in which exposure occurs is important in determining the potential toxicity.

Of the many plant varieties, only a relatively small number are truly poisonous and develop life-threatening effects. A few pose a serious threat to life or health, but among those few are some widely cultivated flowers, vegetables, and ornamental plants. Some of them are household plants in which specimens may come from Maine or California. Together with the toxic flora of field and forest, they represent an abnormal potential for poisoning, particularly in small children who are apt to appreciate beauty by chewing on it.

Species of the plant, its stage of growth, and the season of the year are all important factors in evaluating potential plant poisonings. In some cases, the part of the plant that is eaten also matters. For example, all portions of the potato plant…except the actual potato…are poisonous; the leaves of a rhubarb plant are highly dangerous despite the fact that the stalks make delicious pies. The only reliable rule is not to eat anything that you're not absolutely sure is safe! [3]

And while human beings are a major focus of toxicology, animals that are outdoors and those that consume plants as part of their natural dietary intake, are at severe risk for consuming *a salad bar* of potential hazards. Of the approximately 1000 recognized poisonous plants that grow in North America, all are unique in becoming toxic to animals and particularly livestock that managers commit to pastures and ranges for their dietary needs. Each year significant economic loss occurs to these animals. Estimates of specific losses are difficult to ascertain, but estimates of the losses caused by poisonous plants in only the 17 western states are dramatic. One percent of the death loss in cattle is attributed to toxic foliage; one percent of the loss of calves from abortions or illnesses surrounding births is estimated due to plants; 3.5% death loss is estimated produced in sheep by plants; and 1% loss of pregnancy potential and newborn lambs is estimated to occur in sheep from poisonous plants. A total economic loss of over $250 million is the best estimate available for poisonous plant damage produced in our 17 western states [4]. When one appreciates the fact that poisonous plants do not always produce death, but depending upon consumption of varying amounts may produce no clinically observable damage but yet reduce weight gain and economic productivity, the losses estimated may reasonably be expected to be considerably greater.

So what are the types of chemicals involved in potential poisonous plant chemistry and toxicity, and what are the plants that produce poisoning if ingested or placed in contact with the human body?

Although it is unusual to have a natural occurring chemical present in only one plant, so too it is equally unusual for any one toxic substance to be uniquely found in only one plant species. Indeed, it is the common finding that most plants have several toxic components present. It is only the distribution of those materials and their concentration in various parts of the plant and at various stages of growth that present the hazard. Consuming berries at an early stage of development may be highly toxic, while once the berries are ripe and are in full bloom toxicity may be reduced. The concentration of the toxin may have distributed from the unripe berry to the stems or to the roots. Not to be outdone, the plant that previously was highly toxic in the young stage by consuming leaves, may now be innocuous when the leaves are consumed, but when the seeds are digested acute toxicity may occur. In each of these examples, the concentration and the type of poison vary with each environmental circumstance. Hence, as consideration is given to the various

chemicals present in plants, one must recognize the content of more than one toxic factor being present and the likely toxicity is dependent upon which toxin is present in the highest concentration and which portion of the plant is consumed by the potential victim.

Toxic Chemical Principles in Plants

The toxic principles in poisonous plants carry many names, some actually present due to plant biochemistry, while others are present in the soil or water nourishing the plant and have been accumulated in the growing foliage. With such a heterogeneous potential for effects, it is understandable that numerous categories of chemical principles and components have been listed as present in plant poisonings. Most contain numerous derivatives and related compounds. A general listing includes alcohols, various alkaloids, amines, anticholinergics, glycosides and glucosides, mechanical injury vectors and associated chemicals, minerals associated with plant growth and incorporated into plants, oxalates, various compounds capable of producing photodynamic pigments and resulting photosynthetization, polypeptides, various resins and resinoids, saponins, and toxalbumens. Others capable of carrying enzymatic properties, hormones, phenolics, and unusual nitrogen compounds are also potentials present.

Alcohols

Alcohols are organic compounds formed from hydrocarbons and founds in species of golden rod and snake root. Tremetol is a specific alcohol that is passed in milk and induces neurological effects in humans and animals consuming the toxin-containing milk.

Alkaloids

The alkaloids are a widely disseminated group of various chemicals found in numerous plants. Their presence in concentration is only mildly influenced by the climate and availability of water, but varies greatly with the species and variety of plants studied. They are widely distributed throughout the plant's structure and only produce strong physiological reactions in consuming individuals. The toxicity induced varies from neurological through liver to kidney damage, and includes the veratrum alkaloids, those found in *Aconitum* species, nicotine, coniine, and those present in *Crotalaria*, *Senecio*, potatoes and tomatoes, lilies, and the highly toxic Japanese yew (*Taxus*). As continuing studies occur on the chemistry of plants, new alkaloid compounds are being discovered and added to this list.

Amines

Amines are only a small number of the toxins found in plants, but many foods contain pressor amines, which constrict blood vessels and increase blood pressure. Individuals medicated with tranquilizers or antidepressants, which inhibit monoamine oxidases and thus induce clinical problems. Generally, the toxic amines affect skeletal or nervous tissue and are found in sweet peas (*Lathyrus*) and mistletoe (*Phoradendron*). A number of plants exhibit anticholinergic properties through their content of atropine or scopolamine. These principles are commonly found in deadly nightshade (*Atropa belladonna*), Sacred datura (*Datura metaloides*), Jimson weed (*D. stramonium*), Trumpet lily (*D. arborea*), Angel trumpet (*D. candida*, *D. suaveolens*), other *Datura* spp., Henbane (*Hyoscyamus niger*), Matrimony vine (*Lycium barbarum*), and Mandrake (*Mandragora officinarum*). The entire plant is toxic, although the flowers, fruits, and seeds are especially high in the atropine-like principles. Agitation, hallucinations, flushed skin, dry mucous membranes, elevated heart rates, and dilated pupils are common effects. These plants account for many admissions to critical care units and are often associated with patients who have ingested seeds or brewed tea made from the seeds. Although death is rare, serious injuries result from the impaired judgment and functionality present in affected victims.

Glycosides

Naturally occurring glycosides are widely distributed in the plant kingdom as probably the most common and largest group of toxic compounds found in plants. Although many are nontoxic, wide varieties do have toxic effects upon ingestion or other exposure. The amount of a particular glycoside present again depends on the plant genetics, the portion of the plant being sampled, and the plant's age, as well as factors such as climate, soil fertility, and available moisture. Toxicity from this group is a function of the aglycone component or a part of it. Numerous groups of toxic glycosides have been identified: coumarin and vanillin glycosides (sweet clover, laurel, horsechestnut); the cyanogenetic glycosides that contain or liberate each hydrocyanic acid (velvet grass (*Holcus lanatus*), hydrangea (*Hydrangea* spp.), flax (*Linum* spp.), cassava (*Manihot esculenta*), lima bean (*Phasecolus lunatus*), cherries (*Prunus* spp.), apple (*Pyrus malus*), sudan grass, Johnsongrass, etc. (*Sorghum* spp.), poison suckleya (*Suckleya suckleyana*), white clover (*Trifolium repens*), arrowgrass (*Triglochin* spp.), vetch seed (*Vicia sativa*), maize, corn (*Zea mays*)); gloiterogenic glycosides [chard (*Beta vularis*), rape seed or meal (*Brassica napus*), black mustard seed (*Brassica nigra*), kale (*Brassica oleracca* var. *acephala*), Chinese cabbage (*Brassica pekinensis*), turnip root (*Brassica rapa*), soybean (*Glycine max*)); irritant oils derived from mustard oils (horseradish (*Armoracia*), white mustard (*Brassica hirta*), Indian mustard (*Brassica juncea*), wild radish (*Raphanus raphanistrum*), fanweed (*Thlaspi arvense*)), and those containing the volatile, unstable protoanemonin oil released from the glycoside ranunculin (marsh marigold (*Caltha palustris*), buttercups (*Ranunculus* spp.)); the steroid glycosides, which contain the poison category of cardiac toxins capable of stimulating or disrupting cardiac function (dogbane (*Apocynum* spp.), lily-of-the-valley (*Convallaria majalis*), foxglove (*Digitalis purpurea*), oleander (*Nerium oleander*), squill (*Urginea maritima*))—the plants containing grayanotoxins (rhododendrons, azaleas *Rhododendron*, sheep laurel, mountain laurel (Kalmia), and andromeda *Pieris*) are also potent cardiotoxic containing plants but not

from steroid glycosides—and noncardioactive saponin steroid glycosides that are not easily absorbed but have additional toxins that injure the digestive tract to allow entrance into the blood stream (corn cockle (*Agrostemma githago*), English ivy (*Hedera helix*), alfalfa (*Medicago sativa*), pokeweed (*Phytolacca americana*), bouncing bet, cow cockle (*Saponaria* spp.), coffeeweed, rattlebox (*Sesbania* spp.).

Mechanical Injury

Some plants may inflict injury through mechanical processes that then allow additional adverse effects to occur. The nettle (*Urtica chamaedryoides*) is a common example as it bears stinging hairs that contain significant amounts of acetylcholine and histamine that induce serious reactions. Other plants having such mechanisms include anemone (*Anemone patens*), poverty grasses, awns (*Aristida* spp.), squirreltail barley, barbed awns (*Hordeum jabatum*), foxtail grasses (*Setaria lutescens*), needle grasses (*Stipa* spp.), cocklebur (*Xanthium* spp.), and burdock (*Arctium lappa*).

Mineral Toxicities

Plants may become toxic secondarily through accumulation of minerals found in soil at high concentration. Chief among those elements are nitrogen, which is accumulated in those plants as nitrate, and selenium, which has varying amounts in soils depending on geologic formation and rainfall. Plants that frequently contain toxic concentrations of nitrates include pigweeds (*Amaranthus* spp.), bishop's weed (*Ammi majus*), tarweed (*Amsinckia* sp.), pigweed, lamb's quarters (*Chenopodium* spp.), Canada thistle (*Cirsium arvense*), poison hemlock (*Conium maculatum*), bindweed (*Covolvulus* spp.), Jimson weed (*Datura* spp.), fireball (*Kochia scoparia*), sweetclover (*Melilotus officinalis*), smartweeds (*Polygonum* spp.), dock (*Rumex* spp.), Russian thistle (*Salsola pestifier*), annual sage (*Salvia reflexa*), elder (*Sambucus pubens*), nightshades (*Solanum* spp.), goldenrods (*Solidago* spp.), and Johnsongrass (*Sorghum halepense*). In addition, certain crops will also accumulate exceedingly high levels of nitrate depending upon the environmental conditions and the availability of nitrogen. Some of these are oat hay (*Avena sativa*), beet and mangoid (*Beta vulgaris*), broccoli, kale, etc. (*Brassica oleracea*), turnip (*Brassica rapa*), soybean (*Glycine max*), barley (*Hordeum vulgare*), sweet potato vines (*Ipomoea batatas*), alfalfa (*Medicago sativa*), radish (*Raphanus sativus*), sudan grass (*Sorghum vulgare*), wheat (*Triticum aestivum*), and corn (*Zea mays*).

Plants taking up selenium are of significant concern to livestock and also to humans who might utilize these high selenium-containing forages. Crops that may do so include poisonvetches (*Astragalus*), prince's plume (*Stanleya*), goldenweeds (*Oonopsis*), woody asters (*Xylorrhiza*), aster (*Aster* spp.), saltbushes (*Atriplex* spp.), gumweeds (*Grindelia* spp.), snakeweed (*Gutierrezia* spp.), tansy aster (*Machaeranthera* spp.), and beard tongue (*Penstemon* spp.).

Other elements found in soil or the environment may contaminate plants or produce toxicity. Such elements include cadmium taken up from fertilizers in which the metal impurity is present, copper from copper-rich soils or copper-containing pesticide use, fluoride from fluoride-containing soils, rock formations or industrial influence contaminating the soils, lead from surface contamination from smelters or lead-containing mine debris, and molybdenum found in soils abnormally high in this element.

Oxalates

The organic acid oxalates found in plants occur in the form of soluble sodium and potassium salts or insoluble calcium oxalates or acid oxalate. Although small amounts of oxalates are found in many plants, several of them will have increased oxalate content depending on the season and geographic location. Highest oxalate concentrations are in late summer and fall in the leafy stage of plant growth. The resulting toxicity may occur from the soluble oxalate salts that enter the bloodstream and later are precipitated in the kidney, or as firm calcium oxalates or acid oxalates that irritate oral membranes on contact, producing burning and irritation upon chewing. The common plants containing variable amounts of soluble oxalates include beet, mangold (*Beta vulgans*), lamb's quarters (*Chenopodium album*), halogeton (*Halogeton glomeratus*), sorrel, sour sob (*Oxlais* spp.), pokeweed (*Phytolacca americana*), rhubarb (*Rheum rhaponticum*), sorrel, dock (*Rumex* spp.), greasewood (*Sarcobatus vermiculatus*), and spinach (*Spinecia oleracea*). Common plants containing the insoluble oxalates producing irritation on mucous membrane contact are jack-in-the-pulpit (*Arisaema* spp.), caladium (*Caladium* spp.), dumbcane (*Dieffenbachia* spp.), philodendron (*Philodendron* spp.), skunk cabbage (*Symplocarpus foetidus*), and caladium (*Xanthosoma* spp.).

Photosensitivity-Inducing Plants

Photosensitivity from plant substances is an interesting phenomenon in which individuals become hypersensitive to sunlight due to the presence of plant-originating material in the peripheral circulation. This may be produced by plants carrying specific photosynthesizers in themselves that directly produce the hypersensitivity, plants that have additional compounds that by themselves produce liver dysfunction with no photodynamic activity but the damage resulting retards elimination of plant materials and allows them to reach the peripheral circulation thus inducing photosynthetization, or due to congenital metabolic defects that allow the accumulation of photodynamic pigments within the individual circulation. Plants producing these types of photosynthetization risks include buckwheat (*Fagopyrum sagittatum*), St. Johnswort, Klamath weed (*Hypericum perforatum*), lechuguilla (*Agava lechaguilla*), cultivated rape (*Brassica napus*), lantanta (*Lantana* spp.), horsebrush (*Tetradymia* spp.), water bloom (*Species of bluegreen algae*), oats (*Avena sativa*), milk purslane (*Euphorbia maculata*), alfalfa (*Medicago sativa*), smartweeds (*Polygonum* spp.), summer cypress (*Kolchia scoparia*), sudan grass (*Sorghum vulgare* var. *sudanese*), clovers (*Trifolium* spp.), and vetches (*Vicia* spp.).

Phytotoxins

Phytotoxins are large, complex molecules similar to bacterial toxins in structure and reactions. They are antigenic with protein-like characteristics that have resulted in the terminology "toxalbumins" used as a designation for this group of plant toxins. Some of these plants have the highest concentration of the poison present in the seeds, such as in castor bean (*Ricinus communis*) from which commercial castor oil is produced. Such plants include precatory bean, rosary pea (*Abrus precatorius*), castor bean (*Ricinus communis*), and black locust (*Robinia pserudoacacia*). The toxin ricin has been specifically isolated as the toxin in castor bean. The highly potent nature of these phytotoxins has attracted attention from defense groups dealing with potential use by terrorists.

Polypeptides

Only a small number of plants containing poisonous polypeptides are currently known. These include mushrooms, blue-green algae, soybeans, potatoes, lima beans, kidney beans, unripe bananas, mangos, and some legumes. Significant consumption for several days is usually required to induce toxicity.

Resins and Resinoids

This heterogeneous group of compounds has the physical characteristic of being solid or semisolid at room temperature, easily melted or burned, being soluble in organic filaments and not containing nitrogen. They tend to be oily or greasy upon extraction and induce variable degrees of toxicity in both potency and clinical effects. Such compounds are found in milkweed (*Asclepias* spp.), hemp, marijuana (*Cannabis sativa*), water hemlock (*Cicuta* spp.), iris (*Iris versicolor*), laurel (*Kalmia* spp.), Japanese pieris (*Pieris japonica*), pine (*Pinus* spp.), laurel, rhododendron (*Rhododendron* spp.), and Japanese wisteria (*Wisteria floribunda*).

Others

There are various other poisonous principles that produce toxicity in often unique ways specific to that individual plant. Some contain enzymes that induce neurological deficits or cancer (bracken fern (*Pteridium aquilidum*); horsetails (*Equisetum* spp.); male fern (*Dryopteris felix-mas*)). The polyphenolic gossypol is found in the seed of cotton (*Gossypium* spp.) and is responsible for commercial applications in human medicine as well as toxicities in all species. Unusual nitrogen compounds are found in some of the legume family foliages (creeping indigo (*Indigofera spicata*); sweet pea (*Lathyrus*); Koa hade (*Leucaena leucocephela*)) producing specific effects on pregnancy, the nervous system, hair follicles, and general health. Estrogenic factors are found in subterranean clover (*Trofolium subterraneum*). The toxic factor found in the cotyledon stage of cocklebur (*Xanthium strumarium*) produces rapid weakness and even death in consuming individuals.

As clinical observations become more extensive and astute, and as analytical chemistry become more specific, no doubt additional toxic components will be identified and related to the varying illnesses and conditions associated with plant exposure and consumption.

Organ Systems Affected

The complexity and variability of the various compounds present in plants result in most plant varieties being capable of inducing more than a single effect and producing several system-specific adverse changes. Depending upon the toxin's presence and their individual concentrations, such harmful effects may influence every organ system in the body individually or as a complex combination of various systemic effects.

Gastroenteritis-Inducing Plants

The majority of plant intoxications occur from the ingestion of plants containing gastroenteric irritants. Symptoms range from burning in the mouth and throat when chewing the leaves of such common household plants such as the vine growing on a windowsill (*Philodendron* spp.) or the dumbcane (*Dieffenbachia* spp.) in the big pot by the front door to severe vomiting, intestinal cramping, and purging diarrhea from the fresh roots and stems of the pokeweed (*Phytolacca americana*), Wisteria seeds, the berries of the spurge laurel (*Daphne* spp.) or the leaves of buttercups (*Ranunculus* spp.).

The onset of the irritant's response is variable since it depends upon the nature of the toxin, possible requirement for activation of the irritant, and the various mechanisms that may illicit emesis or gastroenteritis, thus modifying the clinical response. Plants that cause minor abdominal discomfort in an adult may provoke profound emesis and diarrhea in a small child. Since dehydration and electrolyte imbalance can develop rapidly, it may be necessary to institute replacement therapy promptly to prevent shock.

Serious intoxications are produced by plants containing toxalbumins, for example, the rosary pea (*Abrus precatorius*) and the castor bean (*Ricinus communis*). Following ingestion of the chewed seeds of either of these plants, there may be considerable delay prior to the onset of effects; this is related to the quantity of material present in the chewed seeds. Typically the latent period is about 2 h, but it may be a day or more. The clinical picture is one of severe hemorrhagic gastroenteritis with persistent nausea, emesis, colic, and profuse diarrhea. Dehydration may result in oliguria and cardiovascular collapse. In nonfatal cases, the period of illness varies from 2 to 10 days.

Rosary pea seed, precatory bean (*Abrus precatorius*), contains a phytotoxins that is related to the botchulinum toxin. It produces nausea, colic, diarrhea, weakness, and trembling with a hemolytic anemia and ultimately uremia in severe cases.

Poinsettia (*Euphorbia pulchenima*) has irritating saps in the leaves and stems that lead to vomiting and delirium in patients who are ill for several days.

Philodendron (*Philodendron* spp.) has calcium oxalate crystals that severely affect kidney function and induce listlessness and potentially uremia in severely intoxicated patients.

Dumbcane (*Diffenbachia* spp.) contains insoluble oxalate crystals that are not absorbed but are a severe contact irritant. Swollen mucous membranes of the mouth and throat result that may induce suffocation from the respiratory tract swelling that occlude airways.

Ivy (*Hedera helix*) contains saponic glycosides in the berries. This plant then induces severe diarrhea and may contribute to excitement and excessive nervousness in poisoned individuals.

Lantana (*Lantana camara*) has an alkaloid in the unripe berries and clippings. It is a commonly available plant that in low doses of intake produces mainly digestive tract irritation with some photosensitization leading to sunburn and dermatosis. If large amounts are consumed, circulatory collapse and muscle weakness develop that borders on life-threat.

Daffodil (*Narcissus*) is a common plant with a prominent bulb-like root. It has a potent alkaloid in high concentrations in the bulb. Ingestion or chewing on this bulb induces repetitive and prominent vomiting and digestive tract irritation.

Rhubarb (*Rheum rhaponticum*) is well known for containing high levels of oxalic acid in the leaf blades. This acid is highly irritating and produces severe abdominal pain that proceeds to vomiting, convulsions, anuria, coma, and death as the oxalate crystals occlude to the tubules, ultimately producing uremia.

Pokeweed (*Phytolacca americana*) has a prominent alkaloid (phytolaccatoxin) in all plant parts. Although the roots are most toxic, the prominent deep purple berries fortunately contain lower concentrations of the alkaloid; however, because of their attractiveness, the berries are most frequently eaten. The alkaloid produces gastrointestinal signs that often extend to inducing spasms and convulsions with respiratory paralysis occasionally developing into anoxia.

Death camas (*Zigadenus* spp.) is another plant with a bulb-like root that resembles an onion. It is frequently collected as a *wild onion* and then incorporated into cooked stews and soups. Unfortunately, the alkaloid is not significantly changed by the heating process and prominent gastrointestinal effects result from consumption. The vomiting and diarrhea it produces is often lifesaving by rapidly removing the alkaloid from the intestinal tract.

Mayapple (*Podophyllum peltatum*) contains a resinoid in high concentrations in the root. The rest of the plant is nontoxic and is the most commonly utilized portion. If the root is used as a food material, violent purgative effects result from the resinoid properties.

Castor bean (*Ricinus communis*) has the prominent phytotoxins ricin in the seeds. This is a plant protein that induces all the effects that protein materials induce: gastrointestinal effects are prominent, yet fever, red-blood cell hemolysis, and with high doses, severe convulsions are induced.

Elderberry (*Sambucus americana*) is prominently known for inducing gastrointestinal involvement, yet the toxic principle has not been specifically identified. Based upon exposures, it is apparently present in all parts of the plant but remains an unknown. Elderberry also contains some cyanide that fortunately is volatilized by heating or through the fermentation process.

Daphnea (*Daphne* spp.) contains irritating glycosides, particularly dihydroxycoumarin, in the berries and bark of the plant. These compounds produce severe gastrointestinal irritation and bloody diarrhea is a common effect from significant ingestion.

Wisteria (*Wisteria sinensis*) is another plant with an unidentified toxin in its seed. What is clear is the gastrointestinal irritation that results from ingestion and the neuromuscular collapse that occasionally follows with initial consumption or frequent repeated exposures.

Black locust (*Robinia seudoccacia*) has a heat labile phytotoxins and glycoside in all plant parts. The seeds and the bark are the most commonly involved source of poisoning, although other less-available plant portions also induce risk. The gastrointestinal signs are complicated by additional incidences of depression and neurological effects.

The ability of different plant chemicals inducing similar digestive tract involvement and then associated various systemic effects highlights the numerous and various types of plant toxins that are capable of inducing similar yet complex toxic syndromes. The clinical effects of plant toxins are not simple and accurate diagnosis and therapy is largely based upon history of exposure so that appropriate management can be pursued. In the absence of a history of specific plant ingestion, general management and nursing care is required to alleviate the clinical effects.

Digitalis-Containing Plants

An important clinical group of plants are those with cardiotoxic properties. Many of these cardiotoxin-containing plants induce fatal toxicity by individuals eating the berries or chewing on leaves or flowers. Occasionally, the consumption of water from vases containing the flowers of digitalis-containing plants has been incriminated in poisonings. The initial syndrome from these ingestions is usually local irritation to the mouth followed by emesis. In contrast to toxicity from pure cardiac glycosides, poisoning from these plants will also be associated with diarrhea and abdominal pain due to the multi-presence of other irritants and saponins.

Mistletoe (*Phoradendron serotinu*) has toxic amines in its berries. There are sudden gastrointestinal effects with cardiovascular collapse similar to that seen from the ingestion of digitalis overdosage.

Lily-of-the-valley (*Convallaria majalis*) has cardioactive glycosides in its leaves and flowers that induce irregular heart rates and vomiting.

Foxglove (*Digitalis purpurea*) has significant concentrations of the digitalis glycosides in its leaves. This is the plant from which digitalis was originally isolated and serves as the template for the cardioactive plant poisons. Following initial gastrointestinal irritation and its effects, poisoned individuals develop headaches, irregular heart rates, and cardiac arrhythmias that often lead to tremors and neurological disturbances.

Oleander (*Nerium oleander*) has a cardioactive glycoside very similar to digitalis in all its plant parts. It is a potent toxin vehicle that has produced poisoning from minimal ingestion. One leaf has been accused of inducing life-threatening toxicity. Initial effects are those of nausea, abdominal pain, and bloody diarrhea that lead rapidly to circulatory irregularities, unconsciousness, and coma with death due to respiratory failure.

The cardioactive plant toxins have served as models for potent forensic situations used for suicide or for murder. The complexity of the plant chemistry has for many years confused identification of the toxic vehicle; however, current instrumentation and available chemical knowledge has now produced the ability to recognize, characterize, and identify the presence of these cardioactive glycosides in suspected cases of intoxication.

Nicotine, Cytisine, and Coniine-Containing Plants

These are neurological alkaloids that all exert similar actions. Numerous fatal poisonings have occurred because wild flora often contained complexes of these plants. Skin rashes often occurs in individuals who harvest tobacco leaves (*Nicotiniana* spp) and much more serious toxicity can occur if the tobacco leaves are consumed. Cytisine poisoning is most commonly resulting from the consumption of seeds from the pea-like pods of the golden chain tree (*Laburnum anagyroides*) while intoxications from Coniine usually occur from nibbling of the parsley-like leaves of poison hemlock (*Conium maculatum*) or from eating the seeds from that same plant. This alkaloid is related to nicotine, and all parts of the poison hemlock plant are toxic. It produces an ascending paralysis of the nervous system and is the plant that is famous in Greek history as the source of the fatal poisoning of Socrates.

The toxicity from these plants is usually initiated by vomiting that most commonly begins within 15 min of ingestion and is accompanied by a profuse salivation. Abdominal cramping and diarrhea are rare, yet the gastrointestinal syndrome is always present within 1 h of ingestion. This is followed by confusion, hyperpyrexia, incoordination, occasional mydriasis, and then tachycardia that can lead to fatalities from respiratory failure. Serious intoxications result from this group of plants.

Atropine-Containing Plants

Although a number of plants contain atropine and its related alkaloids, by far the most common and important poisoning is from jimsonweed (*Datura strammonium*). As an abundantly found wild weed, this plant affects persons who eat the seed or suck the flowers, often when they are attempting to use the plant as a hallucinogen. The early symptoms are mydriasis and dryness of the mouth which chronically presents itself as the poisoned individual indicating excessive thirst. The skin becomes hot and dry with redness and rash-like dermatosis occurring around the head and neck of the victim. In severe intoxications, there is pronounced hyperpyrexia, delirium, and hallucinations. These hallucinations may lead to dangerous behavior and activities that become life-threatening. Convulsions may appear in severe overdoses and ultimately coma can develop.

Atropine poisoning mimics a variety of pathologic states such as encephalitis, meningitis, and uremia. Atropine poisoning produces consistently equal and bilateral dilation of the pupil, a hot dry skin and significantly increased heart rates. Young children are more sensitive to atropine poisoning because of their lower tolerance to elevated body temperatures. Fatalities due to atropine-containing plant toxins are uncommon and recovery is usually complete within 24 h. Other than the much longer persistent papillary dilation, the other clinical effects resolve within 12–24 h.

Jimsonweed (*Datura stramonium*) contains at least three alkaloids (hyoscyanine, atropine, scopolamine) in all its plant parts. There are considerably more poisoning in humans than in animals from this plant due to the frequent and often habitual use of jimsonweed seeds and flowers to induce various hallucinogenic states. Ingestion of the alkaloids induces prominent thirst, delirium, and convulsion and in unfortunate states can lead to coma.

Belladonna (*Atropa belladonna*) contains most prominently the atropine alkaloid. **Potatoes and tomatoes** (*Solanum* spp.) contain the solanine alkaloid in the leaves, in green tomatoes, and in the sprouts of potatoes. In addition to the atropine-like effects, gastrointestinal irritation is usually seen and significant neurological involvement occurs.

The **nightshades**—black nightshade, Jerusalem cherry, bull nettle—(*Solanum* spp.) also have the solanine alkaloid in the berries and fruits. The unripe berries of this group of nightshade plants are deadly and responsible for numerous hospitalizations and near fatalities. In addition to the gastrointestinal signs, neurological effects with hot skin and pupil dilation are prominent. This entire group of atropine-like plants induces similar signs that make their clinical recognition of important medical value.

Plants Producing Convulsion and Changes in Nervous System

The principle groups of plants responsible for producing convulsions as primary toxic manifestations are those of the water hemlocks (*Cicuta* spp.). They are found only in wet, swampy areas, making them very lush and attractive to individuals looking for their carrot-like appearance. Usually within 15 min to 1 h after ingestion, the affected individual experiences nausea, salivation, emesis, and tremors. This is quickly followed by multiple grand mal seizures with death occurring secondarily due to prolonged anoxia encountered during the severe tonic muscular contractions.

Water hemlock (*Cicuta maculata*) is the most common of the *Cicuta* species and the most often involved in toxicity. The plant contains an unsaturated alcohol resinoid that is rapidly absorbed, quickly enters the nervous systems, and induces acute nervous signs, violent spastic convulsions, and death from respiratory depression. This plant produces such violent convulsions that fractures of jaw bones or limb structures are commonly found on post mortem in individuals who have died from this poisoning.

Members of the **heath family** (laurel, *laurus nobilis*; rhododendron, *Rhododendron* spp.; azaleas, *Rhododendron* spp.; pieris, *Pieris japonica*) all contain a hydrocarbon resinoid (andromedotoxin) in all their plant parts. This makes them extremely hazardous to produce the characteristic gastrointestinal irritation, mental and muscular depression, paralysis, lowered blood pressure, and eventual coma.

Crocus (*Colchicum autumnale*) has alkaloids in all parts of the plant. The compound is heat stable and uniquely is excreted in milk but slowly. Vomiting is characteristic as an initial effect followed by nervous signs of tremors and seizures.

White snakeroot (*Eupatorium rugosum*) contains a unique 16 carbon alcohol entitled trematol that has been historically a severe risk in the early days of Midwest settlements in the United States. Cattle grazing pastures that had this plant typically would develop ketosis with neurological signs characterized by muscular trembling. This became a serious human problem when the milk from these animals was consumed by families depending upon the nourishment of this product. Trematol is excreted in the milk in reasonable concentrations and thus induces similar *trembles* in children and adults drinking the milk.

Yew (*Taxus* spp.) is an attractive plant that unfortunately induces acute cardiac and gastrointestinal problems if the leaves are chewed. The highest concentration of the alkaloid is in the foliage with minimal amounts in the attractive red berries. This reduced concentration in the berries is often lifesaving since children are attracted to the colorful fruit and consumption is not uncommon. Following the typical gastrointestinal response of vomiting, individuals rapidly become weak and have cardiac rhythm changes that induce serious life risks, which are followed by convulsions that include depression of the respiratory center. Death within a few hours of consumption of this plant is typical.

Locoweed (*Astragalus* spp.) has been used for centuries by Native American tribes during their ceremonial rituals. The plant has several compounds present, all of which produce mind-altering hallucinogenic states. While minimal amounts are used in the ceremonies, larger consumption may lead to violent behavioral changes that can be life-threatening.

Cyanide-Containing Plants

A number of plants and their fruits contain cyanide or cyanide precursors that release cyanide upon enzymatic digestion either in the digestive tract or under appropriate environmental conditions. These cyanide compounds are usually in highest concentration in the pits or leaves of the respective plants; the cyanide released from seeds or pits require that the seed coat be broken by chewing that allows the enzymatic juices of the intestinal tract to complete the conversion to the highly toxic cyanide. Classical onset is extremely acute, resulting in the rapid onset of rapid respirations, collapse due to cellular anoxia and the development of bright-red blood and terminal seizures leading to death usually within 10–30 min of ingesting of the freely available active toxin. Fortunately a commercial *cyanide* kit is available and present in every emergency room. The treatment with intravenous sodium nitrite and sodium thiosulfate is highly effective, but must be initiated before cardiac function ceases.

Apples (*Malus* spp.) have a cyanogenic glycoside in the seeds; **cherries** (*Prunus* spp.) have the glycoside in pits and leaves; other *Prunis* species (**peaches** and **apricots**) have the cyanogenic glycoside in their pits and less concentrations in the leaves.

Hydrangea (*Hydrangea macrophylla*) is typical of a toxic plant in having a potent cyanogenic glycoside present in combination with irritating chemical principles that aggravate the clinical toxicity.

Plants Producing Dermal Contact Effects

Depending upon the chemical principle or structure involved, adverse effects from the consumption of these plants are rare, but may occur under unique conditions. In general, skin irritation is the effect with blistering or mechanical injury from hairs or spines that penetrate the skin.

Poison ivy (*Toxicodendron radicans*) and **poison sumac** (*Toxicodendron vernix*) are well recognized as having an allergic sap (3-*n*-pentadecylcatechnol) that is famous for the vesicant dermatitis produced. The fact that some individuals do not respond to contact from this plant while others show violent reactions attests to the allergic nature of this compound.

Snow-on-the-Mountain (*Euphoria marginata*) contains an irritating vesicant sap that is highly irritating to the conjunctiva of the eye and locally irritating to the skin and gastrointestinal tract mucosa if consumed.

Nettles (*Urtica* spp.) are clearly irritating from the prickly hairs on the plant's surface that produce an irritant reaction upon skin contact. The hairs also provide mechanical irritations that further accentuates the irritating nature of the toxin by allowing some skin penetration.

All these irritant-containing plants are contact toxicants that allow instantaneous removal of the irritant if abundant soap and water are used within 5–10 min of contact to wash off the toxicant. The instantaneous use of this cleansing will also prevent spread of the toxicant to other areas of the body upon itching and rubbing. Medications are available that will relieve the irritation and discomfort and may be used to prevent further mechanical spread of the compound. Unfortunately, there is no neutralizing material available once the compound has come in contact with the skin, thus strong and abundant washing is the best relief to reduce the effect of the irritating compounds.

Cactus (*Pedilanthus* spp.) has strong spines capable of penetrating skin. The penetration is worsened by any force that drives the thorn-like spines further into the flesh. Rapid removal of the spines is important to avoid ultaneous granulomas from developing. These body reactions are attempted to wall off the irritation from the spine, but often produce ulcers and highly inflamed lesions in several parts of the body that had contact with the cactus spines.

The signs of most plant ingestions are gastrointestinal involvement, with neurological effects, depression, seizures,

and coma as occurring if severe intoxication occurs. The history of consumption of plants or the proximity to plant materials is most helpful in diagnosis and is an important phase of the problem-solving aspects of dealing with plant poisonings. Very few direct antidotes are available for treatment, although the *cyanide* kit is an exception and has historically been lifesaving in numerous cyanide-containing plant exposures. In general, the treatment of most plants is to remove as rapidly as possible the plant material from the digestive tract through inducing emesis or hastening a laxative effect, and the use of intestinal decontamination through binding agents such as activated charcoal to minimize the absorption of the plant's toxicity. In severe cases where prolonged vomiting and diarrhea have been present, the use of fluids and electrolyte infusions are helpful to maintain hydration and often are lifesaving. General symptomatic care to relieve pain and discomfort are also employed and found valuable by the affected victims.

A poisonous plant glossary (Table 19.1) provides the common names, scientific names, and general toxic effect of each of the toxic plants of concern discussed earlier. This table can be useful to identify from all the many plants in the environment those which are a significant hazard and the type of toxicity or adverse effect that each could produce. Note that many of those listed affect more than one organ system.

Laboratory Identification of Plant Toxins

The classification and identification of plant compounds associated with cases of poisoning is often subjective; indeed, in many cases plant chemicals are deemed toxic by association with poisoning episodes. They are often the major compound found in a plant known to be poisonous and associated with related compounds that have been shown to produce similar symptoms of poisoning. Years of analysis of plant toxins and the more recent development of specific and sensitive instrumentation have found that plants contain many chemicals that are potentially harmful.

Some plant constituents are not particularly toxic in the form in which they exist in the plant, but on ingestion, they undergo transformations to more toxic principles. Thus, when investigating the effect of plant toxins in mammalian systems, metabolites of the toxin have often been the only detectable compounds. With some groups, such as alkaloids, there is considerable variability within a species in the level and composition in the toxic fractions, depending on location, stage of growth, and other environmental conditions [5].

Analytical resources available to analyze for plant toxins vary significantly from laboratory to laboratory, yet there are analytical procedures to identify the numerous categories and wide spectrum of potential plant toxins. In addition to the classic analytical techniques used (e.g., thin-layer chromatography, high performance liquid chromatography, gas chromatography), other methodologies are also employed. Solvent extraction techniques, super critical fluid extraction, lead acetate precipitation, droplet counter current chromatography, centrifugal thin-lay chromatography, and reverse-phase low-pressure chromatography columns are also employed. Even more recently, the wide application of mass spectrometry and its various combinations with chromatography methods have allowed for identification of alkaloids and other plant toxins with minimal plant sample available (see Chapter 41). Even more recent advances have suggested that capillary electrophoresis and further combinations with mass spectrometry and specific detector systems will improve the sensitivity and detection of illusive plant toxins.

The range of specific plant toxic chemicals now detectable include the following [5]: pyrrolizidine alkaloids; piperidine alkaloids; pyridine alkaloids; indole, tryptamine, β-carboline and related alkaloids; quinolizidine alkaloids; steroid alkaloids; diterpene alkaloids; indolizidine alkaloids; tropane alkaloids; isoquinoline alkaloids; other alkaloids such as colchicine, sesbanimide, and dioscorine; cyanogenic glycosides; glucosinolates; phenolic glycosides; saponins and cardiac glycosides; nitropropanol glycosides; other glycosides such as azoxyglycosides, naphthalenes, anthracenones, and anthraquinones; lectins or hemagglutinins including abrin, jatrophin, momordin, phoratoxin, and ricin; enzymes such as thiaminase; amino acid analogues, and nonprotein amino acids, such as hypoglycin, β-cyano-L-alanine, and S-methylcysteine sulfoxide; the phenylethylamines, including tyramine, N-methyl-β-phenethylamine, hordenine, β-phenylethylamine, and galegine; selenium compounds usually stored as amino acids (selenocystine, methylselenocysteine, γ-L-glutamyl-Se-methylseleno-L-cysteine; sesquiterpene lactones; diterpenes such as croton oil, ingenol, and tigliane esters, simplexin, and the grayanotins I, II, III, IV, and XIV; terpenes (trematol) and hydrocarbons (tanacetine, malvalic acid, cicutoxin, and crepenynic acid); and the numerous oxalates, nitrates, sulfides, and organofluorine compounds that exist seemingly throughout the plant kingdom as soluble acid salts, insoluble salts of calcium and magnesium, and as numerous disulfide and similar inorganic forms [5]. Such a wide spectrum of organic and inorganic chemicals challenges the analytical chemist and provides continuing excitement for toxicologists.

Plant Toxins Present in Milk [6]

Mammary gland secretions are one of the frequent ways in which chemicals are eliminated from a consuming individual's body. Compounds from plants are particularly suited for excretion via this route since many are organic in nature and have considerable lipid partitioning into the mammary gland secretions. Exposures may occur directly from mothers having recently consumed plant materials and suckling infants receiving the milk. All species may be affected by this route of exposure. In commercial dairy herds, animals pasturing on plants that contain compounds likely to appear in milk offer hazard for the general public purchasing such processed milk and milk products. Fortunately, when the milk from one herd exposed to toxins excreted in milk is mixed with the large volume of milk from nonexposed cows, dilution results

TABLE 19.1
Poisonous Plant Glossary

Common Name	Scientific Name	Toxic Effect
Apple	*Malus* sp.	Cyanide
Apricot	*Prunus* sp.	Cyanide
Azalea	*Rhododendron* sp.	Neurological
Belladonna	*Atropa belladonna*	Atropine-like
Black locust	*Robina pseudoacacia*	Gastrointestinal
Black nightshade	*Solanum nigrum*	Atropine-like
Bull nettle	*Solanum* sp.	Atropine-like
Buttercup	*Ranunculus* sp.	Gastrointestinal
Cactus	*Pedilanthus* sp.	Dermatological
Castor bean	*Ricinus communis*	Gastrointestinal, convulsive, tremors
Cherry	*Prunus* sp.	Cyanide
Crocus	*Colchicum autumnale*	Neurological
Daffodil	*Narcissus* sp.	Gastrointestinal
Daphnea	*Daphne* sp.	Gastrointestinal
Death camas	*Zigadenus* sp.	Gastrointestinal
Dumbcane	*Diffenbachia* sp.	Oxalate
Elderberry	*Sambucus Americana*	Gastrointestinal, cyanide
Fescue	*Festuca prateusis*	Gangrene, hormonal, reproductive
Fireweed	*Kochia scoparia*	Liver
Foxglove	*Digitalis purpurea*	Cardiac, digitalis
Greasewood	*Sarcobatus*	Oxalate
Halogeton	*Halogeton glomeratus*	Oxalate
Hydrangea	*Hydrangea macrophylla*	Cyanide
Ivy	*Hedera helix*	Gastrointestinal
Jerusalem cherry	*Solanum pseudocapiscum*	Atropine-like
Jimsonweed	*Datura stramonium*	Atropine-like
Lamb's quarters	*Chenopodium album*	Oxalate
Lantana	*Lantana camara*	Gastrointestinal, photosensitization, neurological
Laurel	*Laurus nobilis*	Neurological
Locoweed	*Astragalus* sp.	Convulsive, tremors
Lily-of-the-valley	*Convallaria majalis*	Cardiac, digitalis
Lupine	*Lupinus argenteus*	Liver
Marijuana	*Cannabis sativa*	Neurological
Mayapple	*Podophyllum peltatum*	Gastrointestinal
Milkweed	*Asclepias* sp.	Gastrointestinal, neurological
Mistletoe	*Phoradendron serotinum*	Cardiac, digitalis
Mother-in-laws tongue	*Sanseivera* sp.	Oxalate
Mountain laurel	*Kalmia latifolia*	Neurological
Mustards, crucifers	*Brassica kaber*	Gastrointestinal
Nettles	*Urtica* sp.	Dermatological
Oak	*Quercus* sp.	Kidney
Oleander	*Nerium oleander*	Cardiac, digitalis
Peach	*Prunus* sp.	Cyanide
Philodendron	*Philodendron* sp.	Oxalate, kidney
Pieris	*Pieris japonica*	Cyanide
Pigweed	*Ameranthus retroflexus*	Kidney
Poinsetta	*Euphoribia pulchenima*	Gastrointestinal
Poison hemlock	*Conium maculatum*	Neurological
Poison ivy	*Toxicodendron radicans*	Dermatological
Poison sumac	*Toxicodedron vernix*	Dermatological
Pokeweed	*Phytolacca americana*	Gastrointestinal
Potato	*Solanum tuberosum*	Atropine-like
Precatory bean	*Abrus percatorius*	Gastrointestinal
Ragwort	*Senecio* sp.	Cardiac, digitalis

TABLE 19.1 (continued)
Poisonous Plant Glossary

Common Name	Scientific Name	Toxic Effect
Red maple	*Acer rubrum*	Hemolytic
Rhododendron	*Rhododendron* sp.	Neurological
Rhubarb	*Rheum rhaponticum*	Oxalate
Rosary pea seed	*Abrus precatorius*	Gastrointestinal
Senna	*Cassia fasciculata*	Gastrointestinal
Snow-on-the-mountain	*Euphorbia marginata*	Dermatological
Sorghum	*Sorghum* sp.	Cyanide
St Johnswort	*Hypericum perforatum*	Photosensitization
Tobacco	*Nicotiana* sp.	Nicotine-like
Tomato	*Lycopersicon lycopersicum*	Atropine-like
Veratrum	*Veratrum californicum*	Teratology
Water hemlock	*Cicuta maculate*	Convulsive, tremors
White snakeroot	*Eupatorium rugosum*	Neurological
Wild Indigo	*Baptisia* sp.	Gastrointestinal
Wisteria	*Wisteria sinensis*	Gastrointestinal
Yew	*Taxus* sp.	Convulsive, tremors

in considerable protection; however, the potential risk is still there. This is especially true if home-produced dairy products are consumed from individual herds in which dilution of the plant toxins is unlikely. Although excretion of natural toxins via the milk is not considered a major route of excretion, the milk emulsion of lipids in an aqueous solution of proteins may contain mixtures of virtually any toxin or compound that is in solution in the mother's or cow's body. The toxins may be bound to blood proteins, be in solution in the circulating lipids, or be freely circulating in the plasma. All of these forms can cross mammary cell membranes, generally by simple diffusion.

Some poisonous principles can be excreted very readily in milk. These principles are usually those of high fat solubility that are concentrated in the lipid portion of the milk, have long biological half-lives, and are frequently present in milk at high concentrations and for long periods of time. Suckling offspring or consumers of milk from cows excreting such toxins are especially vulnerable for toxicity. White snake root (*Eupatorium rugosum*) and rayless goldenrod (*Haplopappus heterophyllus*) contain trematol, which is probably the best example of a natural plant toxicant transferred via milk. It induces a condition in cattle called *trembles* and in humans causes a serious debilitating disease referred to as *milk sickness*. Two plants notorius for having that toxin present and available for milk contamination are white snake root (*Eupatorium rugosum*) and rayless goldenrod (*Haplopappus heterophyllus*). Both are widely disseminated in the Northeast and Midwest, offering ample opportunity for inclusion of the toxin in milk products.

Pyrrolizidine alkaloids have been shown to be transferred in the milk of cattle grazing on pastures infested with plants containing these toxins. The important species of toxic plants containing pyrrolizidine alkaloids are *Senecio, Crotalaria, Heliotropium, Trichodesma, Amsinckia,* and *Echium*. Of these, the *Senecio* species are the most potentially hazardous, particularly tansy ragwort (*Senecio jacobaea*) and threadleaf groundsel (*Senecio douglasii*). In addition to their contamination of milk, plant toxins from this group also have potent hepatotoxic risks.

Glucosinolates occur in the Cruciferae family and also may appear in milk. The genus *Brassica* includes cabbage, broccoli, kale, rape, mustard, turnips, and other plants. Other glucosinolates-containing plants include meadowfoam (*Limnanthes*), watercress (*Nasturtium*), radish (*Raphanus*), horseradish (*Amoracia*), and stinkweed (*Thlaspi*). Piperidine alkaloids are widely spread and are found in poison-hemlock (*Conium maculatum*) and in many plants of the genera *Nicotiana, Conium, Lobelia, Pinus, Duboisia, Sedum, Withania, Carica, Hydrangea, Dichroa, Cassia, Prosopis, Genista, Amondendron, Lupinus, Liparia,* and *Collidium*. Lupines (*Lupinus* spp.) contain the quinolizidine. Other members of this group include scotch broom (*Cytisus*), golden chain (*Laburnum*), and mountain thermopsis (*Thermopsis*).

Locoweed (*Astragalus lentiginosus*), used in ritualistic ceremonies, contains the indolizidine alkaloid swainsonine that is transferred in milk. *Oxytropis serecia* is another swainsonine-containing plant in the locoweed category. Grasses of the lush green pastures and other plants that have high chlorophyll content are metabolized to indole and 3-methylindole in the rumin of cattle consuming these forages. These metabolites of L-tryptophan are also transferred in milk to individuals consuming such. Colchicine is a poisonous alkaloid in autumn crocus (*Colchicum autumnale*) that is stable after drying, heating, or storage; it is also excreted primarily in lactating animals via the milk. Because of its slow excretion, a cumulative effect is likely from even small doses received daily over time.

Numerous other plants, particularly those on the western ranges of the United States [6a], are capable of accumulating

high levels of selenocompounds that are not only hazardous to consuming animals, but also are excreted in cow's milk and concentrations in direct proportion to the selenium intake. Certain plant species of *Astragalus* and *Stanleya* have this selenium-accumulating capability.

The presence of other compounds in milk may induce a disagreeable taste or odor. Plants of the genus *Allium* include species of onion and garlic that taint the milk produced. The sesquiterpene lactones (*Tenulen*) in sneezeweed and bitterweed impart a bitter flavor to the milk of lactating animals grazing these plants. Bracken fern (*Pteridium aquilinum*) produces milk from cows fed this plant that induces urinary carcinomas.

With increasing concern for food safety, recognition and appreciation of the potential for plant toxins contaminating human milk supplies is timely. The fact that plant alkaloids are frequent in these situations and are quite basic, allows them to accumulate in milk. The combination of the basicity and fat solubility of the chemical produces accumulation of the plant toxins in the milk with reduced excretion by other natural processes. Other factors to be considered in evaluating the human health risk from plant toxins or from the heavy consumption of milk by infants and young children, who are already more susceptible to the toxins, is the consumption of milk produced by lactating mothers who use herbal remedies, the availability of these toxic plants to lactating dairy animals, and the consumption of milk from point sources where free grazing milking animals have access to toxic plants. The fact that few laboratory methods have been developed for monitoring natural toxicants of plant origin in milk and milk products heightens the need to assess and monitor their potential danger to human health and safety.

Some Interesting Plant Toxicoses Observations

Plants are capable of adversely affecting many specific organ system targets. One of the most common is skin, resulting in dermatitis as a result of hypersensitivity to plant toxins or direct contact with irritant saps or spines. Mucus membrane irritation is a close second due to contact or consumption of plants with irritant saps or containing oxalate crystals and/or enzymes. Gastrointestinal reactions are by far the most common adverse effect to ingestion of a plant part. Indeed, it is almost a rarity to observe a true plant poisoning without some form of gastrointestinal irritation, which may be severe and may require aggressive fluid therapy. Several plant groups and individual plant species are capable of causing liver damage, and although it usually results from the long-term use of plants for various purposes, some plant products produce a relatively acute hepatotoxicity. Renal impairment after the plant ingestion is rare and is usually confined to associations with dehydration or profound shock. Some plants, such as rhubarb leaves, are noted to cause kidney failure due to the systemic absorption of soluble oxalate salts. Involvement of the hematopoietic system is not uncommon and often involves intravascular hemolysis, methemoglobinemia formation, and breeding tendencies caused by coumarins in herbal teas or from penny royal oil. Parts of many plants, including pears, peaches, apples, apricots, cherries, certain lima beans, and hydrangea contain the amygdalin glycoside that can release cyanide. These are acute episodes and in some cases have been related to ingestion of health food supplements.

Other common plants contain cardiac glycosides that are responsible for effects and in some cases death include fox glove (*Digitalis purpurea*), oleander (*Oleander nurium*) lily-of-the-valley (*Convallaria majalis*), and red squill (*Urginea maritima*). Other less studied plants are reported as *depressing the heart and slowing the pulse*. These effects may be secondary to other organ effects. The ergot alkaloids used for migraine headache control and other purposes may cause severe arterial vasospasm. The autonomic nervous system effects are well described for anticholinergic toxicity from plant products such as Jimson weed (*Datura* spp.) along with members of the Solanaceae family that includes deadly nightshade (*Atropa belladonna*) and henbane (*Hyoscyamus niger*). Nicotine is present in various members of the *Nicotiana* plant family and causes peripheral ganglionic stimulation with seizures and death possible.

Central nervous system changes, including depression and stimulation, may also be frequent plant effects. Many plants, such as tobacco, coffee, tea, marijuana, cocaine, and others, are actively *harvested* to achieve such central nervous system alterations. More severely neurotoxic plants and their derivatives are water hemlock (*Cicuta maculata*) and poison hemlock (*Conium maculatum*). The former is well recognized to cause immediate seizures after ingestion while poison hemlock causes death through paralysis [7].

Despite the widely recognized concern for problems from plant ingestions, some studies have concluded that most plant and herb exposures are mild and do not need aggressive treatment. This conclusion was reached despite the fact that complications were detected in this study, especially following intentional and/or chronic cases of plant ingestion [8].

The circumstances and resulting acute toxicity from ingestion of water hemlock (*Cicuta birosa*) is evident in the report of two individuals who viewed the plants growing in a marshy area and believing it to be an edible species, such as wild parsnips, artichokes, celery, sweet potatoes, or sweet anise. They ate several bites of the root and 20 min later became nauseous, experienced salivation and stomach cramps, and vomited repeatedly before developing grand mal seizures and convulsions leading to coma. Prompt attention at an emergency center resulted in recovery by the 11th day [9].

The toxicity and high allergenic and anaphylactic response from castor beans (*Ricinus communis*) and their dust presents similar risk to exposed individuals. The acute gastroenteritis, gastrointestinal bleeding, hemolysis, hypoglycemia, and fluid and electrolyte depletion are common effects from exposure to these plant materials. Mortality rates of almost 10% have been reported [10].

Acute atropine poisoning from the ingestion of Jimsonweed (*Datura* spp.) is a significant risk to children from their ingestion. An average of almost 80 admissions per year occurred to hospitals in New Zealand with poisonings

from these plants related to their belladonna, hyoscine, and atropine content. Although clinical poisoning results, fatalities are rare but do occur [11].

Juniper tar (cade oil) is distilled from the branches and wood from *Juniperus oxycedrus* containing etheric oils, triterpene, and phenols. It is a frequent constituent of many folk medicines and is used for various antipruritic, keratolytic, and antimicrobial benefits in human and veterinary dermatology. Significant anti-inflammatory activity is also noted. The tar is ingested as a liquid extract of the tree by individuals in the Mediterranean region, yet has caused fever, severe hypotension, renal failure, hepatotoxicity, and cutaneous burns when applied to the face. Using supportive and symptomatic care, a recent patient improved but required 11 days to return to good health [12].

Similarly, the inclusion of plant materials in a Chinese vegetable entrée at an international buffet was associated with multiple cases of burning and facial edema after lunch in an office cafeteria. Pain and stinging/burning of the oral mucosa and potential airway obstruction due to the severe edema resulted from the presence of plant material containing raphides such as plants of the genera *Dieffenbachia philodendron* and *Brassica* that contain needle-shaped crystals of calcium oxalate in their specialized cells. These raphides are excreted in response to mechanical pressure causing irritation and subsequent injury, which may be life threatening if the respiratory passageways become swollen [13].

Not all such toxicities result from direct plant ingestion. The rhododendron (*Rhododendron ponticum*) grows extensively on the eastern flat sea area of Turkey. This plant species contain toxic diterpenes (*Grayanotoxins*) in their nectar, which is then used by bees in the area to generate honey from which commercial products are developed. Ingestion of the honey derived from this plant cause profound hypotension and bradycardia, allowing it to be known locally as *madhoney* [14]. Although a prominent problem in Turkey and the Mediterranean bordering countries, honey intoxications from grayanotoxin contamination have also been reported as early as 1794 in the Great Smoky Mountain region of the United States and northward, probably associated with mountain laurel (Kalmia latifolia*)* and sheep laurel (Kalmia augustifolia*)* pollination. What may seem to be a regional concern has more global dietary safety potential, and the availability of imported honey further suggests the need for continuing vigilance [15].

The oleanders (pink oleander, *Nerium oleander*; yellow oleander, *Thebetia peruviana*) contain cardenolides that exert positive ionotropic effects on the hearts of consuming animals and humans. Toxic exposures to humans, domestic animals, and wildlife from these oleander toxins occur with regularity throughout the geographic region where these plants grow. Although the human mortality associated with oleander ingestion is generally low due to the purgative action that occurs rapidly following ingestion, small children and domestic livestock consuming reasonable amounts of the plant are at increased risk of oleander poisoning [16]. The wide distribution and the fondness of individuals to decorate their surroundings with this attractive yet toxic plant provides an interesting example that the *one medicine* concept even applies to plant toxicities.

TOXINS FROM ALGAE

Algae produce toxins; their presence in water should alert one to potential hazards. Toxic blue-green algae (cyanobacteria) are commonly found growing in fresh and salt water in temperate areas worldwide. They are commonly found in fresh water lakes, livestock ponds, rivers, streams, canals, and ditches. Under the appropriate warm environmental temperatures, calm weather, and stagnate nutritionally rich conditions, very rapid growth of blue-green algae results in blooms that accumulate on the surface of the water and provide opportunity for neurotoxins or hepatic toxins to be produced. These blooms of blue-green algae often occur in the northern United States in the late summer or early winter, and are associated with hot, calm weather, decreased rainfall, and increased nutrients in the water from fertilization or animal wastes; winds that blow in concentrate the algae along the shorelines allow ample opportunity for exposure. In the southern states, algal blooms occur all year long when the optimal environmental conditions are met. The blue-green algae *Microcystis* and *Nodularia* produce the cyclic peptide hepatotoxins microcystin and nodularin, while the algae *Anabaena, Aphanizomenon,* and *Oscillatoria* produce the potent neurotoxins *Anatoxin-A* and *Anatoxin-A(s)* [17].

The neurotoxin anatoxin-A is a bicyclical secondary amine that causes deep polarization of nicotinic membranes, acting as a potent postsynaptic neuromuscular blocking agent. The deep polarization of neurononicotinic membranes is rapid and persistent and leads to respiratory paralysis. The related neurotoxin anatoxin-A(s) inhibits acetylcholinesterase in the peripheral nervous system. Fortunately, this toxin does not seem to cross the blood brain barrier, but the neurological effects on skeletal muscle dysfunction are obvious. Cyanobacteria ingested with water are rapidly broken down in the gastrointestinal tract environment. In the stomach acids, the organisms are lysed with the resulting release of toxins that, when free, are rapidly absorbed further from the small intestine. The hepatotoxins microcystin and nodularin are transported to the liver hepatocytes through a specific carrier-mediated uptake mechanism. There they inhibit protein phosphatasis, which initiates a cascade of events leading to rapid massive hepatocyte necrosis, intrahepatic hemorrhage, and shock.

Effects of Algal Toxins

When the blue-green algae grow in polluted nutrient-enriched waters or ponds, they can appear and disappear in a matter of days with the varying temperatures and weather conditions. The results can be the rapid development of varying toxins depending upon the algal growth present, and the clinical effects may similarly be varied depending upon the

presence of the select toxin. Generally, all the toxic effects may be grouped into those that relate to nervous effects producing relatively rapid death, or more slow clinical damage that may require several days to weeks to resolve. The underlying feature of the latter group is that liver damage is the target effect, and if the victim survives the early dramatic hepatic injury or if small to moderate exposures occur over a relatively few days, the individual may develop subtle or subclinical hepatotoxicosis that may be health-depriving for several weeks.

Neurotoxins

The two primary groups of algal neurotoxins are the anatoxins produced by filamentous *Anabaena flosaquae* and the aphantoxins from *Aphanizomenon flosaquae*. The anatoxins are potent postsynaptic depolarizing neuromuscular blocking agents that affect the acetylcholine receptors producing staggering, muscle fasciculations, gasping for breath, convulsions, and even opisthotonus. Death is by respiratory arrest, which may occur within minutes or a few hours, depending upon the dosage and the species involved. Victims need only to ingest a few milliliters of the toxic surface bloom-contaminated water to receive a lethal dose. Some victims also demonstrate salivation, laceration, urinary incontinence, and defecation prior to death by respiratory failure.

The aphantoxins neurotoxins inhibit nerve conduction by blocking sodium channels, yet induce very similar clinical damage as the anatoxins.

Hepatotoxins

Microcystin is the fast death factor produced by *Microcystis aeruginosa*, identified as a cyclic heptapeptide. This cyclic peptide structure is transferred to its morphologic appearance in waters where it characteristically shows up as small packets of glistening spheres under the microscope. Its unique microscopic appearance has been valuable for identification by public health officials and biologists in pollution situations.

Another low molecular weight heptapepide hepatotoxin with similar microscopic appearances is cyanoginosin (BE-4 toxin), a product of a unique strain of *Microcystis aeruginosa* WR-70 [18]. Characteristic liver injury is produced either acutely or more subtly with limited dose exposure.

Other hepatotoxic toxins have been found generated by *Nodularia spumigena*, *Cylindrospermopsis raciborskii* (Anabaena raciborskii), and by varieties of *Oscillatoria agardhii*.

All the microcystin hepatotoxins cause death by hypobolemic shock resulting from interstitial hemorrhage into the liver. The rapid and extensive centrilobular necrosis of the liver comes from extensive fragmentation and vesiculation of hepatocyte cell membranes. It has been suggested that instead of destroying the hepatocyte cell membrane directly, the hepatoxins affect a cytoskeletal component, which, depending upon dosage, may result in sudden loss of total organ function or more prolonged effective damage [18].

Other Algatoxins

Scytophycins produced by certain strains of *Scytonema pseudohofmanni* are strongly cytotoxic in cell cultures. *S. hofmanni* produces cyanobacterin that has strong anticyanobacterial activity and is considered an algaecide for cyanobacteria. A cytotoxic alkaloid has been isolated from *Hapalosiphon fontinalis* strain V-3-1 that has broad antialga and antimycotic activity. All these toxins have relatively low lethal toxicity in laboratory studies, but when combined with all the other cyanobacteria that are found in almost all fresh water algal poisoning reports, potent neurotoxic alkaloids, hepatotoxic peptides, and these several less lethal but more selective cytotoxic compounds demonstrate the unique combination of hazardous effects possible from this group of toxins [18].

The individual cyanobacteria with their associated toxins may produce a spectrum of toxicity determined by the specific population of algae present and the concentration of their respective toxins. Acute death may occur with few clinical signs. Other victims may present with muscle tremors, rigidity, lethargy, convulsions, and death from respiratory paralysis within minutes to hours following onset of effects. Another cascade of signs consistent with inhibition of cholinesterase includes salivation, urination, laceration, and defecation, together with tremors, convulsions, and respiratory difficulty; death from respiratory arrest may occur again within a few hours. Finally, a myriad of signs generally related to liver damage may present a cascade of depression, vomiting, diarrhea, gastrointestinal slow down, weakness, and anemia. While death often occurs within several hours, it may be delayed for several days. Individuals who survive the initial toxicosis may develop secondary photosensitization due to the compromised liver function and presence of photodynamic sensitive metabolites being retained.

Public Health Significance

The potential for mass populations of toxin-producing cyanobacteria in natural and controlled water bodies presents a serious public health awareness to avoid such entities, or if circumstances permit their development to respond appropriately with clinical management, chemical detection, and control of human and animal exposure. Toxic cyanobacterial populations have been reported in fresh waters in more than 45 countries and in numerous brackish, coastal, and marine environments. The biological and circumstantial variations that occur in the hazards of natural origin clearly suggest that uncertainties and numerous gaps in knowledge are present. However, the importance of identifying the exposure media of potable and recreational waters, as well as animal and plant foods that may contribute to the human population exposure is paramount. Steps to develop and implement risk management strategies for cyanobacterial toxins in water bodies are recalled with each outbreak of toxicity, and the gradual recognition by public health communities of the collective experience and wisdom is slowly building [19].

Paramount in the health detection for dealing with outbreaks of algaintoxication is the detection of the cyanobacterial agents present. Their wide variety and structure has led to the common practice of reporting the presence of *toxins* as microcystin-LR equivalents, regardless of which hepatotoxic variant is present [20]. The increased availability of high pressure liquid chromatography with online mass spectral analysis has facilitated more accurate detection of toxic variance, but due to several microcystins sharing the same molecular mass, definitive identification may be difficult. A further difficulty is the requirement for sample processing before analysis. Recent new technologies employing recombinant antibodies and molecularly imprinted polymers are now being extroided to develop assays and biosensors for these algatoxins. Their advantages in being highly sensitive, not requiring sample processing, and offering simple and less expensive alternatives to existing analytical techniques is encouraging [20].

An additional hurdle is the identification of some of the diverse groups of cyanobacteria being able to produce a wide range of toxic secondary metabolites. These have been classified as neurotoxins, hepatotoxins, cytotoxins, dermatotoxins, and irritant toxins. Cyanobacterial brooms are particularly hazardous due to this production of secondary metabolites and their endotoxins that may be broadly toxic to humans, animals, and plants. These compounds differ in mechanisms of uptake, affected organs, and molecular mode of action. Investigating and understanding the environment and effects of these toxins on the organisms receiving these, as well as their basic toxic mechanisms, continue the challenges raised by the presence of the cyanobacteria organisms in water supplies [21].

Prevention and Control

The wide extremes in adverse health effects resulting from cyanobacteria contact in humans highlight the importance of public health measures to prevent and control the annual often unexpected development of toxicity scenarios. The neurotoxic effects from the anatoxins that mimic the effects of organophosphate insecticides, the dramatic hepatic and digestive tract injuries produced by the microcystins capable of producing acute death or lingering hepatic failure, and the even more diverse health ravaging protein synthesis inhibition of cytotoxins, the skin and mucus membrane irritation and tissue inflammation from the irritant algatoxins, and the confusing effect of cyanobacteria lipopolysaccharides in inducing a toxic shock like syndrome cries out for measures to prevent and control future episodes induced by these organisms.

To halt nutrification of ponds and lakes in natural wildernesses and agricultural and industrial areas is indeed difficult, and merely placing cautionary notices and signs to prevent human exposure to *blooming* waters is a challenge for the human nature. Education programs to alert rural inhabitants and campers seeking to enjoy the pleasures of nature are a start but still do not avoid the human fragility of oversight. Continuing observation and recognition of circumstances and environmental conditions that spawn the rapid development of the cyanobacteria toxins are currently the most commonly used preventive measure by public health and regulatory agencies.

Individuals who have become intoxicated from organisms through the public water supply or through contaminated waters used in preparing various commercial or medicinal formulations are particularly vulnerable. Recently, three human probiotics, *Lactobacillus rhamnosus* strains GG, *Lactobacillus rhamnosus* strain LC-705, and *Bifidobacterium lactis* strain Bb12, were found to bind the microcystin cyanobacteria peptide found in water solutions. As much as 46% removal occurred and offered the possibility of utilizing incubation of sensitive water materials with these probiotics to markedly reduce potential toxicity [22].

The increasing recognition of cyanobacterial toxins in the drinking water from water treatment plants [23] and the dramatic documentation of a variety of cyanobacteria and microcystin concentrations in public water supply reservoirs [24] sends up red flags to heighten and intensify the control measures in place to prevent and avoid contamination of these human water sources. The finding of six toxic genera of cyanobacteria with a greater than 40% frequency of hepatotoxic strains provides sufficient recognition that the development and application of detection and control measures for the avoiding of widespread consumption of these contaminated waters is necessary.

TOXINS FROM MUSHROOMS AND TOADSTOOLS

If the rapid and reasonably accurate identification of poisonous plants is difficult, the identification of toxic mushrooms through merely visible examination is virtually impossible. The old wives' tales that suggest that if the skin of a mushroom is easily removed over the cap…or of the mushroom will not blacken silver when cooked…or if the mushroom's gills are grey, the mushrooms are nontoxic, are simply not true! Ultimately, there are no hard-and-fast rules to distinguish the delicacy from the deadly.

Mushrooms are the fruits of fungi that are made up of multiple threads called hythae. Mushrooms grow on several different types of materials, such as wood, ground, plant or animal material, or dung. The spores of mushrooms are a valuable identification aide. Spores are used to form *prints* as part of the identification process. Many different shapes are described for the various parts of mushrooms, and when put together, they assist in identification. Putting all these items together requires experience, knowledge, and skill—all of which are necessary for the proper identification of genus and species and the determination of whether safety or toxicity is present.

Although *Aminita* (e.g., *A. muscaria, A. phalloides*) is the most toxic genus of mushrooms, the specific toxic principle varies significantly with the specific species of mushroom

collected and ultimately consumed. These toxic principles produce clinical signs that vary from excitement and hallucinations…to severe gastrointestinal involvement with few fatalities…to jaundice and explosive liver damage…to circulatory failure…to coma and death within hours to days. Clinicians recognize the importance of not relaxing treatment measures until the patient is fully recovered from any one of the varied and complex clinical syndromes produced by ingestion of toxic mushrooms.

Clinical Syndromes

The mushroom so frequently pictured in story books with the red cap covered in polka dot fashion with white spots is *Amanita muscaria*. Mushrooms in this group produce maniac excitement and hallucinations within 2–3 h after ingestion. This is usually preceded after an initial period of drowsiness leading to the more excitable phase. The central nervous system excitement does not usually exceed more than 3–4 h in duration.

There is a specific group of muscarine-containing mushrooms that will produce sweating, salivation, and colic that begins within 15 min of ingesting. Pulmonary edema and moist rales are frequently present. Fortunately, this toxin does not cross the blood-brain barrier in sufficient concentration to produce central nervous system effects.

Another subgroup of toxic mushrooms produces severe gastroenteritis that develops with a rapid onset of action. Whereas wild mushrooms eaten by adults invariably have been cooked, a process that markedly reduces or inactivates several irritants and even some major toxins, this is not the case with children, which may account for some of the pediatric fatalities following the ingestion of uncooked *nontoxic* species.

The most serious mushroom poisoning is that in which there is a latent period of about 12 h prior to the onset of symptoms. These intoxications have a grave prognosis and consume considerable therapeutic resources. The mushrooms involved in this syndrome belong to the *Amanita phalloides* group. They contain two classes of thermostable cyclic polypeptides: the phallotoxins and amatoxins. Each of which has distinct pharmacological properties. The phallotoxins act initially at the end of a brief latent period by producing nausea, vomiting, painful colic, and a watery profuse and sometimes bloody diarrhea. Following an apparent recovery and a symptom-free period of 3–5 days, during which symptoms are relieved, the amatoxins exert their hepatorenal toxicity in very evident fashion. Jaundice develops, abdominal pain and weight loss become obvious, and a severe extension of the initial poisoning occurs. If only a small amount has been ingested, only renal tubular necrosis may occur. Large mushroom ingestions often produce fatal hepatic necrosis even before the kidney damage becomes obvious.

Although both of these clinical syndromes are seen, the hepatorenal effects terminating in liver failure and produced by intermediate dosages of mushrooms are the more commonly accounted phenomena. An especially high mortality is associated with mushroom intoxication in children.

Specific Mushroom Toxins

So what are the various chemicals specifically toxic in mushrooms? There are various categories and types of mushroom toxins, most of which have a characteristic set of signs. Animals as well as humans are poisoned by mushrooms, and the ingestion of one fungal species by an animal does not make it safe for human consumption.

Cyclopeptides [25]

Amanita and *Galerina* species of mushrooms are most commonly involved in producing anatoxins. These toxins inhibit mammalian nuclear RNA polymerase B forms, which are involved in transcription of DNA to messenger RNA, thus causing inhibition of protein synthesis at the ribosome level. Their primary target organs are the gastrointestinal tract, kidney, and liver.

After the ingestion of these toxins, there is usually a latent period during which little happens for several hours. Then vomiting, diarrhea, and intense abdominal pain occur for several hours. This then subsides and the patient appears almost normal for a few days. The toxins gradually produce hepatic and kidney failure, which may occur within a few days if the dose received was high enough. Hepatic liver enzymes are elevated; the patient is jaundiced and anuric. Tachycardia, acidosis, hypotension, dehydration, electrolyte and blood coagulation abnormalities, hypoglycemia, hepatic coma, and shock usually occur. If *Amanita smithiana* is the ingested mushroom, primary renal tubulonecrosis may occur with minimal hepatic damage.

Treatment of cyclopeptide toxic ingestion is one of the few mushroom poisonings in which extensive therapies have been tried. Silymarin, derived from the milk thistle *Silybum marianum,* has offered some hope as a free radical scavenger and inhibitor of the penetration by amatoxins into the liver cells. Therapy is required for 4–5 days at 20–50 mg/kg of body weight per day intravenously or 1.4–4.2 g per day orally. Often Silymarin is used together with penicillin G. Penicillin G at 300,000–40,000,000 units per day seems to be of some benefit in treating this mushroom intoxication if given within a few hours of ingestion. The antibiotic seems to reduce hepatic uptake of the toxin, but unfortunately such early therapy following mushroom ingestion is often not possible because of the several hour latent period following ingestion. Thioctics acid has been recommended in the past, but it is difficult to obtain and has had mixed success.

Fairly classical additional treatments have met with moderate success in mushroom toxicities from cyclopeptides. Decontamination of the gastrointestinal tract within a few hours of ingestion using emesis and/or gastric lavage along with activated charcoal administration is classic. Multiple doses of the charcoal given several hours apart are felt useful due to enterohepatic circulation of the toxin. Crucial is the supportive care required to maintain appropriate organ functioning and to avoid renal failure, hepatic coma, blood coagulation problems, acid–base abnormalities, decreased blood glucose, and dehydration. Because of the prominent

liver damage, liver transplants have been done in humans suffering from this specific mushroom intoxication.

Orelline and Orellanine

Species of *Cortinarius* mushrooms contain the *orrellanine* renal toxin. Since clinical signs may not appear for several days following ingestion. Although with larger doses, the clinical signs appear earlier. Anorexia, gastrointestinal disturbances, oliguria, and renal failure are the usual sequence. Although there is no specific antidote, decontamination methods are useful if ingestion is recognized as occurring within the first few hours after the event. Affected kidneys generally recover slowly if the mushroom dose is insufficient to produce cellular death. Dialysis is very useful during the period of renal failure.

Muscimol and Ibotenic Acid

Dramatic and mind-boggling clinical effects occur within a few hours after ingestion of *Amanita muscaria, Amanita pantherina, Amanita strobiliformus,* or *Tricholoma muscarium* mushrooms. The effects begin with drowsiness followed by giddiness, often a maniac behavior, hallucinations and derangement of senses, and signs that resemble those seen in alcohol intoxication. These neurologic effects are complicated by nausea and vomiting, as well as salivation and diarrhea, which may further complicate the clinical picture. The patient becomes confused, disoriented, delirious, and may experience visual hallucinations, muscle spasms and twitching, and occasional convulsions. The periods of drowsiness and agitation often alternate and enthusiastic exaggerated physical activity may be seen. The effects are usually maximum within 3 h after ingestion, but fortunately mortality is minimal, ranging up to 12%.

These variable effects suggest that the toxin concentrations in the mushrooms are quite variable and have been confirmed by chemical analyses. Muscimol and ibotenic are derivatives of gamma-aminobutyric acid and exert their effects on the central nervous system. They both appear to cross the blood-brain barrier, probably by means of an active transport system. Although clinical effects are usually still present more than 5 h after the peak excretion in urine, existing blood levels do not correlate with the clinical effects on the central nervous system. With no specific antidote available, care is generally symptomatic and supportive to avoid self-inflicted injuries due to abnormal behavior. The extreme drowsiness phase frequently requires observation to avoid lack of attention to dangerous behavior.

Monomethylhydrazine

Several mushroom groups carry this toxin. *Gyromitra gigas, Gyromitra esculenta, Helvella elastica, Verpa bohemica, Piziza badia,* and *Gyromitra infula* contain a monomethylhydrazine precursor that when developed as a metabolite becomes toxic. The clinical signs, which are mostly gastrointestinal, occur several hours after the mushrooms are ingested and may not show up for 2 or more days. Once developed, the clinical syndrome is sudden with a bloated sensation followed by vomiting and prominent watery diarrhea. Abdominal cramping, abdominal pain, headaches, and general depression follow and may last for 2 days. Some individuals may develop jaundice, methemoglobinemia, and hemolysis. In the later stages, the clinical effects may include dizziness, loss of muscle coordination, seizures, and coma.

The prominent gastrointestinal effects produced require that affected patients be monitored for dehydration, electrolyte imbalances, red blood cell destruction, and methemoglobinemia. If ingestion is recognized prior to clinical signs developing, gastric decontamination is highly recommended. Although no directive antidote is available, the range of clinical effects (i.e., central nervous system signs, red blood cell alterations, blood glucose depression) and the mushrooms' effect on several organ systems suggest that hepatic and renal function should be monitored for several days.

Muscarine

Prominent cholinergic effects result from consumption of *Clitocybe dealbata, Clitocybe truncicola, Inocybe lacera, Inocybe Pudica,* and *Entoloma rhodopoilum* mushrooms. These effects occur within 30 min to 2 h after ingestion, although when high concentrations of muscarine are present in the consumed mushrooms, signs may appear in as little as 15–20 min. Affected individuals experience blurred vision, excessive perspiration, salivation and lacromation, decreased heart rates, lowered blood pressure, increase peristalsis, mild hypotension, urinary urgency, nasal discharge, pulmonary congestion, and difficult respirations. The presence of excessive perspiration, salivation, and lacromation are generally only seen with this type of mushroom poisoning. Patients may also exhibit flushing of skin, vomiting, abdominal pain, and watery diarrhea.

The toxin is readily absorbed and binds effectively to acetylcholine receptors, initiating what are well-recognized acetylcholine effects at muscarinic sites: the cells are glands, cardiac muscles, and smooth muscle. The effects are peripheral since muscarine has ionic character, which prevents it from crossing the blood-brain barrier. Emesis should always be performed if ingestion is observed. Activated charcoal may be of some value, but the life-threatening cholinergic signs require that atropine be administered. The atropine dose should be repeated until atropinization is present, since the end point for appropriate reversal is the cessation of secretions such as salivation.

Psilocybin and Psilocin

The mushroom genus of *Psilocybe, Panaeolus, Gymnopilus,* and *Conocybe*, and some *Stropharia* species contain these toxins. Effects often are initiated within minutes after ingestion, but may be delayed for a few hours. The effects last up to 4 h with peak activity occurring about 1 h after consumption. The psychoactive effects from these compounds may last up to 15 h, with other nonpsychological effects such

as mydriasis, hypertension, and drowsiness persisting even longer. These potent neuroactive toxins reduce mood swings from euphoric to apprehensive. Visual hallucinations, sweating, yawning, flushing, tremulous speech, incoordination, paresthesias intensified hearing, and unmotivated compulsive body movements typically occur. Nausea and vomiting are present in only about 20% of ingestions.

From 1 to 20 mushroom caps are required to produce the psychological effects, including flashbacks. Psilocybin and Psilocin are four-substituted tryptamine derivatives, which are active when reaching the brain. The effects of psilocin and LSD on brain serotonin are similar. Both psilocin and psilocybin have serotonergic interactions in the peripheral and central nervous system. Most cases are effectively managed when placed in low-stimulus environments and managed with appropriate supportive care. If panic or hyper reactions develop, diazepam therapy is beneficial. Phenothiazine tranquilizers are counterindicated.

Irritants of the Gastrointestinal Tract

Although few fatalities have been reported from mushrooms that produce gastrointestinal irritation, the presence of nausea, vomiting, abdominal pain, and diarrhea soon after ingestion are dramatic effects. Additionally, weaknesses, dizziness, paresthesias, sweating, and varying degrees of headaches have been associated with the gastrointestinal syndrome. *Agaricus xanthodermus, Amanita volvata, Boleus satanus, Chlorophyllum molybdites, Entoloma lividum, Gomphus floccosus, Lampteromyces japonica, Rhodophyllus rhodopolius,* and *Rhodophyllus sinatus* are the mushroom varieties associated with the gastrointestinal disturbances. In total, the onset of clinical signs may be variable, being as early as 15 min or in a few hours. The actual chemicals involved in this digestive syndrome are diverse. It is unknown what specific contributions each of these compounds make to the gastroenteritis. Fortunately, these effects usually resolve spontaneously within a few hours although some may last 24 h or more. There are no serious aftereffects and general symptomatic care is adequate to ensure recovery.

Hypersensitivity Reactions

Various types of hypersensitizations may occur after exposure to mushrooms. These include gastrointestinal, dermatological, and respiratory effects that may be associated with the actual mushroom, with smuts, rusks, sly moles, or dry rotten fungi. Species of *Agaricus* produce an upset stomach for some individuals. Even *Agaricus bisporus*, the mushroom most commonly found in United States grocery stores, has produced temporary gastrointestinal upset in some people who are particularly sensitive to this mushroom. Respiratory and dermal reactions to fungi and fungal spores are fairly common. Since these are usually short-term effects, no specific treatment except prevention by avoiding future exposure is applied. In rare cases where clinical signs are particularly significant, brief corticosteroid administration may be applied. In instances of hepatic failure from mushroom intoxication, recent advances in liver transplantation have proven successful in restoring liver function in most patients.

Treatments

The diverse nature of these mushrooms often precludes the rapid application of therapy. No specific antidotes are available, but if treatment is sought within a few hours after ingestion, the use of emetics to empty the stomach followed by the administration of activated charcoal is a common practice. The use of atropine is significantly avoided. Additional treatments are symptomatic to relieve the uncomfortable effects of the mushroom ingestions and to support the patient during the recovery or healing process. If neurological effects are present, appropriate seizure or nervous control is applied, and diuretic fluid administration and appropriate electrolyte infusions are used to assist with excretion of the toxins and correction of dehydration and electrolyte imbalance. Careful patient monitoring and awareness of potential liver or kidney after effects are necessary nursing steps to assure a satisfactory prognosis and recovery.

An exceptional effort has been made to identify useful therapy for the ingestion of the cyclopeptide antitoxins commonly found in *Amanita* and *Galerina* species. The application of silymarin, derived from the milk thistle *Silybum marianum*, has been periodically favored as a specific treatment for these cyclopeptide intoxications. The flavanoligans in silymarin have been found protected in animals, and are thought to be free radical scavengers and inhibitors of amnitoxin penetration into liver cells. When used for 4–5 days at 20–50 mg/kg of body weight per day intravenously or 1.4–4.2 g/day orally, often in combination of penicillin G, the combination comes as close as practical to a specific antidotal regimen for mushroom toxicity.

The common sense guideline of "Don't pick the mushrooms yourself—and if unsure of a mushroom's toxicity—don't!" provides often critical clinical-sparing guidance. Paramount in the basic toxicology syndrome is the recognition that dose is still a factor. A little taste might satisfy the yearning, while an abundant appetite may lead to serious consequences.

ANIMAL TOXINS

Toxins are represented by an impressively wide spectrum of compounds in numerous botanical species; however, nature's evolutionary chemistry is equally impressive in its complex synthesis of toxins and their diversity and distribution in the world's animal phyla and taxa. From the oceans and continental landmasses of all geographic regions and climates, toxins have been discovered in an array of aquatic and terrestrial animals. The prominent animal kingdom phyla associated with toxins are the Cnidarins, Platyhelminthes, Echinodermata, Mollusca, Annelida, Arthropoda, and Chordata [26].

What Is an Animal Toxin?

An animal toxin can be a protein, enzymatic protein, a small peptide, or homogeneous in nature and is produced by living cells or organisms. Toxins may derive their chemistry from their environment or diet, while the production of some toxins requires the codependence of associated microorganisms and the host animal. Additionally, the chemical make-up of specific animals' toxins can be influenced by geographic variation, seasonal climatic factors, and the influence of these on the animals' habitat. Toxins, biologically and chemically, range from quite simple to complex compounds. Certain animal species may possess only a single toxin within a specific anatomical site in their body. The effect of a single toxin can be like a biochemical bullet that precisely finds its specific target. However, mixtures of toxins in animals are not uncommon, and each single toxin in the mixture may have its individual specific target. When a toxin is introduced into the tissues of another living organism, an adverse physiological event results from the inhibition, or promotion, of another substance or biochemical reaction. In certain circumstances, once a toxin has been introduced into other organisms' tissues, an immune response is elicited, triggering the induction of neutralizing antibodies or antitoxins.

Venoms and Poisons

Some animals, such as amphibians and reptiles, are known to possess a mixture of different toxins usually associated with, and harbored in, highly evolved toxin delivery systems or glands. These toxin cocktails, collectively, are referred to as venoms or poisons, depending on their origin in the animal species. Thus, venoms and poisons are usually mixtures of toxins. Venoms and poisons can elicit a cascade of adverse pharmacological events in their victim. Venoms, being a mixture of toxins, are biologically manufactured or synthesized de novo in a well-developed secretory gland or organ associated with a venom delivery anatomical structure. A poison is generally considered to be the terminal toxin product of a metabolic process, a toxin product of an organism within the animal due to a symbiotic relationship, or toxin product resulting from the biodegradation of a substance. Thus, toxins not directly synthesized by an animal, but which are present in the animal tissues render the animal poisonous. The differentiation of venomous and poisonous animals is most applicable to creatures in marine ecosystems. Given these simplistic definitions, venomous animals are poisonous, but many poisonous animals are not venomous [27].

Toxin Functions

Toxins, venoms, and poisons function by acting as a single agent or working in concert to repel other organisms or more effectively achieve a desired adverse end effect on another organism. Commonly, toxin functions are loosely related to their effects on a given physiological system, resulting in relative terms such as neurotoxins, myotoxins, cytotoxins, and/or hemorrhagic toxins. Regardless of the physiological system affected, animal toxins appear to primarily exist for two important reasons. First many toxins are useful in subduing prey for food, and secondly, toxins often serve in a role of self-defense for the animal in which they are present. Both of these functions, independently or together, provide for the self-preservation and sustained survival of the animal species. However, there are circumstances where a toxin is present in an animal as a result of being passively taken into the body, causing no apparent adverse effects to the animal, and having no apparent function. It is difficult to know if a mollusk harbors a toxic alga for the purpose of protection from predators or for predation of prey food versus simply being present due to the alga's proximity in the mollusk's environment. Thus, a toxin can enter the food chain without having specific function for the animal harboring the toxin.

Bioaccumulation and Bioamplification of Animal Toxins

Animals can accumulate toxins as they are transferred up the food chain. This can be an active or passive bioamplification process as animals ingest, filter in, or absorb certain toxins or toxin-producing organisms, with the end result being a passively acquired toxicity for the host animal. In turn, these animals may, or may not, become poisonous to larger animals that prey on them [28]. This particular scenario is prominent in marine biology as many species of fish are the top consumers or predators of various toxic marine organisms. For example, a mollusk can filter feed on toxin-producing algae and then itself be eaten by a fish that survives the ingestion without complication. A human then consumes this fish with its burden of bioaccumulated toxin. Whether the mollusks' or fish's not being affected is the result of tolerance to the algal toxin or natural resistance to the toxin's effects is variable depending on the species. When humans or other mammals ingest these toxin-laden fish, severe toxicological medical consequences can occur. Animals may also acquire their toxins from a wide variety of toxin-containing plants on which they feed as part of their diet. A more thorough discussion of toxins in the food chain is discussed in Chapter 14.

Toxin Delivery Systems

Toxin delivery to a specific target can only occur following its introduction onto or into another living organism, and in this respect the animals of the natural world have proven to be quite accomplished, reflecting traits of great creativity. The introduction of a toxin, venom, or poison can occur via absorption onto, or across a barrier, and by injection into, or through a barrier. Usually, this barrier is anatomically dermal in nature and not readily penetrated by most nocuous external environmental substances. Toxin application and penetration processes are achieved by specific mechanical strategies such as superficial mucous secretions, bristles and hairs, glandular skin secretions, barbs, spines, and teeth. Pharmacological or toxicological actions of toxins following

TABLE 19.2
Interrelationships of Animal Phyla, Environment, Toxins, and Toxin Targets

Phylum	Class	Common Names	Toxin	Toxin-Target
Mollusca	Marine	Blue-ringed octopus (*Hapalochlaena lunulata*)		
Chordata		Puffer fish (*fugu* sp.)	Tetrodotoxin	Na^+ ion channel
Chordata	Terrestrial	Rough-skinned newt (*Taricha granulosa*)		
		Neotropical toad (*Atelopus carbonensis*)		
Cnidaria	Marine	Sea anemone (*Aiptasia pallida*)	Phospholipase A_2	Cell membranes
Arthropoda	Terrestrial	Honey bee (*Apis mellifera*)		
Mollusca	Marine	Cone snail (*Conus geographus*)	Omega-conotoxin	Ca^{++} ion channel
Arthropoda	Terrestrial	Funnel web spider (*Agelenopsis aperta*)	Omega-agatoxin	
Mollusca	Marine	Cone snail (*Conus purpurascens*)	*K*-conotoxin	
Cnidaria		Sea anemone (*Bundosoma granulifera*)	BgK	K^+ ion channel
Chordata	Terrestrial	E.African green mamba (*Dendroaspis augusticeps*)	Dendrotoxin	
Arthropoda		Yellow scorpion (*Leiurus quinquestriatus*)	Charbytoxin	
Chordata	Marine	Broad-banded sea snake (*Laticauda semifasciata*)	Erabutoxin	ACh receptor
	Terrestrial	Black-necked cobra (*Naja nigricollis*)	Neurotoxin-alpha	
		E.African green mamba (*Dendroaspis augusticeps*)	Fasciculin 1	ACh-esterase

their topical deposition on body surfaces can cause superficial or severe localized reactions. This topical delivery process can be enhanced, dependent on the chemical properties and pharmacological actions of the toxin, inducing the toxin's transdermal absorption, and result in systemic toxicity. Toxin delivery into the systemic processes of another living organism can cause effects ranging from limited physiologic responses to severe systemic physiological alterations, resulting in the death of the unfortunate toxin recipient. Airborne toxin delivery is a method frequently employed by some insects and is achieved by the spraying of a toxin topically onto dermal or ocular surfaces, or even spraying in the oral cavity of predators. More advanced and direct methods of toxin delivery are achieved by injection via a bite or string. These may be associated with an anatomically sophisticated delivery system such as that present in the fang-venom duct-venom gland complex present in solenoglyphic venomous snakes, or the pulsating stinging apparatus of the common honeybee.

Complementation and Diversification of Animal Toxins

The sharing and interrelationships of phyla, toxins, toxin structures, and toxin functions exist across marine and terrestrial environments. Distinct animal species from different phyla, from widely separated geographic regions and habitats, have developed toxins with a variety of chemical-structural and functional relationships. Combinations of animal phyla, animal toxins, and the associated toxin structure–function relationships has yielded four basic schemes in nature: (1) interphylogenetic species producing a common toxin that acts on a common target, (2) interphylogenetic species producing different toxins with chemically related structures that act on a common target, (3) interphylogenetic species producing toxins with unrelated chemical structures that act on a common target, and (4) intraphylogenetic species producing toxins with similar chemical structures, or toxins with unrelated chemical structures, and acting on the same target, or different targets (Table 19.2). The interrelationship of these factors provides evidence for divergent evolutionary function in toxin-producing animals, between phyla or within a phylum, and for a certain degree of convergent evolutionary function with respect to toxin properties and function.

The K-conotoxin from the cone snail, *Conus purpurascens*, phylum Mollusca, is quite different in its chemical configuration compared to that of charbytoxin from the yellow scorpion, *Leiurus quinquestriatus*, phylum Arthropoda, yet both of these toxins target the potassium ion channel [26]. An alternate path in this relationship is observed between toxins from the same phylum when toxins of similar chemical structure are produced, but the toxins act on separate targets. Toxins such as neurotoxin alpha in the venom of the black-necked cobra (*Naja nigricollis*), and fasciculin 1, from the venom of the east African green mamba, are structurally similar, but neurotoxin alpha acts on the acetylcholine receptor while fasciculin 1 acts on acetycholinestease. Omega-conotoxin from the cone snail, *Conus geographicus*, phylum Mollusca, is similar in chemical configuration-structure to omega-agatoxin from the funnel web spider, *Agelenopsis aperta*, phylum Arthrpoda, and both act on calcium ion channels [29]. These examples are illustrative of the evolutionarily diverse creatures that generate similar

chemically configured toxins, resulting in similar pharmacological actions. Thus, toxins from quite evolutionarily different animals, or evolutionarily similar animals, can exhibit both qualitative and quantitative similarities and differences, in their effect. However, there are also toxins, like tetrodotoxin, which are widespread in the animal kingdom (found in the skin of amphibians, fish organs, and blue-ringed octopus) that have a common chemical structure and common pharmacological site of action [30].

Mechanisms of Animal Toxin Actions

Biodiversity of organisms provides for a diverse spectrum of how animal toxins act pharmacologically to do what is essential for the continued survival of their host animal, whether they are needed for acquiring or digesting food, or providing defense. In certain circumstances, this also includes how toxic effects are exerted in animals that have ingested a toxin-containing organism and inadvertently become victims of poisoning. Whether the toxins effects are neurotoxic, myotoxic, cytotoxic, or coagulopathic, these effects are usually due to toxin proteins, enzymatic proteins, and peptides, acting either singly or collectively, as in the case of some venoms, to create a single action or a cascade of reactions that result in toxicological consequences to a living organism.

Toxicity resulting from the pharmacologic actions of different toxins can reflect both qualitative and quantitative differences. A toxin's ability to elicit a toxic effect by acting at a single target may be quantitative due to the resultant toxicity being dependent on the dose of toxin reaching the target, or reaching subtargets within a target. Receptors may structurally possess a single binding site for a toxin, or more than one binding site for the toxin. The degree of toxin binding may be limited by conformational characteristics of the binding site, but binding to a single site or more than a single site, may still depend on the dose of the toxin. If the dose is low, there may be minimal binding at only one site on the receptor, and this may not result in any measurable toxicity. If the toxin dose is large, increased binding may occur at a single site, or at more binding sites on the receptor, resulting in significant toxicity. Thus, with different animal toxins and their quest for different receptor targets or subtargets, there potentially exists a resonance of flexible stoichiometric relationships. All these factors, in addition to the toxin's own chemical configuration, can affect what binding site a toxin is specific for. Subtle differences in how these factors are related is especially important as slight chemical and structural differences in toxins cannot only provide for a specific toxin's binding the same receptor as another toxin, but also allow for the toxin to bind at different site on the same receptor.

Additionally, there may exist binding sites within a given target, such as a cell membrane, which allow the binding of different toxins. This multiple toxin–multiple target relationship is commonly observed with snake venoms. These multiple toxins found in venoms may also function sequentially via synchronized pharmacologic actions when injected into another organism, with one toxin leading to improved bioavailability of another more potent toxin to its target.

Although toxin dose is an important component in the development of toxicological effects, the inherent affinity of toxins for given targets relating to toxin specificity, is also a factor that influences the potency of a given dose of toxin. Animal toxins can compete as agonists or antagonists with endogenous neurotransmitters in synaptisomal clefts, and may either act presynaptically or postsynaptically. Toxins that target the acetylcholine receptor, or neuromuscular junction, are well-established among the different animal phyla. There also exists a prevalence of toxins that target voltage-gated ion channels, primarily sodium, potassium, and calcium ion channels. Again, nature has provided some unique features of select pharmacological effectiveness of toxin specificity as is observed by the selective binding of toxins such as beta-scorpion toxin versus tetrodotoxin from newts, which bind to different sites on the sodium ion channel.

Toxins may also digest or directly inactivate neurotransmitters. Phospholipase A_2 is an enzymatic protein with wide representation in the animal kingdom, and known to exist in greater than 100 isoforms. Not surprisingly the various forms are capable of exhibiting diverse pharmacologic activities and physiological properties ranging from the destruction of cell membranes to acting as presynaptic neurotoxins [31,32]. Other animal toxins can act on a variety of cell membranes, altering cellular membrane integrity, and inducing secondary toxic effects from a toxin's cytolytic, myotoxic, and coagulopathic effects.

Collectively, toxin effects, whether myotoxic, agonistic, or antagonistic to receptors, ion channel altering, or neurotoxic based on their properties such as affinity and specificity, conformational structure/specificity, or length of amino acid sequences contribute to varying levels of toxicity, diversity of pharmacological function, and the extent of pharmacological function leading to a toxicological end effect. A role of animal toxins in nature that has become confounding has arisen from the increasing interface between humans and animals in all ecosystems. How nature's animals use their toxins has been expanded due to the behavioral nature of humans in pursuing activities that cross all ecosystem barriers. Consequently, animals have been forced to use their toxins and venoms in defensive circumstances far beyond their intended protective use against natural predators. The dynamics of these animal–natural predator, and animal–human, relationships will be further discussed with respect to toxinology: specifically, animal phyla, their specific toxins or venoms, toxin functions in the natural world, and their impact in biomedical research and clinical toxicology.

Marine and Freshwater Ecosystem Animal Toxins

Greater than two thirds of the planet earth is covered in ocean saltwaters that are home to diverse and highly integrated marine communities. The remaining one third of the planet's landmasses is dissected by rivers and scattered with lakes, comprising freshwater aquatic ecosystems that also

contain integrated animal communities of great diversity. Similar to the relationship of venomous versus poisonous animals, all marine animals are aquatic, but not all aquatic animals are marine. In organisms from marine environments alone, the chemical structures of greater than 5000 natural products, as single chemical classes or mixed biosynthetic compounds, have been elucidated [33–35]. This number is probably far less for compounds derived from freshwater species because of the considerably smaller freshwater habitat on earth. These organism-taxonomic and toxin-chemistry relationships are complex, and the biogenetic diversity of animal toxins in these habitats is great, and the quest to dissect out animal communities and the specific sources of animal toxins is a continual and ongoing search for scientists.

Invertebrate Marine and Freshwater Animal Toxins and Venoms

Multiple genera representing 11 different classes, derived from 5 different phyla, comprise the phylogenetic roots for thousands of invertebrate species in marine ecosystems (Table 19.3). Although marine invertebrates are rather simplistic in form, ranging from simple hydrozoan corals to more complex forms such as gastropod cone snails, many possess some form of toxin or venom as a means for acquiring a meal, or as a deterrent against predators. However, in addition to toxin effects on other organisms, a remarkable number of marine invertebrate toxins are also harmful, or even lethal, to humans.

Annelida

Bristle worms and fire worms, class Polychaeta, that possess toxins are reported for several genera (Table 19.3) [36]. Known by a variety of common names they share the common feature of having parapodia, leg-like appendages on each of their body segments, with hollow monofilament-like bristles, called setae, attached. In many cases, the bristles may have a feathery appearance. Despite anatomical likenesses, the different species can display a range of appearances reflected in their common names such as palolo worms, featherduster worms, sea mouse, or lug worms. Some such as *Eunice gigantea* have been reported to grow up to three meters in length [36].

Eurythoe complanata, a species indigenous to the tropical Pacific Ocean and Gulf of Mexico has spiny, barbed bristles that it promptly flares when threatened. If bristle contact occurs by a predatory animal, or human touch, the barbed glassy spines are readily released into tissues of the offender. However, this action results in a mechanical or physical insult causing a localized dermal inflammatory response and paresthesia that may last for weeks, but is not believed to be associated with delivery of a toxin. Embedded bristles may be partially removed by the application of adhesive tape over the affected area. Topical use of a steroid cream and oral or topical antihistamines may be a beneficial symptomatic treatment, and severe cases of secondary infection or even gangrene is possible [37,38].

Importantly, certain species such as *Eunice*- and *Glyceria-species* have jaws associated with a defined venom gland and a fang-like proboscis that delivers a large molecular weight, ion channel forming, alpha-glycerotoxin [39]. Clinical symptoms from the bite show it to be quite painful with blanching around the punctures, followed after several days with persistent itching. However, uneventful recovery generally follows.

The medicinal leech, class Hirudinea, is a freshwater annelid with a quite interesting history, as it has been used by the lay public for self remedies, and by physicians since 200 BC, for a variety of medical purposes, including its use as a

TABLE 19.3
Marine and Freshwater Invertebrate Animals: Venomous or Toxin-Producing

Phylum	Class	Common Names	Genera (Toxin-Producing)
Annelida	Polychaeta	Bistleworms, fireworms	*Chloeia, Glycera, Eunice, Eurythoe, Hermodice*
	Hirudinea	Medicinal leech	*Hirudo*
Cnidaria	Anthozoa	Anemones	*Actinia, Aiptasia, Antheopsis, Condylactis, Entacmaea, Halcuriasp, Heteractis, Stomolphus, Tealia, Urticina*
		Corals (soft, stoney, hard)	*Alcyonaria, Gorgonaria, Heliopora*
	Cubozoa	Box jellyfish	*Chironex, Chiropsalmusa, Carukia, Carybdea, Physalia, Tamoya, Tripedalia*
	Hydrozoa	Fire corals	*Millepora*
		Portuguese man-of-war	*Physalia*
		Sea fans	*Aglaophenia, Halecium, Lytocarpus, Macrorhynchia, Nemalecium, Thecocarpus*
	Schyphozoa	True jellyfish	*Cassiopea*, *Cyanea, Chrysaora, Pelagia, Rhizostoma*
Echinodermata	Echinoidea	Sea urchins	*Asthenosoma, Aerosoma, Diadema, Phormosoma, Tripneustes,Toxopneustes*
	Asteroidea	Starfish (sea stars)	*Acanthaster*
	Holothuroidea	Sea cucumbers	*Bohadschia, Thelonota*
Mollusca	Gastropoda	Cone snails	*Conus* sp.
	Cephalopoda	Blue-ringed Octopus	*Hapalochlaena*
Porifera	Demospongiae	Sponge	*Fibula, Lissodendoryx, Microiona, Neofibularia, Tedania*

postoperative aid in microvascular surgery patients [40,41]. These creatures have two suckers, which lie at the anterior and posterior ends of the head, with the mouth being in the anterior sucker where three jaws of teeth are located. Once attached, to the often unknowing victim, blood is sucked out as the leech uses rhythmic contractions of its pharynx to deliver the blood to its crop for storage until digestion takes place. The leech can ingest a blood volume nearly 10 times its weight, and as much as 15 mL of blood may be removed [42]. The anticoagulant effect of leech venom stems from the toxin hirudin, an antithrombin substance, but the venom also contains hyaluronidase, proteolytic enzymes such as bdellins and elgins, fibrinase, collagenase, and salivary apyrases [43,44].

The most common effects of leech bites are the depletion of blood volume in small animals from which the leech draws its nutrition, and the continued oozing of blood from the bite site following detachment. It has been reported that oozing may continue for up to 24 h after leech removal [45]. Leeches have caused hematuria due to urinary tract invasion, and hemoptysis form upper airway attachment [46].

Treatment of leech bite is not standardized and a myriad of means for leech removal have been proposed. Cocaine has been used to paralyze leeches, resulting in their release from their victim, but would certainly not be recommended in cases of a leech-lodged airway. The use of forceps to hold the leech until it releases has been effective, and one individual required anesthesia with direct visualization for leech retrieval [47]. Regardless of the leech removal method, once removed, it is recommended that the wound be irrigated, pressure applied and gelatin sponge used if bleeding persists, and evaluation for wound infection followed up [45].

Cnidaria

The Cnidaria, also known as Coelenterates, include anemones, corals, jellyfish, and hydrozoans (Table 19.3). This phylum is unique for being the only entirely venomous phylum in the entire animal kingdom. The variety of creatures in these classes appear to be quite different visually, yet they frequently share a commonality in their toxin-delivery mechanism, a process that involves stinging organelles known as nematocysts, sometimes referred to as cnidocysts [48,49]. These microscopic structures are organelles contained within specialized stinging cells, or cnidocytes, which, in the resting stage, appear like a well-inflated balloon with a small spike protruding. The spike continues to the interior as a barbed luminous thread that spirals like a spring within a venom capsule. If a significant osmolar or pressure change occurs, the nematocyst explosively discharges its inverted, barbed filament into the tissue of its victim with a simultaneous injection of venom [50]. The discharged nematocyst then appears, grossly, as a slightly deflated balloon attached to a string [36]. Nematocysts are primarily located on tentacles or along folding anatomical ridges, with thousands of these organelles present, allowing for the delivery of considerable quantities of toxins. Interestingly, some creatures have extended the use of nematocysts and their toxins via an interesting phenomenon in which noncnidarian marine creatures such as the octopus, *Tremoctopus violaceus*, attach fragments of Portuguese man-of-war tentacles to their own tentacles as both offensive and defensive armaments [51].

Toxins that have been identified in cnidarians stem from a variety of peptides and proteins that possess neurotoxic, hemolytic, or cytolytic properties [52]. Although the number of species of Cnidaria has been estimated at 10,000 or greater, the number of species potentially dangerous to humans is not known, but believed to be relatively low [53]. Regardless of the unknown number of species dangerous to human health, the medical problems associated with cnidarian envenoming are global in nature [54].

Anthozoa

Anemones are one of the nature's most beautiful and graceful sea life forms, yet these mostly sedentary cnidarians can readily deploy their paralyzing toxins from nematocysts into potential predators that brush against their tentacles, or into the unknowing prey animal that seeks shelter among their tentacles. However, not all sea anemone toxins have been confirmed to be actually located in tentacle nematocysts, as the contracting, intact body of some anemones will secrete cytolytic toxins with mechanical stimulation [55,56]. Anemones can also retract their tentacles under stress conditions, resulting in the secretion of large quantities of cytolytic or hemolytic toxins in mucus, rather than by nematocyst release, that can induce osmotic cell lysis via pore-forming ion channels in membranes. Interestingly, these toxins that serve to acquire prey, may also aid in the self-preservation of an individual that possesses resistance to its own cytolosins, within an anemone colony by allowing intraspecific spatial competition [57].

An additional complexity concerning this marine life form is the interrelationship between some anemone genera (*Entacmaea* and *Heteractis*) and species of fish in the genera *Amphiprion* and *Premnas* that demonstrates a symbiotic relationship in which anemone toxins provide protection to fish hiding among the nematocyst burdened anemone tentacles [58]. The exact reason fish survive in this symbiotic relationship has not been confirmed to be universal, as some fish species are protected by a mucus coating of their skin while others may have acquired immunity to certain sea anemone toxins [59].

An array of toxins has been discovered in anemones, as lethal cytolytic proteins and peptides have been reported in more than 32 species of sea anemones in the order Actinaria alone [60]. Roughly 30 different pore-forming toxins, known as actinoporins, have been characterized in greater than 20 sea anemone species [61]. However, there are several anemone genera that have been reported to be toxin producing (Table 19.3), and several toxins have been extensively characterized, such as sodium/potassium ion channel blockers [62], crustacean/vertebrate lethal cytolysins, and a cardiostimulatory protein, lacking cytolytic, activity has been isolated from *Urticina piscivora* [63].

Some toxins such as, tealiatoxin produced by *Tealia felina*, exhibit both hemolytic and histaminolytic activities [64].

Although a multitude of toxins have been identified, the medical consequences of most anemone stings to humans are fairly similar. The common symptom is a painful, stinging cutaneous lesion, which is localized to the area of tentacle contact [36]. Severity is proportional to the number of discharged nematocysts in the skin. Nematocysts can be inactivated by, *fixing* with the application of 5% acetic acid (vinegar), or a 70% isopropyl alcohol solution. Removal of remnant tentacles and nematocysts can be accomplished by the unidirectional scraping of the affected area with a knife blade or the edge of a plastic card. The lesion should then be washed with antibacterial soap and generously rinsed with water. Topical steroid cream may be useful in reducing local inflammation [65].

Rare acute envenomations have resulted in symptoms of severe pulmonary edema and cardiac arrest and have been the cause of eight deaths following envenomation by *Stomolphus nomuri* [66]. A species of *Condylactis* is believed to have been responsible for the death of a young male due to rapid hepatocellular failure [67].

Cubozoa

The box jellyfish is probably the most well known of the jellyfishes because of their unique anatomy and the potentially severe toxicity that can result from their painful stings. Although closely related, their distinct anatomy separates them from true jellyfish. They are four-sided, cowbell-shaped, translucent, gelatinous creatures that have muscular pedalia at each corner, where tentacles 10 or more times the height of the bell, and have been reported to be in excess of 60 m, stream in the underwater currents [68]. They inhabit mostly subtropical and tropical waters, and the coasts of Australia are notoriously famous for the presence of dangerous jellyfish. *Carybdea* species have been occasionally found in temperate waters [69]. Cubozoans possess four photosensory structures known as rhopalia, which each contain four simple eyes and two complex eyes. This vision system makes cubozoans exceptionally phototactic as evidenced by their light-sensitive behavior of descending to deeper water during bright sunlight, and moving toward the surface in early morning, late afternoon, and evening.

In contrast to the majority of jellyfish, box jellies do not drift with the underwater currents and are not planktonic in nature. They are strong swimmers and some species such as, *Chionex fleckeri*, the dangerous Australian species commonly referred to as the sea wasp, have been reported to move at 3–6 m/min [70]. The Cuboidal jellyfish from Australian waters is associated with interesting and unique local nomenclature such as the *Irukandji* (*Carukia barnsei*), the *jimble* (*Carybdea rastoni*), and the *morbakka* (*Tamoya* spp.) [71–73].

Cubozoan tentacles are laden with nematocysts of several different types, which collectively form a complement of nematocysts referred to as a cnidome [68]. Differing severity of toxicity between different genera may correlate with the nematocyst concentration present in the tentacles of a given species, which is also related to cubozoan species size [74]. However, studies using antibodies against box jellyfish have shown lack of cross-reactivity with other jellyfish venoms, and clearly suggest toxicity difference is also the result of different toxins in the venoms from different genera [75]. Venom of *Chironex fleckeri* is proteinaceous and possesses dermonecrotic, hemolytic, and myotoxic toxins that can work synergistically to cause lethal cardiorespiratory effects [76,77]. Myotoxins of 150 and 600 kDa have been isolated from *C. fleckeri* and shown to be lethal in animal studies, but characterization of other venom components have been illusive [78]. Commonly, the result of envenomation is a hypercatecholanaemia symptom profile, but this does not hold for all species, as the effects of venom from *Chriopsalmus* sp., a species responsible for many fatalities in the Philippines, does not respond to receptor antagonists, or antibodies, that are effective in reversing *C. fleckeri* venom-induced symptoms [79,80]. In cases of severe envenomation, death has occurred within minutes, and there have been at least 67 documented deaths [76].

In general, Cubozoan envenomations do not result in severe complications, but those inflicted by *Chironex fleckeri* can cause death as evidenced by the numerous fatalities reported in Australian waters [81]. Treatment of cubozoan envenomations, without severe symptoms, is accomplished with the use of acetic acid to retard nematocyst firing, and symptomatic support. However, more severe envenomations have required support with mechanical ventilation, which has still been ineffective in cases of profound cardiotoxicity [82]. Antivenom for *C. fleckeri* envenomation treatment has been developed, but there is only limited documentation as to its effectiveness in humans with severe cardiorespiratory symptoms [83].

Irukandji syndrome is a severe form of toxicity resulting from the sting of the small *Carybdea barnesi* jellyfish. It is a species of undefined geographic origin that has primarily been reported off the coasts in the northern-half of Australia, yet an Irukandji-like syndrome has been reported in Hawaii [84]. Although cardiopulmonary complications are predominant, the exact underlying mechanism is unclear. Victims exhibit distress, generalized pain, nausea, frequently severe hypertension, and pulmonary edema. The clinical profile suggests excess catecholamine [85]. Treatment is generally supportive, requiring analgesics, antihypertensive drugs, ventilation, and pressor drugs, with recovery in several (3–4) days [76].

Hydrozoa

Hydrozoa are primarily colonial benthic cnidarians, with the exception of floating *Physalia*, which are simple marine life forms that, like all other members of cnidaria, possess nematocysts. Four different morphological forms of nematocysts are known to be present in Hydrozoa: atrichous isorhiza, microbasic euryteles, microbasic mastigophores, and stenoles [53]. They differ in the arrangement of spines on the shaft, which may result in varying degrees of anchoring to tissue and venom delivery into the victim [86]. Thus, when contact with these organisms occurs, there is mechanical

injury in addition to toxicological injury. Although the number of species in the class has been estimated at greater than 10,000, the number of species known to be potentially dangerous to humans is small [76].

Fire coral, *Millepora* sp., is a hydrocoral rather that a true coral, and is familiar to many reef divers as it is frequently encountered among colonies of true corals. Antler-like in appearance, the velvety tines are covered with nematocyst-containing hydranths that look like small, segmented hairs with umbrellas at the tip. The venom contains thermolabile, proteinaceous components possessing both dermonecrotic and hemolytic properties. Toxicity studies in small rodents have shown it capable of causing death [87]. When contact with fire coral occurs, there is an immediate burning and stinging sensation that progresses rapidly to a painful pruritus. Wheals develop and the area of contact becomes edematous and erythematous. Pain symptoms generally resolve within an hour or two, while dermatological symptoms may persist for up to a week, with pigmentation lesions persisting for 1–2 months [88]. Amelioration of symptoms usually requires only the application of a skin cream, but, in more severe cases, may require deactivation and removal of nematocysts, followed by the application of hydrocortisone cream. It has been reported that papain-containing *meat tenderizer* may be useful in recovering ingrained nematocysts [65].

Portuguese man-of-war, *Physalia physalis*, is a nondiving, oceanic, jellyfish-like, hrydrozoan with a global geographic distribution. Their gas-filled bladders, actually modified medusae, allow them to drift with wind and surface water currents. Their numerous tentacles may be long, trailing 13 m or more into the depths, and can deliver potent venom from their associated nematocysts [89]. A small, morphological form of this genus, *Physalia utriculus*, commonly called the blue bottle, is indigenous to the Micronesian waters of the Pacific, but lesions resulting from its stings are less extensive than those of *P. physalis* [36]. Venom from *Physalia* has not been fully characterized, but a glycoprotein structured toxin, physaliatoxin, has been isolated and described as possessing strong cytolytic and hemolytic properties [90]. Additionally, enzymatic proteins with cardiotoxic effects have been isolated, and whole venom can elicit rapid histamine release from mast cells [91,92]. Complications to humans from the stings of *Physalia* can range from acute pain in tentacle contact areas, to migratory joint pain, and in rare circumstances death from rapid respiratory failure followed by cardiovascular collapse [93]. Unlike the inhibitory response to firing nematocysts of Chironex with acetic acid, this therapy has been reported to induce further nematocyst discharge in cases of *Physalia* sting [94]. Cold packs may reduce the superficial inflammatory and pain response of stung tissues, and papain solution has been useful for inactivating nematocysts [95]. The use of topical lidocaine preparations may also be beneficial in reducing localized inflammation and pain [96]. Antivenom for treatment *Chionex* stings provides no paraspecific coverage or protection to victims of *Physalia* envenomation, as it is not cross-reactive with *Physalia* venom [97]. Thus, there is no definitive treatment in cases of life-threatening envenomation, and basic cardiopulmonary resuscitation and support measures are essential if recovery is possible.

Sea fans are some of the more elegant appearing hydrozoans with their feathery, fan-leafed ferns look. These benthic hydrozoans are found in shallow Atlantic, Indo-Pacific, and Australian waters. Despite their harmless appearance, their nematocysts can deliver painful stings, associated with erythematous and edematous whealing, when contact occurs [54]. The best-known genera for delivering venomous stings are the *Aglaophenia* and *Lytocarpus*, yet other genera may also cause dermal contact-related problems (Table 19.3). Little is known of sea fan venoms, yet in recent years, the discovery of new venomous species has occurred by accident as case reports of stings find their way into the literature. One such species, *Nemalcium lighti*, was found to be capable of delivering pruritus and burn-sensation inducing stings to an unfortunate snorkel victim that persisted for days [52]. Sea fan envenomations are not life-threatening, but basically an aggravating discomfort, and their superficial symptoms respond to deactivation of nematocysts still in contact with skin, and the application of anti-inflammatory creams [65].

Schyphozoa is the class representing the true jellyfish, which are probably the most frequently encountered jellyfish by beachgoers. They tend to be radially symmetrical with their gelatinous medusal forms swimming in varying currents from the surface waters down to the abyss of the oceans, depending on the species [36]. They have a variety of interesting and descriptive names: upside-down jellyfish (*Cassiopea xamachama*), sea nettle (*Chrysaora quinquecirrha*), hair jelly (*Cyanea* sp.), described by Halstead as *mop hiding under a dinner plate*, and mauve stinger (*Pelagia noctiluca*) [36,98]. Although Schyphozoan nematocysts are capable of delivering irritating stings that may rarely cause muscle cramps and unconsciousness, their venoms are not as dangerous as those of their Cubozoan relatives, as no cases of death are found reported in the literature [99].

Chrysaora quinquecirrha crude venom (CQV) possesses a bioactive hyaluronidase, an enzyme thought to aid in the spread of venom, that is similar to the hyaluronidase found in the venom of the five pace snake (*Agkistodon acutus*), again demonstrating the similarity of toxins across phyla in the animal kingdom. Further studies showed CQV to exhibit hemolytic activity [100]. Animal studies have predominantly supported that fatal reactions to jellyfish envenomation have been the result of cardiac, respiratory, or renal toxicity, but CQV studies also suggest the venom is capable of inducing hepatic lesions, subsequent to lethal cytotoxicity, that may explain fatal reactions as a result of delayed hepatic failure [101].

Cassiopea xamachana, from Puerto Rican coasts, can cause painful stings, and recent research showed their venom (CxTX) contains a low molecular weight, <10 kDa, fraction that binds to acetylcholine muscarinic receptors and demonstrated strong hemolytic activity, in an animal model. Additionally, a phospholipase A_2 (PLA_2) fraction contributed significantly to its paralytic activity and lethality [102]. It is

likely that CxTX toxicity is the result of venom toxins acting synergistically. The degree to which Schyphozoans share similarities in their toxins remains to be elucidated, but toxin effects appear to cause a similar clinical profile in humans who have had an unfortunate contact. The varying severity of envenomation apparently is dependent on the extent of the tentacle contact area, and the duration of contact [36]. Treatment of Schyphozoan envenomation is similar to that recommended for Cubozoan stings, and with good supportive care, a favorable outcome is to be expected.

Echinodermata

The phylum Echinodermata is perhaps most familiar to people of all ages because of the amazing starfish, class Asteroidea. For many years these creatures have been taken from the world's oceans, dried and sold as curios, to be appropriately placed on desktops, library shelves, and used in unthinkable numbers for school *show and tells*. Sea urchins, class Echinodea, are also widely recognized due to the fear of a painful encounter with one of their spines. Many children and amateur snorkelers have paid the price for that tempting moment of curiosity, when the extended finger has tried to gently touch one of these creatures, and been rapidly withdrawn in profound pain, or the unwary wader plants a foot on the needle-like spines. Unlike these better-known Echinoderms, the sea cucumbers (class Holothuroidea) possess a more fragile, large sluggish, appearing form that lacks the mechanical injury-inducing abilities of other Echinoderms. Although these creatures are reported to number approximately 6000 different species, only about 1% is believed to be venomous [103].

Sea urchins, class Echinoidea, represent the majority of venomous echinoderms and are painfully familiar to most people who have spent any time swimming, beach walking, surfing, or diving and snorkeling in subtropical or tropical coastal waters. They have a pincushion appearance with stiff, bristle-like spines that are structurally a calcium carbonate matrix, which may be hollow or solid, blunt or sharp, and may possess a venom-producing gland at the spinal apex [104]. They also may introduce venom via pedicellariae, which are jaw-like structures used to grasp unsuspecting prey. Urchin venoms comprise cholinergic substances, hemolysins, proteases, serotonin, and sterol glycosides [105].

Most urchin species are incapable of effectively injecting venom into humans and the resultant sting is primarily a mechanical injury. However, there are two Indo-Pacific ranging species, the Needle Spined Sea Urchin (*Diadema setosum*) and the Flower Sea Urchin (*Toxopneustes pileolus*), that are known to be capable of inflicting serious envenomation symptoms [89]. The severity of symptoms varies depending on the number of penetrating spines, and the associated pain is greater than that of mechanical injury alone. In rare cases of severe envenoming to humans symptoms of bronchospasm, cranial nerve dysfunction, hepatitis, hypotension, palpitations, and syncope have occurred [104,105]. *Toxopneustes* envenomation has even been reported to have caused deaths in Japan [106]. Treatment of urchin envenomation symptoms is ill-defined, and complicated by the fragile nature of spines frequently broken off in the wound. Methods of extricating spines have been relatively ineffective, and appropriate wound care may involve warm water immersion, analgesic drugs, and the breaking of large embedded spines into smaller fragments so they may be more readily absorbed or extruded [37]. Thus, the mainstay of treatment is symptomatic and supportive as needed.

Starfish, class Astoidea, although comprising many harmless species, has one major species responsible for envenomations to humans, the crown-of-thorns starfish (*Acanthaster planci*) of the Indo-Pacific region [36]. The crown-of-thorns starfish, as its name suggests, is an ornately pigmented, and large starfish that is found among the colorful coral reefs where their feeding on coral polyps can destroy the fragile coral reef structure [107]. Like urchins, it is the spines of this starfish, which may grow to 60 cm in diameter and possess 13–16 rays with spines up to 6 cm in length, that inflict a painful wound [36]. Venom is believed to lie in the epidermal cell layer of the spines where acidophilic glandular cells produce venom, which has been shown to have hemolytic and lethal activities in a rodent model [108]. Crude venom has also been shown to exhibit edema-forming and capillary permeability inducing properties [109]. More recent studies have revealed the venom contains a 15.5 kDa, phospholipase A_2 that can induce skeletal muscle myonecrosis in animals [110]. Their venom has also been reported to contain a basic glycoprotein of 20–25 kDa molecular weight, with lethal properties when injected into mice, and hepatic and hypotensive effects have also been observed in animal experiments [111,112].

The symptoms of crown-of-thorns starfish stings are quite similar to those observed from urchins, but the pain subsequent to venom release from embedded spines has been described as extremely intense with throbbing and burning sensations, which may last for a month [37]. Treatment is also similar to that for urchins with hot water submersion being of benefit in relieving pain initially, with prudent follow-up wound care, observation for secondary infection, and use of steroid creams and antibiotics as indicated by symptoms [37].

Sea cucumbers, class Holothuroidea, in stark contrast to their spiny bodied urchin and starfish relatives, are soft-bodied, blob-like creatures with an amazing array of color patterns that are secretive in nature. They have no true appendages other than small tentacles around their mouth, and internally harbor organs referred to as Organs of Cuvier, also known as Cuvierian tubules [89]. Occasionally, divers and beachgoers will handle sea cucumbers causing them to release a glycoside, known as holothurin [113]. This toxin is a saponin produced in the Cuvierian tubules that are readily deployed from the cloaca when contact is made with the offending organism, has a glue-like consistency and can be most inflammatory to skin and cause conjunctival complications to the eye [114]. To remedy symptoms, victims should decontaminate the areas of contact with gentle and voluminous rinsing and washing with mild detergent, and ocular exposures require normal saline irrigation [113].

Mollusca

The cone snails, class Gastropoda, superfamily Conidea, are the dangerously beautiful, paramount toxin-producing representatives of the phylum Mollusca. As a genus, *Conus* represents the largest genus of living marine invertebrates, and it encompasses more venomous species than any other single genus in the entire animal kingdom, with greater than 500 species that display an array of brilliant color patterns [115]. Their venom components have primarily been associated with modest-sized, disulfide and cysteine-rich, peptides, collectively known as conotoxins [116]. Currently, the number of different conotoxins is believed to be greater than 50, 000, and within this number are a plethora of peptides and proteins, with diverse structures, and toxicological activities that target a wide range of ion channels and receptors [117,118]. Not surprisingly, given their venom diversity, cone snail toxins and their derived peptides have a potential role in the development of therapies for a variety of medical disorders.

Cone snails inhabit tropical waters of the world and are predatory, possessing a hollow, harpoon-like radular tooth within its extensible rostrum that can be rapidly deployed for envenomating prey [103]. Prey is usually engulfed and simultaneously envenomated. They have been roughly divided into three groups depending on their preferred prey: molluscivorous (feeding on gastropods, making them somewhat cannibalistic), piscivorous (feeding on fish), and vermivorous (feeding on polychaete worms). They have also been known to prey on bivalve mollusks, octopus, and hemichordate worms [119].

Cone snail toxins have been classified based on their structure, principally their cysteine framework, which is based on the distribution and number of cysteines in the primary sequence [120]. Two major groups of toxins that have been intensely studied are the conotoxins with multiple disulfide bonds, and the much less prevalent *Conus* peptides that possess only a single, or no disulfide linkages. Superfamilies of these peptide toxins most extensively researched are the A—(alpha-conotoxins, alphaA-conotoxins, and kappa conotoxins), M—(μ-conotoxins and psi-conotoxins), and O—(omega-conotoxins, delta-conotoxins, μO-conotoxins, and kappa-conotoxins) superfamilies. Those in the A-superfamily act as nicotinic acetylcholine receptor antagonists, and voltage-gated potassium channel blockers, while the M-superfamily toxins impair voltage-gated sodium channels and act as noncompetitive nicotinic acetylcholine receptor antagonists. The O-superfamily conotoxins possesses the greatest range of function pharmacologically with actions to block voltage-sensitive calcium channels and the ability to block both sodium and potassium voltage-gated channels. The nondisulfide *Conus* peptides such as conantokins, contalakins, conopressins, exhibit M-methyl-D-aspartate receptor antagonism, neurotensin-like peptides, and vasopressin antagonism, respectively while the pharmacological role of contryphans remains to be determined [117].

Cone snail related fatality to humans was first reported by Rumphius in 1705, and the fatality rate in more recent times has been reported to be 70% [121]. Of all the cone snails, it is the Geography Cone (*Conus geographicus*) that most frequently causes envenomation to humans, and is responsible for most fatalities. However, *C. aulicus, C. gloriamaris, C. marmoreus, C. omaria, C. striatus*, and *C. tulipa* have been reported to cause death in humans [121,122]. Victims of *C. geographicus* and *C. obscurus* envenomation experienced a radiating numbness while contrastingly, victims of *C.aulicus, C. textile*, and *C. tulipa* reported intense pain. Acute envenomation symptoms involve muscular paralysis, respiratory distress, loss of gag reflex and ability to swallow, and blurred or double-vision. Timely treatment involving cardiopulmonary support is essential as no antivenom currently exists [117].

The blue-ringed octopus, class Cephalopoda, is a small octopus species native to shallow coastal and intertidal waters of Australia. Two similar appearing species, *Hapalochlaena maculosa* and *H. lunulata*, are native, and as their common name implies, they have yellowish-gold bodies with dark mottled bands and blue rings, which appear to light-up a brilliant fluorescent blue color when the animal is disturbed or frightened [89]. They have eight arms, are carnivorous, and use their two beak-like jaws to introduce venom via the salivary duct with the inflicted bite, but have been observed to subdue prey by releasing their saliva into water surrounding the prey [123].

The venom of *Hapalochlaena* sp. has had two main toxins identified, hapalotoxin and tetrodotoxin, with tetrodotoxin having a defensive role against predatory fish. Additional venom components such as histamine, serotonin, and tyramine have been found in the salivary glands [124]. Originally, the tetrodotoxin was called maculotoxin, but careful spectral analysis using nuclear magnetic resonance showed that it was identical to tetrodotoxin [125]. The bite of the blue-ringed octopus may not elicit a significant pain response, and it may go unnoticed until symptoms of paresthesia around the face and neck are felt. Envenomation to humans has proven fatal, and death has been reported to occur within 30 min of having been bitten. Since no antivenom is available, the rapid use of standard life support measures, with emphasis on respirator-supported breathing being maintained until spontaneous breathing returns [126].

Interestingly, there is recent documentation indicating that saxitoxin is present in *Abdophus* sp. of octopus from the northern coastline of Western Australia. The source of this curare-like neurotoxin, normally produced by dinoflagellates, in the diet of this octopus is uncertain, but the tentacle toxin concentration is believed to be capable of causing profound paralytic shellfish poisoning [127].

Porifera

Sponges, class Demospongiae, are the simplest multicellular marine life forms, having no defined organ systems. They are sessile creatures with amorphous and radially symmetrical shapes that grow attached to nearly any substrate, and are found from deep ocean bottoms to the shallow coastal

waters. There are four classes of Porifera, but the class, Demospongiae, has been most studied. The exact number of sponge species is still a major question, but at least 7000 species are reported in the literature (far fewer are freshwater species), and it has been speculated that the number may be greater than 15,000 [128].

Some have sharp spicules, but these are not believed to deliver toxin, and it may be that potential toxins are associated with the solid body rather than spicules [129]. Although detailed knowledge of sponge chemistry and pharmacology is in its infancy, the number of pharmacologically active compounds being discovered, such as the antitumor macrolides (spongistatins), is rapidly increasing [130]. Toxins specific to sponges have not been identified, but it is clear that adverse dermal reactions can result when dermal contact occurs.

Skin contact with the Southern Australian toxic sponge, *Neofibularia mordens*, can cause severe dermatitis that may last for months, and does not respond well to symptomatic medication. *Tedania* sp. and *Lissodendoryx* sp. have also been reported to cause an irritating dermatitis, but of shorter symptom duration [89]. Ice packs and cooling solutions, with camphor and menthol, have been reported to ameliorate the irritated skin, and hot water and alcohol are to be avoided as they intensify the agitation [37].

Briefly, freshwater sponges such as the *pipera-coora* (*Ephydatia* sp.) from Australia and the *Spongilla lacustris* from Europe may cause irritation with handling, and undefined substances from both were found to cause diarrhea, respiratory distress, and death in animals [89].

Vertebrate Marine and Freshwater Animal Toxins and Venoms

Marine and freshwater vertebrate animal toxins are present in some of the world's creatures most commonly familiar to humans (Table 19.4). Whether it is a stingray in the sandy beach waters, a catfish caught on the fishing line, or sea snake or sea turtle swimming in the coral reef, these are all descriptions for which we can easily conjure up images. However, these conjured images are linked with the fact that stingrays, venomous fish, and sea snakes are the marine vertebrates most responsible for significant envenomations to humans.

Fish are vertebrates represented by 22,000 species, accounting for half the world's vertebrate population [131], and greater than 200 species are venomous [132]. Interestingly, it has been speculated that the evolution of the venom apparatus in a large number of these fish is a result of their nonmigratory, slow moving habits, and their liking of sheltered shallow water environments [133].

It is these various vertebrate marine forms that necessitated the need for a more distinct differentiation between the terms poisonous and venomous. Marine animals that are considered poisonous generally possess an *acquired* toxicity as a result of their having ingested other toxin-producing organisms while venomous animals manufacture their venoms in a distinctive venom gland primarily from their own endogenously produced toxins, and deliver their venom via barbs, fangs, spines, or teeth that cause trauma in addition to the toxicity associated with venom. The collective toxins that make up of venoms tend to be heat labile, large–molecular-weight

TABLE 19.4
Marine and Freshwater Vertebrate Animals: Venomous or Toxin-Producing

Phylum	Class	Common Names	Genera (Toxin-Producing)
Chordata	Chondrichthyes (cartilaginous fish)	Hornsharks, spiny dogfish	*Heterodontus, Squalus*
		Stingray, bat rays, eagle rays, butterfly rays, river rays, whip rays, skate, stingaree	*Aetobatus, Aetomylaeus, Dasyatis, Gymnura, Mobula, Myliobatis, Potamotrygon, Pteromylaeus,Taeniura, Rhinoptera, Urogymnus, Urolophus*
	Osteichthyes (bony fish)	Boxfish, trunkfish	*Lactophrys, Ostracion*
		catfish	*Aurius, Centrochir, Clarias, Heteropneustes, Ictalurus, Liobagrus, Noturus, Pimelodus, Plotosus*, *Pterodoras*, *Tandanus*
		Scorpionfishes	*Centropogon*, *Dendrochirus, Gymnapistes, Helicolenus, Hypodytes, Inimicus, Neosebastes, Notesthes, Pterois, Scorpaena, Sebastes, Sebastodes*
		Stonefish	*Synanceia* (*Synanceja*)
		Toadfish	*Batrachoides, Thalassophryne*
		Blennies	*Meiacanthus*
		Rabbitfish	*Siganus*
		Stargazer	*Uranoscopus*
		Weeverfish	*Echiichthys, Trachinus*
	Reptilia	Sea kraits	*Laticauda*
		True sea snakes	*Acalyptophis, Aipysurus, Astrotia, Disteria, Emydocephalus, Enhydrina, Ephalophis, Hyderlaps, Hydrophis, Kerilia, Kolpophis, Lapemis, Parahydrophis, Pelamis, Thalassophina, Thalassophis*

proteins. Poisons, in contrast, are primarily low-molecular–weight, heat stable substances, and the poisonous animals involved lack an exacting anatomical venom delivery system [134]. Fish of the genera Batrachoides, Echiichtis, and Syanchceia are commonly considered scorpionfishes but are singled out in Table 19.4 because they are prominent venomous piscine genera.

Sea snakes of the Laticauda genus have periods in their reproductive life cycles requiring brief excursions to coastal beaches for egg laying. All other sea snake genera spend their entire lives in water, including giving birth to live young [135]; thus, sea snakes are truly marine animals and are included along with the bony and cartilaginous fishes for presentation and discussion in this vertebrate marine animal section.

Chordata, Marine and Freshwater Venomous Vertebrates

Chondrichthyes

Sharks from only two genera are known to be venomous (Table 19.4), and they are sometimes described as shark-like fish of the subclass, Elasmobranchii [136]. Although it is likely envenomations by these sharks may be more frequent than reported, literature that documents the consequences of envenomation by either genus is lacking. Envenoming by these sharks usually occurs to fishermen as a result of a dermal penetrating puncture with their venomous spines. They have two sharp spines with luminal venom ducts, and no associated integumentary sheath enclosure, that contain a whitish mass with venom-producing vacuolated cells. A single spine is located immediately in front of the anterior dorsal fin, and immediately in front of the posterior dorsal fin [68]. The Port Jackson Shark (*Heterodontus portusjacksoni*) is native to Australian waters, and their spines inflict rough-edged lesions with venom delivery resulting in muscle weakness of the envneomated appendage [137]. The spiny dogfish, Squalus megalops/cubensis, from Brazil known in the local region as *cacao-bagre* caused significant injury to a fisherman while it was being handled. Immediate and intense pain at the site of spine puncture, followed by erythema, edema, and localized kerotosis that persisted for 14 days resulted [138]. Shark spine injuries have the obvious trauma component that accompanies venom effects, and have been treated with wound irrigation, hot water submersion, and antibiotics [36,134].

Stingrays are elasmobranchs, which have representation by at least one genus in nearly all oceans and seas, and the fresh waters of Central and South America. Their dorsoventrally compressed bodies are the perfect design for their cryptic cover while resting in shallow water. The tail extends from the flattened, tetrahedral-shaped body, making them appear like a kite with an attached balancing tail. The tail may possess a single spine or multiple spines with highly serrated edges. Their body size may range from centimeters to greater than three meters, and their barb-like spines have highly serrated edges [134]. Spine morphology varies considerably between different genera and species. The venom is contained within specialized secretory cells inside an integumental sheath that surrounds the caudal spine.

There is little known about the specific chemical make-up of freshwater or marine stingray venoms, but they are thermolabile [139]. Stingray venoms of the genera, *Patamotrygon* and *Urobatis*, have been reported to cause cardiocirculatory toxicity as a result of direct effects on the myocardium [139,140], and neurotoxic effects have also been reported [141]. Recent studies of freshwater stingray venom from *Potamotrygon falkneri* revealed 18 different venom components by acrylamide gel separation, with molecular weights ranging from 12 to 130 kDa, and exhibiting caseinolytic, gelanolinolytic, and hyaluronidase enzymatic activities [142].

Envenomation occurs via the tail whipping the spine forward and upward into a predator or other unfortunate victim, when the stingray is alarmed, frightened, or stepped on causing the simultaneous rupturing of the sheath and secretory cells to release venom. There may be considerable mechanical tissue trauma at the site of laceration or puncture resulting in severe bleeding, and the venom can add further insult by intensifying severe localized pain, erythema, edema, skin necrosis, and ulceration. Systemic symptoms of dizziness, fever, migraine, myalgia, and vomiting have occurred from freshwater stingray envenomations [142]. Victims of marine stingray envenomation have died of cardiovascular failure [143], and in one case, the reported cause of death was the result of cardiac tamponade and myocardial necrosis, following puncture of the thoracic cavity by the stingray spine [144].

Stingray envenomation routinely is inflicted on the lower extremities of humans, and treatment may include immediate attempt in pain reduction by immersion of the affected area in hot water. The wound area may also be anesthetized with 1% lidocaine and irrigated with povidone-iodine, followed by prophylaxis for infection and tetanus. The wound should be carefully examined to confirm that no residual spine fragments or other debris remain, as the removal of these in a timely fashion greatly reduces pain and secondary complications [142]. Severe cases of envenomation, usually those involving puncture of the upper torso, may require careful radiographic evaluation of the puncture site, and advanced life-support measures [144].

Osteichthyes

Venomous bony fishes are primarily inhabitants of tropical oceans, but also exist in some temperate oceans and seas. Additionally, many species have become quite popular and are maintained by fish hobbyists and in private collections. The venoms of the bony fishes are produced, maintained, and delivered by anatomical structures similar to all species. This involves venom production by specific secretory glands within an integumental sheath, associated with spines that are anatomically located in conjunction with fins of the anal, caudal, dorsal, opercular, pectoral, pelvic, or shoulder areas of a given species. When a spine pierces tissue, the integumental sheath's integrity is disrupted and the venom delivered into the wound [145]. It is notable that the inflicted spine

injury from many species may in itself be more traumatic and damaging than the venom.

Fish venoms of nearly all species are used almost exclusively for self-defense, and as such, there is a great degree of similarity in their chemical and pharmacological properties, and their differences are quantitative [146]. Like the venom of their cartilaginous relatives, bony fish venoms also exhibit labile sensitivities, their stability and activity being easily abolished by fluctuations in temperature, heat, and pH [147]. Venom toxins identified for piscine fish range from 15 kDa [148] to 800 kDa [147]. The pharmacological effects of piscine venoms, collectively, are represented by three major observed toxic effects in vivo: (1) their ability to induce serious cardiovascular toxicity, (2) their neuromuscular activity, and (3) their cytolytic activity. Mechanistically, it is thought that both the cardiovascular and neuromuscular effects are the result of the potent cytolytic property, and that a single toxin in the venom is responsible for expression of all these activities [146].

Not surprisingly, given the pharmacological and toxicity similarities for most piscine fish venoms, the treatment of their envenomations is also similar. Early initiation of treatment is important and involves the immediate irrigation of the wound, removal of any easily retrieved material, and immersion of the wound in hot water (not so hot as to cause burns). Analgesic drugs and the use of local anesthetic at the wound site should be instituted. If specific antivenom is available, it should be administered. Timely follow-up with wound care is important to rule out the possibility of retained solid materials in the wound, and evaluate for secondary infection. Marine wound infections, when they occur, are commonly polymicrobial, and appropriate antibiotic administration should be initiated if symptoms are warranting [149].

A few of the more well-known groups and genera of bony piscine fish will now be discussed in more detail.

Catfish—Catfish are an easily recognizable whiskered fish, and approximately 1000 different species inhabit the fresh and salt waters worldwide. They have dorsal and pectoral fin spines, that may or may not be edged with sharp serrations, and are associated with a venom-containing integumental sheath. These spinal fins are usually kept flush with their body; however, when distressed, the spines become locked in an extended position to inflict their wound [68]. Although there are multiple genera associated with toxic reactions (Table 19.3), the main adverse effect of consequence from their inflicted stings is related to the trauma associated with the puncture of tissue.

In North America, catfish of the genera *Ictalurus* and *Noturus* are responsible for most stings [150]. Venom of the white catfish, *Ictalurus catus*, has been reported to contain two to eight lethal factors, and two dermonecrotic factors that was moderately heat stable [151].

In the Indo-Pacific region, the Arabian Gulf catfish (*Arius thalassinus*) and the striped catfish (*Plotosus lineatus*) are notorious for their ability to inflict extremely painful wounds, and deliver several proteinaceous toxins, known as *crinotoxins*, in the stinging process. Both species have also been shown to contain these toxins in their skin secretions as well. Investigation of the activities of *A. thalassinus* toxins revealed they contained vasoconstrictor components that could induce vasoconstriction in vascular smooth muscle [152], and may be related to muscarinic receptor activity [153]. The skin toxin of *P. lineatus* has yielded a hemolysin, two edema-forming factors, and two lethal factors [154]. Venoms of Indian catfish species, *Hereropneustes fossili* and *Plotosus canius*, contain or cause the release of prostaglandins that induce smooth muscle contractions in experimental models [155], and have caused severe neurotoxic symptoms when injected into mice [156]. A lethal component of *C. canius* venom, toxin-PC, acts presynaptically, blocking neurotransmitter release resulting in neuromuscular blockade [157]. Given the array of catfish venom toxinological effects, it is unfortunate that there is little reporting of envenomation cases that would provide a basis to develop optimal clinical management.

Catfish stings and envenomation most often occur to fishermen who are actively pursuing the capture of fish. Symptoms of bleeding, stinging, throbbing sensation, intense pain, and even peripheral neuropathy may result [158]. Treatment comprises judicious wound care and removal of any residual spine fragments in the wound. Secondary infections are a frequent complication with catfish injuries, and antibiotic therapy may be necessary [159,160].

Scorpionfishes—Scorpionfish is a somewhat vague name that is frequently used to encompass a broad general taxonomical classification when referring to any one of a number of genera of anatomically similar fish (Table 19.3). They are commonly known throughout their wide range, as they are present in all oceans of the earth, but venomous species are most prevalent in tropical waters. Out of this diverse group, approximately 80 species have been reported to possess venom toxins or have caused envenomation to humans [161]. They are easily recognized due to their unique and often ornate appearance, an attribute that also makes them desirable by collectors. Some species, such as the lionfish (*Pterois volitans*) and zebrafish (*Dendrochirus zebra*), of the Indo-West Pacific oceans and Red Sea, are highly sought after by aquarists [162].

Scorpionfish venom glands are associated with spines represented in the configuration of 3 anal spines, 10–15 dorsal spines, and 2 pelvic spines. Venom extracts from lionfish (*P. volitans*) and soldier fish (*Gymnapistes marmoratus*) have been found to increase intracellular Ca++ in cultured neurons possibly as a result of pore formation in cell membranes. The increased Ca++ was associated with increased cell death due to cellular swelling and neurite loss [163]. In support of the calcium ion role in toxicity is the observation of both adrenergic and cholinergic cardiotoxic effects from *P. volitans* [164] and *G. marmoratus* [165] crude venoms in animal tissue and cell culture models. Cardiovascular effects of *Scorpaena guttata* venom in rat hearts are biphasic with early reduction in chronotropic and inotropic effects, later

followed by an increase in these effects. Thus, the venom acts on muscarinic and adrenoreceptors, respectively, and this may be the result of endogenous autocoid release [166]. The venom of the bullrout (*Notesthes robusta*), a species native to eastern Australia, contains a 169.8–174.5 kDa protein (*nocitoxin*) that is capable of pain induction in humans via polymodal nociceptor stimulaton [167]. Collectively, the cardiotoxic and neurotoxic effects suggest that scorpionfish venoms act pre- and postsynaptially to induce cell membrane depolarization, and that this action is dependent on extracellular divalent cations [146]. Future research with scorpionfish venoms should provide a number of pharamacologic toxins useful as research tools and possible medical uses.

Envenomation by scoropionfishes nearly always results in profound pain, a possibility of significant swelling, and systemic effects such as nausea, vomiting, diaphoresis, dyspnea, hypotension, weakness, syncope, and collapse have been reported. Systemic complications are possibly related to the intensity of pain rather than true toxic effects of venom [168,169]. Treatment of envenomation is supportive as described earlier; however, stonefish antivenom has been implicated as potential therapy for lionfish and soldierfish envenomations [170].

Stonefish—Synanceia spp., also taxonomically referred to as *Synanceja* spp., are one of the most researched venomous fish genera, and their venoms have yielded unique toxins with a variety of pharmacological activities [171]. The different *Syanceia* species are native to waters throughout the Indo-Pacific oceans, and are known by a variety of local names, such as dornorn or wary-phoul in Australia, devilfish in Java, gofu in Polynesia, sherova in East Africa, and the U.S. name of stonefish is almost universal [134]. These slightly grotesque, but quite interesting looking, fish are morphologically reminiscent of an algae, moss, or barnacle covered rock, and as such, their looks complement their cryptic behavior. Thus, they lay on the bottom waiting to ambush unsuspecting prey, and may also be accidentally stepped on by the unobservant human wading in the shallow waters they frequent.

Stonefish venom glands are closely associated with the 13 dorsal spines, which appear to be covered with rough, thick skin. Two lateral grooves run the length of the spine and the venom glands reside in the mid-third of the spine shaft. The glands are sheathed in a fibrous, multilayered capsule that is vasculature and has a nerve network. Stonefish will release a milky white dermal secretion, independent of their spine venom, when handled or agitated. Crude, fresh Stonefish venom has been characterized as being opalescent in appearance, heat labile, and has a pH of 6.0 [172].

The venoms of three species, *S. horrida*, *S. trachynis*, and *S. verricosa*, have all been found to contain catecholamines [173], exhibit various enzymatic activities [174], and increase vascular permeability [172]. A lethal substance called *stonustoxin* (SNTX) that comprises two large subunits, alpha = 79.4 kD and beta = 79.3 kDa, has been isolated from Indian stonefish, *S. horrida*, venom [175], and induces cardiovascular hypotensive responses in rats, that are thought to be nitric oxide mediated [176]. SNTX also inhibits muscle cell contraction by acting directly on the myocyte rather than neurotransmission blockade [177]. Estuarine stonefish, *S. trachynis*, venom contains a 158 kDa toxin, *trachynilysin* (TLY), that elicits atrial muscle cell membrane hyperpolarizing actions [178], causes massive neurotransmitter discharge at low concentrations, and both muscle and nerve injury at higher concentrations, in tissue preparations [179]. Interestingly, it has been reported that TLY effect mechanisms are quite similar to those produced by alpha-latrotoxin from the black widow spider (*Latrodectus* spp.) [180]. The venom of the Reef stonefish, *S. verrucosa*, also has its own special toxin, *verrucotoxin* (Vtx), a four subunit toxin comprising two alpha (83 kDa) and two beta (78 kDa) subunits [181]. This toxin can also elicit hypotensive [181] and neurotoxic effects in animals [182]. These different stonefish toxins represent the most bioactive components of their respective crude venoms, account for all the suspected lethal activity, and suggest that their venoms comprise a relatively small number of toxins [164]. Their pharmacological and toxicological effects in various cell and animal models may be useful in biomedical research, but it is difficult to clearly define specific functions that impact human health.

Clinical features of stonefish envenoming in humans involve local tissue trauma with gradually intensifying pain at the spine penetration site. Swelling and erythema may progress for up to 24 h. Systemically, the key serious complications are cardiovascular related, with hypotension, bradycardia, pulmonary edema, arrhythmia, and cardiovascular collapse associated with neurologic symptoms of muscle weakness, paralysis, and convulsions, potentially leading to death [183]. Although death has been reported [184], there may be possible inaccuracies with the documentation, and stonefish sting should not be a terminal life event.

Treatment of stonefish envenomated victims is supportive and symptomatic, and specific antivenom produced in Australia (CSL Limited, Parkville, Victoria, Australia) is effective in reversing many of the severe effects of venom toxicity [185]. This antivenom has been considered to possibly provide paraspecific coverage against the toxic effects of *G. marmoratus* and *P. volitans* venoms based on animal studies, but this has not been tested in humans [170].

Toadfish—Thalassophryne spp. are similar to stonefish in behavior and appearance, inhabiting the bottoms of coastal waters worldwide, and some species can use their swim bladders to *sing* their watery trill. They have two dorsal and two opercular spines connected to venom glands, and their venom is delivered with rupture of the integumentary spinal sheath upon stinging [134]. Venom is composed of heat labile, bioactive toxins, range 18–97 kDa molecular weight, that alter arteriolar and venous microvascular hemodynamics, and cause myonecrosis [186]. Envenomation to humans results in severe pain, dizziness, and edema with a rapidly ensuing tissue necrosis [187]. Treatment consists of immersion of the wound in hot water, analgesics or local anesthetics, and supportive wound care [188].

Weeverfish—Echiichthys or *Trachinus* species, weeverfish, predominantly inhabit the flat sandy bottoms of European coastal waters, the Black and Meditteranian seas, Chile's Pacific coast, and South Africa's [68]. Taxonomical confusion may come from the fact that four species of *Echichthys* were previously listed in the genus, *Trachinus* [30]. Although multiple species have been identified, two European species are well known for their toxic stings, the greater weeverfish (*Echiichthys draco*) and the lesser weeverfish (*Echiichthys vipera*) [68]. As their name implies, the greater weeverfish is fairly large, and may reach a half-meter in length. Weeverfish venom delivery is accomplished with any of the five dorsal, or two opercular, spines that have a thin-walled integumentary sheath near their base, covering the venom gland [134].

Weeverfish toxins are quite heat labile, but have been isolated, intact, from *T. draco*, a 105 kDa polypeptide with hemolytic properties called *dracotoxin* [189], and from *T. vipera*, a 324 kDa compound composed of four subunits called *trachinine* [190]. The crude venom of *T. draco* contains histamine, catacholamines, exhibits cholinesterase activity, and produces hypertension with subsequent sustained hypotension in cats, demonstrating a biphasic effect [191].

Weeverfish stings vary widely in their severity from the usual symptoms of intense pain with local inflammation to extremely rare cases of secondary infections associated with fatality [192]. Generally, symptoms resolve in about a week, and treatment is the same as that used for other Osteichthyes envenomations [149].

Sabre-toothed blenny—Meiacanthus nigrolineatus is briefly mentioned here because it is the only piscine fish known to deliver its venom with its grooved canine teeth, in contrast to other piscine fish that deliver their venom with spines. Their venom gland is located in the lower jaw, and there is no information as to the chemical or pharmacological properties of their venom [193]. Their bite to humans is said to be painful (Keyler, D.E. personal communication, 2005.)

Rabbitfish—Siganus spp. are native to the Indo-Pacific oceans and seas. They have 13 venom-delivering dorsal spines, and the associated venom glands are located within anterolateral grooves that run the length of the spine. The four pelvic spines are similar in structural anatomy. This venom delivery and venom gland arrangement is quite similar to that of the lionfish. Like the sabre-tooth blenny, little is known about their venom, but stings are said to be comparable to those of other similar scorpionfish [134].

Stargazer—Uranoscopus spp. have the descriptive name, stargazers, as their small eyes are located on the flat dorsal aspect of their cuboidal-shaped head, making them appear to be gazing upward toward the water's surface. They have a short, but sharp stubby spine with a poorly defined venom gland that surrounds their base on each side of their body in the region immediately above the pectoral fins. Knowledge concerning chemical composition or pharmacological activity of their venom is lacking, and it is only the reporting of human fatalities caused by the Mediterranean specie, *Uranoscopus scaber*, that supports their being considered venomous [194]. Investigators have tested for toxic effects in animals by injecting crude venom extracts from stargazer venom glands, but were unsuccessful in confirming any toxicity [195]. This lack of toxicity confirmation may have resulted from sensitive chemical and toxin instability of the venom following its removal from the venom gland. Treatment of stargazer envenomation should follow that of other piscine envenomations [196].

Boxfish and *Trunkfish—Lactophrys* and *Ostracion* are two genera to be discussed briefly because they produce toxins in their skin, and secrete them rather than deliver them with a specialized apparatus. They are native to the Indo-Pacific and their toxins are used as a defense against other fish predators, and therefore are ichthyotoxic. By releasing these natural fish killing toxins into their immediate environment, they effectively deter their predatory counterparts and survive to live another day [134]. The primary toxin produced by both these genera is *pahutoxin* (PHN), a choline chloride ester of 3-acetoxypalmitic acid that has phospholipid membrane permeability disruptor and surfactant properties [197]. Thus, dual pharmacologic actions of lipophilic and hydrophilic components appear to function synergistically. The regulatory mechanism of this action stems from two distinct fractions in trunkfish skin secretion, and has been proposed to be receptor mediated [198]. The consequences to humans who make contact with these skin secretions are dermal irritation, and persistent complications would not be expected to follow [30].

Reptiia

Sea snakes navigate coral reefs and oceans in the world's subtropical and tropical regions but have been observed thousands of miles distant to these regions, even in the absence of oceanic currents or foul weather. Thus, they are the most widely dispersed venomous reptiles on the earth [199].

Their morphological appearance and physical body structure, although similar to terrestrial snakes, is usually distinguished by the presence of laterally compressed tails with a paddle-like look. Their physiology allows them to dive to depths of 100 m, and stay submerged for greater than 3 h, but they rely on atmospheric air to survive. There are 17 different genera (Table 19.4), and they average approximately 1 m in length, but some species (*Laticauda colubrine*) approach 3–4 m. Sea snakes usually have short (<4 mm), conical, fixed-position fangs, and the fang length varies according to the prey eaten. The fangs are luminal and interface with ducts that connect the fangs to elongated venom glands lying in the upper jaw on each side of the head. Venom glands are fibrous-encapsulated and attach to adductor muscles that contract to express venom through the venom duct network and into prey [135].

Sea snake venoms are complex toxin blends with varying combinations of protein toxin types, are extremely toxic, and their venoms are the most potent of any vertebrate animal on earth. The venom yield delivered from most sea snake species

ranges from 1 to 10 mg, but species such as *Astrotia stokesii* can deliver greater than 20 mg, and as little as 0.04 mg/kg of *Aipysurus duboisii* is fatal to mice. Venom dose-response studies suggest that the beaked sea snake (*E. shcistosa*) delivers enough venom in its maximum yield to kill approximately 53 humans [135]. Whole sea snake venom possesses several enzymes and comprises acetylcholinesterase, hyaluronidase, leucine aminopeptidase, 5'–nucleotidase, phosphomonoesterase, phosphodiesterase, and phospholipase A [200]. Toxins isolated from venom usually contain 60–62 amino acid residues, and a total of 29 different toxins have been isolated from 9 separate species of sea snakes. Purified toxins maintain their integrity as they have been demonstrated to be heat stable, both acid and base pH tolerant, and are more potent than crude venom based on animal LD50 experimental data [201]. The potency of sea snake venoms is linked to both pre- and postsynaptic neurotoxins. An isolated postsynaptic protein toxin of 6–8 kDa molecular weight, which is highly stabilized by multiple disulfide bridges, is a primary pharmacologically active toxin [201]. The postsynaptic toxin binds to the acetylcholine receptor. Venom from the beaked sea snake (*Enhydrina schistose*) contains a phopholipase, and the venom of *Laticauda semifasciata* contains the curaremimetic toxin, *erabutoxin* [202]. A that has a demonstrated presynaptic toxic effect, plus phospholipase activity [202]. Thus, the net effect of venom, on living animals envenomated by sea snakes, is paralysis resulting from irreversible binding of the postsynaptic toxin at the acetylcholine receptor in the neuromuscular junction [203].

Envenomation by sea snakes to humans is a serious medical concern. Sea snake fangs, being small, do not make deep punctures, and fang marks (may appear as scratches) may not be easily visualized. Additionally, the bite is frequently asymptomatic, not even felt by the victim, and local symptoms are usually absent. Envenomation is manifested by diplopia, dysarthria, dysphagia, muscle fasciculations, muscle pain, shock, trismus, and paralysis [204]. Myoglobinuria, myonecrosis, and nephropathy have also been observed [205]. Neurotoxic symptoms may be deferred for up to 3 h, often causing the victim to delay seeking medical attention. Fatalities usually result from paralysis of the diaphragm progressing to respiratory arrest, and lack of timely treatment. A pressure immobilization method of first aid has been found to be beneficial and may be of major importance when a victim is remote to a medical facility [204]. Cardiopulmonary support is effective therapy, and sea snake antivenom (CSL Limited, Parkville, VIC, Australia) is a definitive therapy. Importantly, the antivenom appears to be effective for envenomation by all sea snake species, and it also demonstrates venom similarities of sea snake species [206].

Terrestrial Animal Toxins

The land dwelling animals of the world are associated with all the continental landmasses of the earth, although the diversity and numbers of different species are the greatest in tropical regions. As the latitudinal distance from the equator becomes greater, the diversity and number of animal species decreases. Thus, an inverse relationship exists between the number of species and distance from tropical climates, a relationship that is paralleled with respect to the diversity and number of animal toxins. In recent centuries, a confounding element in these relationships has been the human being. As humans have increasingly traveled the earth, there has been an associated increase in the dispersal of species to regions beyond their natural ranges. Additionally, as the world's population increases and expands geographically into natural environments there is an ever-increasing interface between humans and terrestrial animal species. This leads to human encounters with animals, and consequent envenomations from insects, centipedes, scorpions, spiders, amphibians, lizards, and snakes. The frequency of encounters with these toxin-producing species is probably greatest in tropical third world countries, where the availability of medical treatment is nonexistent or quite limited. Unfortunately, the consequences of humans living in close proximity to potentially dangerous creatures may also lead to the disappearance of animal species over time, and the loss of knowledge related to their natural toxins. The toxins from stinging, biting, and skin secreting terrestrial species, both invertebrate and vertebrate, have received considerable attention in biomedical research for scientific and medical reasons.

Terrestrial Invertebrate Animal Toxins and Venoms

The terrestrial invertebrates capable of delivering toxins via bites, stings, skin secretion, or bristle and hair contact are members of the largest phylum in the animal kingdom, Arthropoda. These relatively small, often thought of as creepy creatures, are probably responsible for envenomations to humans in numbers exceeding the sum of envenomations from all other animal phyla [132]. Arthropods all have legs, and walk, run, or fly, and there are three main classes of toxin- or venom-producing members (Table 19.5).

Arthropoda, Terrestrial Venomous Invertebrates

Arachnida

Arachnids differ from centipedes and insects by possessing eight legs in contrast to the large number on centipedes and the six on insects. They do not have antennae or wings, but can live in a variety of ecosystem environments. Their toxins represent an amazing pharmacological arsenal capable of subduing a spectrum of prey, and multiple arachnid forms pose significant danger to human health in some regions of the earth.

Scorpions

Scorpions are represented by at least 1500 individual species, from multiple genera (Table 19.5) that live in arid and wet ecosystems, are the largest of the arachnids in size, and may possibly be the oldest living creatures on earth (400 million

TABLE 19.5
Terrestrial Invertebrate Animals: Venomous or Toxin-Producing

Phylum	Class	Common Names	Genera (Toxin-Producing)
Arthropoda	Arachnida	Scorpions	*Androctonus, Botothus, Buthus, Centuroides,Heterometrus, Leiurus, Mesobuthus, Opistophthjalmus, Orothochirus, Palamnieus, Scorpio, Tityus*
		Spiders	*Achaearanea, Aganippe, Agelenopsis, Araneus, Arbantis, Argiope, Artosa, Atrax, Badumna, Bothriocyrtum, Breda, Chiracanthium, Cupiennius, Diallomus, Dolomedes, Drassoides, Dysdrea, Eriophora, Fillistata, Hadronnyche, Harpactirella, Hermeas, Heteropoda, Holoplatys, Isopeda, Ixeuticus, Lampona, Lycosa, Latrodectus, Liocranoides, Loxosceles, Missulena, Misumenoides, Neoscona, Nephila, Olios, Opisthoncus, Peucetia, Phidippus, Selenocosima, Steatoda, Tegenaria, Thiodina, Trechona, Ummidia*
		Bird/Goliath spiders	*Ornithoctonus, Theraphosa*
		Tarantulas	*Acanthoscurria, Avicularia, Brachypelma, Megaphobema, Phoeneutria, Phrixotrichus, Poecilotheria, Stromatopelma*
	Chilopoda	Centipedes	*Cormocephalus, Ethmostigmus, Otostigmus, Rhysida, Scolopendra, Scutigera*
	Insecta	Ants	*Mrymecia, Paraponera, Solenopsis*
		Caterpillars	*Doratifera, Euproctis, Lagoa, Lanomia, Thaumetopoea,*
		Bees, bumblebees	*Apis, Bombus*
		hornets, wasps	*Dolichovespula*, *Dasymutilla, Vespa*
		Yellowjackets	*Paravespula, Vespula*
		Sawflies	*Arge, Lophyrotoma, Perreyia*

years) [207]. Scorpions appear like a small, earth-tone colored, lobster carrying a thin tail with an inverted hook, arched over its back. Their body comprises three distinct sections, but the head and thorax are joined to form a cephalothorax, which has pincers, or pedipalps, used for restraining prey and feeding themselves. The pincer-like claws are actually a modification of the first set of legs, and the remaining three pairs of legs are used for walking. The body part most humans and prey are familiar with is the stinger, which is attached to the telson, a terminal distal segment that houses venom glands connecting to two orifices in the stinger. Scorpions sting with this apparatus, and deliver their venom into a variety of prey organisms, including other arthropods and humans. Scorpions are fossorial and reclusive in nature, tending to be nocturnally active.

Scorpion venoms have been the focus of ion channel researchers for nearly 25 years since the isolation of, *Noxiustoxin*, a short chain venom peptide, in 1928 [208]. Their venoms are a mixed combination of approximately 50–100, low molecular weight, polypeptide toxins, and it has been estimated that there are greater than 100,000 different peptides among all the scorpion species [207]. Of this large number of species and toxins, only 30 scorpion species and an associated 200 distinct polypeptides have been investigated, and their functional properties reported [209]. Their venoms contain various polypeptides with an array of pharmacological and physiological actions highly specific for crustaceans, insects, and mammals, and have been of interest in bioinsecticidal research and development [210].

All scorpion toxins are constructed with an alpha-helical segment and two to three anti-parallel beta-sheet folds, tightly held by three to four disulfide bridges that maintain the tertiary conformational structure.

Four main families of scorpion ion-channel toxins have been proposed, with their separation based on their effects on membrane ion channels: Na^+ channels [211], K^+ channels [211], Cl^- channels [212], and Ca^{2+} channels [213]. Of these four ion-channel families, two main groups, based on their pharmacological activity and molecular size have been identified. The short-chain toxins comprise 23–47 amino acids that primarily effect K^+ channels [214] and the long-chain peptides possess 59–76 amino acids that target their effects on voltage-gated Na^+ channels [215]. It is also evident that scorpions, of nearly all the toxin-producing genera, possess varying combinations of ion-channel toxins [216].

Scorpion potassium channel toxins are diverse and have been divided into alpha-, beta-, and gamma-scorpion toxin subfamilies based on their biophysical properties [214], and these subfamilies have been further split based on their voltage-gated and ligand-gated K^+ channel actions [217]. Scorpion K^+ channel toxins from representatives of several different genera such as *Androctonus mauretanicus* (Kalitoxin), *Buthus martensi Karch* (BmTX3), *Centuroides noxius* (*Noxiustoxin*), *Leirurus quinquestriatus* (*Charybdotoxin*), *Hterometrus spinnifer* (HsTX), and *Scorpio maurus* (*Maurotoxin*) have been identified [218].

The scorpion sodium ion-channel, long-chain toxins are important in their toxic effects, as 90% lethality is attributed to these toxins binding voltage-sensitive Na^+ channels in muscle and nerve cells of mammals [219]. Scorpion sodium ion-channel toxins are designated as alpha- or beta-toxins depending on their binding, site of action, in the Na^+ ion channel [215]. Some of these toxins have also been found to be potent arthropod-selective in their toxicity toward crickets, crabs, squids, and triatomides (kissing bugs), yet lacking apparent toxicity in mammals. This has been found

to be the case with the Na^+ ion-channel toxin, *ardiscretin*, isolated from *Tityus discrepans* venom [216]. In contrast, alpha-toxins from *Buthus* sp. venoms have significant toxic effects in mammals [220]. Similarly, the Na^+ ion-channel toxin, Lqh-alpha-IT, isolated from *Leirurus quinquestriatus hebraeus* venom contains an insect-specific alpha-toxin, while an alpha-toxin from *Androctonus australis* affects primarily mammals. Thus, despite between genera alpha-toxin similarities, diversity in effect on target organisms suggests that relatively minute differences in structure are responsible for the expression of different functions, and that alpha-insect and alpha-mammal toxins may have been derived from a common genetic ancestor [221].

Scorpion envenomation is a serious public health problem in the countries of India, Mexico, Mediterranean and Middle East, North and South Africa, and South America, but relatively few species, despite the large number of toxin-producing species, are worthy of medical concern. However, in countries like Tunisia where up to 40,000 scorpion stings occur annually, resulting in 20–40 deaths, medical treatment of scorpion envenomation is important and fairly common [222].

Scorpions will sting when laid on, sat on, stepped on, or agitated by squeeze-contact with clothing and shoes and can deliver a painful sting with their arched telson, directing their stinger into the victim. Stings frequently occur in the evening hours when people are walking around in their bare feet. Although adult humans may suffer severe complications with scorpion stings, it is children that suffer the greatest mortality [223,224]. Given the pharmacological properties of scorpion venom, it is not surprising that stings, depending on the genus and species involved, can cause local pain, autonomic and central nervous system complications, seizures, cardiopulmonary dysfunction [224], with shock and pulmonary edema being responsible for most fatalities [225]. It is also important to note that hemolysis frequently develops in severe envenomations, and secondary nephrotoxic effects can occur [205].

Medical management strategies, which vary with different global geographic regions, leading to successful outcomes in envenomated victims, have revolved around treatment being performed in medical intensive care facilities, the use of various pharmacotherapies, and the administration of specific antivenom [224]. The use of antivenoms and their effectiveness have been a great controversy historically, with treatment having involved large antivenom doses and resultant anaphylaxis and delayed serum sickness [225]. Efforts are currently underway to produce more effective antivenoms with reduced adverse-effect profiles using recombinant technology [222].

Spiders

Greater than 38,000 spider species, comprising 3526 genera, are known [226], and they inhabit nearly all ecosystems, ranging from the arid hot deserts to the rain forests, and from the peaking slopes of Mount Everest [227] to life within the underwater world [228]. Spiders are masters of deception, existing in a wide variety of shapes and colors, and exhibiting equally diverse behaviors. They have been documented to mimic different vegetation forms, insects, bird dung, twigs, and gastropods, and some like the crab spiders can even change color morphology to camouflage with their preferred foliage [229]. Their size ranges from the size of a pin head (*Patu marplesi*—Samoan moss spider) up to the 25 cm leg span of the bird spider (*Therophosa blondi*) [230]. Like scorpions, their head and thorax are combined to form a cephalothorax, but their abdomen is differentiated from this anatomical structure by a narrow restricting band, or pedicel, making the abdomen pronounced. Spiders usually have eight eyes, and some like the brown spider, *Loxoscelles* spp., have only six, and some cave-dwelling species are blind. Eyesight is limited in most species, but some hunting spiders such as the jumping spider (*Portia fimbriata*) has markedly acute vision [231].

Almost all species of spiders have venom glands and produce toxins, but their ability to be of harm to humans or large prey is usually limited by the size of their paired fangs, which reside with the chelicerae at the anterior of their mouth. Spiders are predatory by nature and their venom delivery is quite effective for prey immobilization. They do not swallow their prey following their paralytic envenomation, but rather extract prey contents using their siphoning stomachs. The digestive enzymes used in liquefaction of their prey are not venom components. Two infraorders represent most of the spiders humans are familiar with. Mygalomorphae contains the tarantulas, and venomous funnel-web spiders of the *Atrax* and *Hadronyche* genera, which possess large paraxial fangs that move in a vertical up and down fashion. Fangs of these large spiders can puncture a fingernail [230]. The smaller Araneomorphae represent approximately 93% of the earth's spiders, and have smaller diaxial fangs that move in a horizontal fashion side to side [226]. Although Araneomorph spiders are not as large as the Mygalomorphs, it is Araneomorph species that are responsible for most bites to humans, and are truly dangerous to humans. Thus, an inverse relationship with size and toxicity, as the smaller spiders are generally more toxic. The spider genera known to produce toxic bites to humans are listed in Table 19.5.

The chelicerae/fangs are quite small in the majority of spider species, and as such are of no danger to humans.

Spider venoms are heterogeneous toxin mixtures composed of 3–8 kDa polypeptides, with a few greater than 10 kDa in mass, and the black widow (*Latrodectus* sp.) possesses a 100 kDa polypeptide toxin [232]. They share many pharmacological properties similar to scorpion venoms with respect to ion-channel actions. It has been estimated that the total number of diverse spider venom polypeptides may be in excess of 1.9 million [233]. Spider venom components have been generally identified as neurotoxins that affect glutamaterigic transmission, Ca^{2+}, Na^+, K^+, and Cl^- ion-channels, stimulate neurotransmitter release, and block postsynaptic cholinergic receptors [234].

The chemical configurations of several glutamate receptor toxins have been elucidated, and determined to be primarily acylpolyamines and polyamine amides [235]. A few identified

toxins include *Joro spider toxin* (JSTX) from *Nephila calvata*, *Nephila spider toxin* (NSTX) from *Nephila maculata*, *argiopine* from *Argiope lobata*, *curtatotoxins* from Hololena curta, and *PhTX-4 toxins* from *Phoneutria nigriventer* [234].

Ion-channel spider toxins have been extensively studied, and they have significant neuropharmacological properties that affect the release of neurotransmitters. Ca^{2+} ion-channel venom studies led to the discovery of *omega-agatoxins* from *Agelenopsis aperta*, and pioneered the way for the discovery of *omega-grammatoxin* (*Grammostola spatulata*), *atracotoxin* (*Atrax robusta*), and many other omega-toxins. Na^+ ion-channel toxins impair excitable cell membrane function by altering ligand-gated sodium channels such as nicotinic cholinoreceptors and voltage-gated sodium channels that impact neuromuscular function, making them effective paralytic toxins. Spiders in the *Atrax* and *Hadronchye* genera (funnel-web spiders), and the genus *Phoneutria* (wandering spiders) have venoms possessing important Na^+ ion-channel toxins. K^+ ion-channel toxin actions are similar to those reported for scorpions. K^+ ion-channel venom toxins such as *hannatoxin* from the Rose tarantula (*Phrixotrichus spatulata*), *phrixotoxins* from the Chilean fire tarantula (*Phrixotrichus auratus*), and *heteropodotoxins* from *Heteropoda venatoria* are just a few of the many pharmacologically active potassium channel toxins. Cl^- ion-channel spider toxins although not critical in action potential mechanisms, they are involved in transepithelial transport [236], and involved in cellular excitability as receptor components for inhibitory neurotransmitters such as GABA [237].

Spider toxins and venom fractions that affect cholinergic transmission, in both the autonomic and central nervous system, have been isolated from several species of orb weaving spiders in the genus *Argiope* [238].

Enzymes in spider venoms are important components responsible for a number of different pharmacological functions. Collagenase activity in venom fractions from Australian spiders of the genera *Eriophora* and *Nephila* (orb weavers), and *Isopeda* (huntsman spiders) has been identified [239]. Hyaluronidase, believed to enhance venom spread, has been found in the venoms from the genera *Cupiennius, Lycosa, Loxosceles,* and *Phoeneutria* [234], and metalloproteinases with proteolytic activity have been isolated from *Loxoscelles* venom [240]. Phosphodiesterases that cleave cyclic nucleotides have been found in the venoms from *Atrax robusta, Aphonopelma cratus*, and *Latrodectus mactans* [241], and sphingomyelinase D-type, a powerful necrotizing toxin, has been characterized from *Loxosceles reclusa* venom [242].

The effects of spider venoms and toxins in cases of spider bite to humans are surprisingly limited, and although many spider genera contain species that have bitten humans, the majority of bites are relatively innocuous, with only a few species responsible for significant envenomation profiles (Table 19.5) [243]. *Badumna* sp. (black house spiders), *Lycosa carolinensis* (wolf spider), and *Chiracanthium mildei* (sac spider) can cause pain and erythema, and *Tegenaria agrestis* (hobo spider) necrotic lesions, but all these symptoms generally resolve without long-term consequences [244]. Spider bites, in contrast to many insect bites and stings, are not associated with inducing allergic reactions [243].

Spiders of medical importance, capable of causing significant morbidity or mortality, include the Australian genera *Atrax* and *Hadronyche* (funnel web spiders), Brazilian *Phoeneutrea* spp. (wandering spiders), *Loxoscelles* (recluse spiders), and *Latrodectus* and *Steotoda* (widow and comb-footed spiders) [244].

Funnel web spiders of Australia (*Atrax* spp.) are believed to be the most venomous and dangerous spiders on earth, and although bites are uncommon and severe envenomation infrequent, they can cause life-threatening symptoms and deaths have occurred [245,246]. Symptoms associated with severe envenomation include both parasympathetic and sympathetic effects on both neuromuscular and cardiopulmonary systems, with paralysis, coma, and profound hypotension resulting in general systems failure [246,247]. Fortunately, there is a funnel web spider (*Atrax robustus*) venom-specific IgG antivenom that is effective in reversing venom-induced toxicity. This antivenom is polyspecific, and is also effective in treating envenomations caused by other *Atrax* and *Hadronyche* spp. [244].

Armed, wandering, or banana spider (*Phoeneutria* spp.) envenomations in South America can result in significant medical complications, have caused at least one pediatric death and are responsible for 42% of the 2850 spider bites reported per year in Brazil [248]. These spiders occasionally appear in other parts of the world after they have been inadvertently imported on agricultural produce. Less than 1% of victims had severe complications (pulmonary edema), and the common symptoms of envenomation were pain, and edema. Local anesthetics and oral analgesics are of benefit, and an Fab'_2 antiarachnid antivenom has been used in moderate to severe envenomations, but its benefit is unknown [248].

Recluse or brown spiders (*Loxosceles* spp.) are capable of inflicting severe ulcerating and tissue-necrotizing bites, and considered a serious hazard in Central and South America, as well as south-central and southeastern parts of North America. They have also appeared in Europe, Asia, South Africa, and Australia, most likely having been transported into these countries, and little is known of their bites in these settings [244]. Their venoms are known for the 23 kDa enzyme, shingomyelinase D (which has four isoforms), which is the main pharmacologically active venom component responsible for most toxic effects [249]. Unfortunately, many spider bites are attributed to *Loxosceles* species, but rarely are bites confirmed to be true recluse bites, and misdiagnosis follows [250]. Bites frequently go unnoticed, as they are relatively painless, and victims delay in seeking medical attention.

Envenomation by *Loxosceles* may be dichotomous in effect, as it may cause a dermonecrotic profile, or it may appear as a viscerocutaneous form [244]. A necrotic lesion may take up to a week to express itself, and the resulting ulceration is insidious, and may persist for months. Fever and fatigue may also be present. The viscerocutaneous symptoms of Loxosceles envenomation may be fulminant, with

hemolysis, coagulopathy, shock, renal failure, and multiple systems failure leading to death if untreated [251].

The persistent nature of recluse spider bite lesions has led to considerable controversy as to the most effective treatment, but in general good thorough wound care, following an early diagnosis, leads to a favorable outcome [250]. Therapies ranging from hyperbaric oxygen to nitroglycerin patches have been used with varying effectiveness [252]. Life support measures and good intensive care are best in cases of severe systemic envenoming [252], and although antivenom is available in South America, its benefit is questionable [251].

Widow spiders (*Latrodectus* spp.) are found throughout the world, and as many as 40 species are known [244]. Envenomation may cause significant morbidity, and has resulted in death [253,254]. *Latrodectus* venoms, and clinical features following envenomation, are similar and have a common clinical syndrome throughout all geographic regions [244]. Widow venoms contain latrotoxins, >100 kDa proteins, the best known is alpha-latrotoxin, and they stimulate massive neurotransmitter release from a variety of nerves [234]. The tissue around a bite does not react to envenomation, but local pain may be present. Severe skeletal muscle pain in the abdomen, back and chest, diaphoresis, hypertension, and cramping are symptoms requiring medical treatment [253]. Spiders in the genus *Steatoda* have been reported to have symptoms similar to widow spiders, but less severe in effect. Treatment may include parenteral benzodiazepines or opioids, and antivenoms are available in Australia and the United States. Antivenom, given intravenously, is beneficial in cases of severe envenomation [244,253] The Australian redback spider antivenom has also been used in severe cases of *Steotoda* envenomation [243].

Tarantulas, due to their size and reputation, deserve a final word with respect to spiders. There are approximately 860 known species comprising 107 genera, and they are found in semi-tropical, semi-temperate, and tropical regions around the world. They are predators that inhabit many biodiverse ecosystems, and are capable of physically overpowering and mechanically injuring invertebrate and vertebrate prey. Their uniqueness and notoriety has resulted in their becoming an amazingly significant portion of the world's pet trade, and multiple species of several genera have been afforded legal protection. Their large fangs deliver rapid-acting venoms that have irreversible effects on central and peripheral nervous systems, allowing the induction of rapid paralysis, even to higher vertebrates [255]. However, despite the magnitude of their notoriety, and their anatomical and venom pharmacological traits, tarantulas are not particularly dangerous to humans.

Few details are available concerning tarantula venoms. Crude venom studies involving mice and 55 different tarantula species showed that certain specie's venoms can induce massive neurotoxicity leading to death in 3–5 min, while others had similar, but reduced effects, resulting in death after greater than 2 h. The most toxic venoms come from the arboreal genera *Heteroscodra, Poecilotheria*, and *Stomatopelma*, with their venoms causing death in seconds (Escouba and Rash, 2004). The venom of the Chinese bird spider, *Ornithoctonus huwena*, known as *Dilaohu*—the *earth tiger*—in China contains a series of toxins known as *huwentoxins* [256] These are small (3000–5000 Da) neurotoxin peptides with voltage-gated Ca^{2+} and Na^{+} ion-channel activities, which are a significant portion of crude venom that is capable of causing lethality in mice and birds in less than 2 min [257].

Bites to humans usually cause mild to severe pain, burning sensation, edema, erythema, itching, and joint stiffness. Severe envenomations, as seen with arboreal Asian (*Haplopelma* spp.), Sri Lankan and Indian (*Poecilotheria* spp.), and South African (*Stromatopelma* and *Pterinochilus* spp.) tarantulas, have been associated with more severe clinical problems such as intense pain and muscle cramps of weeks duration, temporary paralysis, and coma [258]. South American tarantulas (*Theraphosa* spp.) possess body hairs (setae) capable of eliciting dermal urticaria [259] and ocular injury [260].

Tarantula bite treatment has not been specifically defined, but symptomatic treatment with analgesics, antihistamines, topical steroids, and supportive care as needed have been used with success, even in cases of severe envenomation such as those involving Sri Lankan species [261].

Chilopoda

Centipedes are arthropods familiar to most people, and it is usually an unpleasant familiarity. Perhaps it is the words of arthropod scientist, J.L. Cloudsley-Thompson, "centipedes seem to exert a weird fascination on the morbid appetites of the hysterical and insane," that best characterize human thoughts on centipedes [262]. They are globally distributed being native to the Americas, Africa, Australia, Asia, and Europe. Centipedes are nocturnally active, fast-moving, have segmented bodies with one pair of legs per segment, compound eyes with up to 200 optical units, range from 3 to 250 mm in length, and have an aggressive behavior [262]. They are carnivorous and use their powerful poison claws, which are actually modifications of their first pair of legs, to deliver venom from an internal sac into their prey causing rapid paralysis [263]. Prey items include other small arthropods, including other centipedes, and they have been known to feed on toads, snakes, and small vertebrates [264]. There are approximately 3000 known species, but those of the Scolopendromorpha and Scutigeromorpha orders are most important clinically [265].

Centipede venoms, like snake venoms, are complex protein mixtures and have been found to contain esterases, histamine, 5-hydroxytriptamine, lipids, polysaccharides, proteinases, and other various enzymes. An acidic, heal labile, 60 kDa protein cardiotoxin (*toxin S*) has been isolated from Scolopendra subspinipes and could induce hypertension in cats [266]. In contrast, extract from *Scolopendra moristans* venom caused cardiac asystole in the toad heart [267]. Studies of centipede venom on other arthropod nerve systems, using *Scolpendra* sp. venom fraction (SC1), revealed the fraction possessed muscarinic agonist properties that acted directly

on an insect muscarinic receptor subtype closely homologous to the M1-M3 (muscarin) receptors found in mammals [268].

Envenomation by Scolopendra centipedes frequently results in pain, and occasionally local necrosis, but generally does not cause serious complications [269]. However, rhabdomyolysis [270] and fatality have been reported [268]. A study of Australian centipede confirmed envenomations, involving *Cormocephalus*, *Etmostigmus*, and *Scolopendra* species, showed bites from all three genera could cause significant pain, erythema, swelling, and itching with *Ethmostigmus* and *Scolopendra* symptoms more severe [265].

Ingestion of *Scutigera morpha* by an infant (confirmed by an intact centipede found in the feces) resulted in systemic effects, evidenced by paleness, muscle hypotonia, and vomiting, followed by spontaneous recovery over the 48 h period following the ingestion [271]. Treatment with ice packs, analgesics, and hot water submersion have been effective interventions [265].

Insecta

Insects are the most ubiquitous of all terrestrial animal forms, and inhabit every ecological niche on the planet earth. They are six-legged, or many more in the case of larval forms such as caterpillars, allowing their ambulation on a multitude of substrates. Many possess wings, they have three hinged body parts (head, thorax, abdomen), and they all are covered with a chitinous exoskeleton. Additionally, they possess a variety of biting mouth parts and venom delivery devices. Although a plethora of insect species are vectors for a large number of infectious diseases, few have truly toxic venoms of concern. Those that do possess toxins in their venoms are incapable, as a single entity, of causing fatality to humans. However, single assaults can cause toxic reactions and elicit immune responses in humans, and multiple concurrent insults by some species (i.e., swarms of Africanized bees) can result in human fatality. In fact the venoms of most insects in the order Hymenoptera, including fire ants, honey bees, and vespids, are highly immunogenic due to venom proteins [272]. Venom doses range from <1 μg for fire ants, 10 μg for wasps, and up to 50 μg for bees [272]. Thus, the allergic reactions resulting from insect stings are a more clinically prevalent problem than complications associated with direct venom toxicity.

Ants

Only a few species of ants are of any significance with reference to venom toxinology, and are capable of inflicting significant bites and stings. While formic acid is a common component of substances in venom sprayed for either defensive or prey-acquiring purposes by many genera (*Camponotus, Formica, Lasius, Poyergus*), it is a chemical toxin of little consequence to humans other than causing mild dermal or ocular irritation. In contrast, South American fire ants (*Solenopsis* spp.) [273], Australian/Tasmanian bulldog and *jack jumper* ants (*Myrmecia* spp.), Costa Rican and South American tropical ants (*Pseudomymex triplarins* and *Pachycondyla goeldii*) can potentially inflict severe stings [274]. Unfortunately, these ants have been relocated to geographic regions far removed from their native lands, and fire ants have been present in the United States since the early 1920s [275].

Fire ant (*Solenopsis* spp.) venom contains the alkaloids, 2-methyl-6-alkyl and alkenyl disubstituted piperidines, commonly referred to as *solenopsins*. These toxins possess cytotoxic activity that alters the functional integrity of mast cell membranes, causing intracellular degranulation and the subsequent release of histamine. The allergenic component of venom is due to low molecular weight proteins, and four are present in fire ant venom [276]. Fire ants have an aggressive stinging behavior with envenomation resulting in a wide range of effects including dermal necrosis, edema, sterile pustules (occurring several weeks post envenomation), urticaria, and in extremely rare circumstances anaphylactic shock and fatality [277]. The quantity of venom delivered by fire ants ranges from 10 to 100 ng [278], and severity of envenomation appears to be dose-dependent [279]. Management of fire ant stings is not consistent, but antihistamines, topical corticosteroids, cold compresses, and antibacterials have been used [275].

Jack jumper ant (*Myrmecia* spp.) venom has enzymatic activities due to its phospholipase A_2, phospholipase B, hyaluronidase, acid phosphatase, and alkaline phosphatase fractions [280]. Venom also contains two basic peptides: Pilosulin 1 elicits cytotoxic effects [281], and pilosulin 2 has antihypertensive properties [274]. Envenomation by the jack jumper ant has shown its venom is a strong insect allergen, with it being responsible for greater than 90% ant venom anaphylaxis cases in Australia, and deaths in Tasmania [282]. The allergic problems associated with *re-stings* in victims became so serious that a major effort was made, and accomplished, for effective immunotherapy in preventing sting analphylaxis [282].

One mimicking insect often confused to be an ant is the solitary female wasp in the genus *Dasymutilla* (Table 19.5). These are truly wingless female wasps that have the morphological, look-alike, appearance of a large ant, and are commonly referred to as velvet ants (*Dasymutilla magnifica*). As their name implies, their bodies are covered in dense brightly colored hair, giving them a velvety appearance. Venom of *Dasymutilla* spp. contains phospholipase and hyaluronidase, but is low in other enzymatic activities [283]. Their sting is one of the most painful of all insects, and for one species (*Dasymutilla occidentalis*), the pain is so severe they have been dubbed *cow killers* [284], although in reality, the sting is not strong enough to kill a cow!

Caterpillars

Caterpillars are the insect larval form of butterflies and moths (Lepidoptera). Familiar, captivating, and wondrous to children all over the non-Arctic regions of the earth, caterpillars are not all innocent, fuzzy or colorful, crawly creatures. In many countries, they are often considered a major economic agricultural pest as a result of their defoliating appetites. Many species probably acquire their toxins from the foliage

they feed on such as *Lagoa* spp. that feed on oak leaves, resulting in high tannin content in their spines [285]. Though most species are harmless, there are a few that should be left untouched. The caterpillars of concern have hairs (setae) on their slug-like or tubular-shaped bodies, sometimes making them appear like a brush-like, furry/fuzzy, or glass-haired cucumber. There are multiple caterpillar species known to have hairs that readily break off when handled, and these are generally considered stinging hair caterpillars because of the needle-prick pain, and burning and itching that occurs from hair contact. In some species (*Doratifera* spp.) stinging groups of hairs are paired, and mounted on body protuberances attached to venom glands [89], and American and European species may have hairs containing complex peptides and proteins [286]. Examples of caterpillar species causing these effects are Saddleback Caterpillar (*Sibine stimulea*), Io Moth (*Auomeris io*), Hagmoth (*Phobetron pithecium*), Buck Moth (*Hemileuca maia*), Hickory Tussock Moth (*Lophocampa caryae*), Silver-spotted Tiger Moth (*Halisidota argentata*), and the Stinging Rose Caterpillar (*Parasa indetermina*) [287–289].

Australian caterpillars, especially, *Euproctis edwarsi* (Mistletoe Browntail moth), have been confirmed to be of medical significance as their venom contains kallikrein-like serine proteases [290], while those causing lesser effects are represented by the genera *Doratifera* (Cup Moths), *Theosa* the genus of the *billygoat plum stinging caterpillar*, *Theretra* (Hawk Moths), and many more [89]. Symptoms vary in severity from intense pain (rarely) to commonly mild/moderate pain, slight redness to wheals, and may be up to 48 h in duration [287].

The puss caterpillars, *Lagoa cripata* and *Megalopyge opercularis* of the southern United States and Brazil, respectively, have caused local pain, regional lymphadonopathy, headache, and inflammatory dermatitis. Despite analytical attempts to elucidate venom components from these species, they appear to be lacking in enzymes, or toxins, with only several amino acids found. However, nongastrointestinal body extracts reveal that high tannin content is present, and this is derived from their diet of oak leaves [285].

Lanomia oblique (Saturniidae Moth) is a caterpillar primarily from southern Brazil, and is a species of caterpillar second to none when it comes to envenomation. In addition to the local symptoms that result from hair contact of other caterpillars, *Lanomia* caterpillar envenomation produces profound burning pain, and can induce major hematological complications. Their sets of hard-bristled hairs contain venom with prothrombin activating (LO prothrombin activator protease), factor X activator, a phospholipase A(2)-like activity (*Lonomiatoxin*) [291], and both nociceptive and edematogenic properties [292].

Severe envenomation from bristle contact with Lanomia results in a major hemorrhagic syndrome [293] with decline in prothrombin and accelerated thrombin generation, manifested clinically by ecchymosis, hematuria, with bleeding of the nose, skin, gastrointestinal tract, lungs, vagina, and pulmonary and intracerebral hemorrhage leading to death [294,295]. This syndrome usually occurs from 6 to 72 h post envenomation.

Caterpillar sting treatment from most species is symptomatic and involves removal of hairs using tape, washing of the skin, ice packs, and antihistamines, leading to resolution of symptoms within hours [287]. *Lanomia* envenomation treatment is supportive, and specific antivenom has been developed [296], but antivenom experience in human victims of *Lanomia* envenomation is lacking.

Bees, Bumblebees, Hornets, and Wasps

These are part of nature's air force, and human beings from all parts of the world are familiar with the consequences of their attack. Like the ants, the primary complication from envenomation by these stinging *Hymenopterans* stems not only from limited venom toxicity, but also from venom toxins being hyperallergenic to humans [297]. However, massive numbers of stings to a victim results in a venom body burden capable of resulting in severe venom toxicity, and even death [298]. Thus, victims may die whether or not they are allergic to the venom.

Bumblebees, honeybees, and vespid wasps can all deliver a painful sting. However, bumblebees tend to be less aggressive, while Africanized (killer bees) honeybees (*Apis mellifera scutellata*) and hornets will aggressively swarm victims, and wasps are aggressive solitary attackers. The bees use their venomized stingers for self-defense or the defense of the colony while wasps will additionally use their venom to acquire prey [299]. There are approximately 200 species of bumblebees between Canada, Europe, and the United States, [300], multiple honey bee (*Apis*) and hornet/wasp (*Vespa*) subspecies. The venom delivery system for all these stinging hymenoptera, is actually an anatomical adaptation of the ovipositor in females, with a lengthy venom gland connected to a channel that runs through the barbed stinger, and as such, it is only females that can sting. Following a sting, a wasp can usually remove its stinger and repeatedly sting its victim. In contrast, the honey bee's stinger has a more barbed anatomy and remains in the victim, along with the muscular venom gland reservoir attached, which continues to pulse venom into the victim until it is depleted or removed [30].

The venom of bumblebees and honeybees is cross-reactive, demonstrating similarities in their composition [301]. The injected dose of venom for a single bee is about 50 μg [272]. Bee venom (*Apis* and *Bombus*) components have been isolated and identified as a melittin peptide and it tetramers (a major constituent of bee venom), 39 kDa acid phosphatase, 38 kDa hyaluronidase, 28 kDa protease, 14 kDa phospholipase, apamin, and mast-cell degranulating peptide [300,302]. The anaphylactic effects of bee venoms associated with IgE antibodies are due to phospholipase, hyaluronidase, and to a lesser degree melittin [302].

Hornets, wasps, and yellowjacket venom immunochemical studies have shown that hyaluronidase, phospholipases, and a substance of unknown function called antigen 5, are their major venom components [303]. Studies of Taiwan yellow-legged hornet (*Vespa verutina*) venom yielded the

identification of three toxins designated as, *verutoxins* (V1), (V2a), and (V2b), and all three possess a phospholipase A_1 with hemolytic activity demonstrated to directly kill mice at doses as low as 0.87 mg/kg [304]. Given that an average vespid delivers approximately 10 μg per sting [272], this hornet species harbors potently toxic venom.

Envenomation to humans from either bees or vespids can trigger rapid acute immune reactions leading to vomiting, diarrhea, hypotension, coma, hemaglobinuria and myoglobinuria progressing to renal failure, and rhabdomyolysis [305,306]. Moderate venom reactions are more common and involve pain, dermal rashes, urticaria, and edema [307]. Toxicity, independent of immune system responses, or in victims without allergic reactions, is due primarily to phospholipase activity.

Treatment of bee and vespid envenomated victims is largely symptomatic and supportive, and requires a timely response in the case of anaphylaxis, with epinephrine, antihistamine, steroids, and life support. Effective antivenoms are not available, but this therapeutic strategy has been investigated [297].

A final comment on Africanized bees, as their aggressive geographical spread from South America to North America, and aggressive behavior has led to a large number of fatalities in the process of their northward migration [308]. The severity of envenomation by *Apis mellifera scutellata* relates to the venom dose injected with each individual sting, and the potentially large number of stings a victim sustains. A killer bee, in contrast to other bees, delivers approximately 150 μg of venom per sting, which is threefold greater than that delivered by other bees. This characteristic, amplified by the large number of stings as a result of a strong swarming behavior [309], makes encounters extremely dangerous, as the cumulative dose of venom, rather than anaphylaxis, can lead to irreversible organ system damage, and death [310]. Thus, it is important to terminate victim exposure from bees rapidly and safely as possible, and seek medical attention.

Sawfly

Sawflies (*Arge, Lophyrotoma, and Perreyia* spp.) cause significant problems for the livestock industry and the veterinary profession. Sawfly larvae have been documented as having been responsible for large numbers of cattle and sheep deaths in Australia (*Lophyrotoma interrupta*), cattle, dogs, and sheep in Denmark (*Arge pullata*), and cattle, pigs, and sheep in Uruguay (*Perreyia flavipes*) [311]. Sawflies will infest certain species of trees and grasses, for the larval phase of their metamorphosis, and with a changing of seasons many are found on the ground where livestock graze. As such they are consumed by livestock and a brief period of time lapses between ingestion, symptoms, and death [312]. Necrotizing hepatotoxicity is the primary toxic insult. Symptomatic cattle have been described to be hyper-excitable and aggressive to humans [312]. The toxins, octapeptide *lophyrotomin* and heptadeapeptide *pergidin*, are unique because the larvae synthesize de novo these toxins, rather than bioaccumulating them from their food sources, and this appears to possibly be a species survival strategy [313]. In feeding trial experiments it was found that even dead larvae were toxic to cattle and sheep, and that oven-drying of larvae at 110°C for 24 h did not abolish toxicological activity. Of interest, is the fact that *pergidin* appears to be the first phosphorylated peptide isolated from an animal species [311]. The sawfly larvae, having been found on three different continents, suggest that there is not a common toxin source from animal food roughage, and that each larval species can synthesize the toxic peptides. Further conjecture rests with the possible migration of these peptides to nonvisceral tissues in produce animals, as they are water soluble and nondegradable, and this would likely result in their having long resident-times in animal tissues.

Terrestrial Vertebrate Animal Toxins and Venoms

The terrestrial venomous or poisonous vertebrates have caused the human mind to conjure up the most dark-sided images of nature. Snakes, lizards, frogs, salamanders, toads, newts, all have been used in the *witches' brew*, and associated with the evil that exists in the natural world. The unusual appearances of these fascinating creatures, and the fact that many do possess potent poisons, has resulted in their biological and chemical traits being associated with great mystical powers historically. Importantly, in today's world, the presence of some of these creatures is a growing medical concern to humans in many countries where human and natural communities are converging. Equally intriguing are the terrestrial creatures, such as certain species of birds and mammals, which are not usually thought of as likely sources of toxins and venoms. Modern analytical and technological capabilities allow more comprehensive analysis of animal toxins, and a better understanding of animal systematics. As a result of the interface between these disciplines, our toxinological knowledge, and its value in biological science, conservation, and medicine, continues to grow.

Chordata

Reptilia

The ancient Greek historian Diodorus Siculus, thought that the sands of the Theban desert in Egypt could *spontaneously* generate serpents [314]. For millennia since ancient times, terrestrial poisonous snakes, such as cobras and rattlesnakes, have consistently maintained a prominent place in the hierarchy of many cultures, religions, and the realm of fascination in the human mind, even by those who fear them most. Less well known, from a global perspective, are the venomous lizards, the Beaded Lizard and Gila Monster, as they are only distributed in a small confined arid geographic region of the southwestern United States, Mexico, and Guatemala [315]. While arachnids account for a large number of envenomations to humans, and a considerable number of marine animals inflict envenomations as well, the majority of consequences from these envenomations are associated most frequently with morbidity. As such, snake bite has been reported to be the most significant *toxin-related* disease,

envenomation, on earth because of its quantitative contribution to morbidity and mortality, particularly in the Americas, Africa, and Asia [316].

Historical studies of mortality from snake venom poisoning are limited, and the only global comprehensive review revealed an average of 50,000 deaths annually, based on hospital records from 1945 to 1949 [317]. The current number of venomous snake bite envenomations has been estimated to be 2.5 million, and mortality to be 125,000 deaths [318]. Thus, venomous snake bite is still a medical concern of appreciable significance in the world today.

Although the problem with snake bites is important, it should not overshadow the role venomous snakes have in the balance of the natural world. Venomous snakes, using their venom for its primary biological function, readily consume millions of pests that invade human food supplies in many countries of the world. They not only reduce the loss of food supplies keeping pest numbers in check, by keeping populations from exploding, but may also reduce the spread of disease carried by their prey. The impact of their elimination from world ecosystems would be noticed. Furthermore, venomous snakes synthesize an array of toxins that possess a spectrum of pharmacological properties and act on the physiology of numerous animal species. Their venoms provide the natural templates from which biochemical and biomedical research can generate a plethora of useful therapeutic agents.

Snakes

There are approximately 2900 recognized species of snakes, comprising approximately 420 genera [319]. Venomous snakes are found in arid, subtropical, tropical, and temperate regions, ranging up to polar ecosystems on six of the planet earth's continents, excluding Antarctica [316]. They inhabit arboreal, aquatic, desert, mountain, subterranean, and diverse terrestrial microhabitats. Snakes are the most common predator of other vertebrates, and most venomous snake species feed on other reptiles, amphibians, birds, and mammals, with their venoms being highly selective for a given species' preferred prey [319].

Classification and division of venomous snakes into various groups has been a constantly evolving taxonomical process. Generally, there are four main groups of venomous snakes: Atractaspididae, the advanced snakes of the super family Colubridea, the Viperidae, which is a family divided into vipers and pit vipers, based on the presence of an infrared heat-sensing pit between the eye and nasal opening in some vipers, and the Elapidae (Table 19.6). Sea snakes are unique members of the family, Elapidae, as they spend the majority of their lives in oceanic waters, and therefore, are discussed with the marine vertebrates. However, they are true snakes, and a subfamily of Elapidae, the Hydrophiinae [315].

Venomous snakes have evolved several anatomical features with a variety of functions important to their survival. The structures of importance in venomous snakes are those relating to their venom, and its production and delivery. Biomanufacturing of venom is primarily accomplished via ophidian oral glands of two types, although a variety of subtle modifications exist. A salivary system modification, frequently referred to as the Duvernoy's gland, is the toxin-secreting (venom) structure of colubrid snakes, and is a seromucus cell, secretory structure with a weakly defined lumen-like duct, that in some species produces toxic proteinaceous substances [320]. A quite similar structure is found in elapid snakes [319]. The exception to this similarity is the elongated, compressor muscle enclosed venom gland of the Asian coral snakes [314], and some Burrowing and Night adders, which have a venom gland approaching half their body length [319]. Venom glands in viperid snakes are slightly more sophisticated as the musculature, derived from the jaws, encompasses a well-defined duct that extends to the base of the accessory gland that interfaces with the fang sheath, and terminates at the proximal fang-tissue juncture [27].

Fangs of venomous snakes generally are one of four basic dentition types: (1) Aglyphous (conical fangs with no groove, typical of some colubrids); (2) Opisthoglyphous (enlarged teeth with a groove in the back, commonly in colubrid rear-fanged snakes and atractaspids); (3) Proteroglyphous (large anterior, fixed-position fangs with deep frontal grooves, such as cobras); and (4) Solenoglyphous (large tubular, canaliculated, fangs with a hinge-like action due to their attachment to the highly flexible maxillary bone, such as observed in Gaboon vipers) [319]. Both the production of toxic saliva, or venom, and the delivery systems for these different dentitions are key to the acquisition of prey food, such as centipedes, fish, frogs, birds, mammals, and other snakes, and as a defense for self-preservation from predators.

Snake venoms, make up nature's true biochemical cocktails, and are composed of a greater number of toxins than those of other venomous or toxin-producing species of animals, making them the most complex of all poisons [321]. The venoms of the four major groups of terrestrial snakes (Table 19.6) possess activities that affect several physiologic systems in animals. Venoms primarily affect the central and/or peripheral nervous systems, blood coagulation system, cardiovascular system, musculoskeletal system, and renal system [316]. Some venom components, such as the purine and pyrimidine nucleosides, adenosine, guanosine, and hypoxanthine, seem to be present in nearly all elapid and viperid venoms, and act as multifunctional toxins believed to be important in prey acquisition [322]. The net toxic effect, or effects, may be a result of direct toxin toxicity on a target within a single system, but usually results from a combination of effects caused by different toxins acting directly, or secondarily.

Atractaspididae

Mole vipers, burrowing adders, or sometimes referred to as stiletto snakes, comprise the fourteen genera, and 65 species of the fossorial- and subterranean-inhabiting family, Atractaspidae, and are not actually vipers [319]. They are small, dark, glossy scaled, with virtually no distinction between their head and neck, and have small bead-like eyes. Native to Africa, the Arabian Peninsula, and Israel, they have the appearance of a nonthreatening little snake.

TABLE 19.6
Terrestrial Vertebrate Animals: Venomous Snakes

Phylum	Class	Family		Common Names	Genera
Chordata	Reptilia	Atractaspididae	African	Burrowing, mole, stiletto vipers	*Atractaspis, Macrelaps*
		Colubridae	Americas	Bimini racer, parrot, vinesnakes	*Alsophis, Leptophis, Oxybelis*
				Hognosed, wandering garter snake	*Heterodon, Thamnophis, Philodryas*
			African	Boom slang, twig snake	*Disphlodidus, Thelotornis*
			Asian	Keelback, tree snakes	*Rabdophis, Ahaetulla*
		Elapidae	African	Cobras, nambas	*Naja, Hemachatus, Dendroaspis*
			Americas	Coral snakes	*Micrurus, Micuroides*
			Asian	Cobras, coral snakes,	*Naja, Calliophis, Maticora*
				king cobra, kraits	*Ophiophagus, Bungarus*
			Australian	Black/brown snakes	*Pseudechis, Pseudonaja*
				copperheads, death adder	*Austrelaps, Acanthophis*
				taipans, tiger snakes	*Oxyuranus, Notechis*
		Viperidae		*Crotalinae (pit vipers)*	
			Americas	Bushmaster	*Lachesis*
				Cantils, copperheads, cottonmouth	*Agkistrodon*
				Lanceheads, Fer-de-Lance, urutu	*Porthidium, Bothrops*
				jumping vipers, montane vipers	*Atropoides*, *Cerrophidion*
				Eyelash viper, palm pit vipers	*Bothriechis, Bothriopsos*
				rattlesnakes	*Crotalus, Sustrurus*
			Asian	Bamboo, green tree vipers, habu	*Trimerersurus, Protobothrops*
				Hundred pace snake	*Deinagkistrodon*
				Malayan pit viper, mamushi	*Calloselasma, Agkistrodon*
				Temple pit viper	*Tropidolaemus*
				Viperinae (vipers)	
			African	Bush, saw scale, montane vipers	*Atheris, Echis, Montatheris*
				Desert, horned vipers, night adders	*Macrovipera, Cerastes*
				Puff adder, gaboon, rhinoceros	*Bitis*
			Asian	Fea's, leaf-nosed, Russell's vipers	*Azemiops, Eristicophis, Daboia*
				Desert, false horned vipers	*Macrovipera*, *Pseudocerastes*
			European	Asps, adders, mountain vipers	*Vipera*

However, local names in Africa like, *father of blackness*, and *shroud bearer* reflect its potential danger to humans [319]. When scared, the snake will form a buried-head-coil and release like an unwinding spring to deliver a slashing bite. Strangely, each of the four different dentition patterns is represented by at least one genus in the family. Most species in the family are rear-fanged, but a notable exception is the front-fanged assembly of those in the genus *Atractaspis*. Fangs are usually grooved, connecting to an elongated venom gland, and biting is accomplished with little opening of the mouth, as a single fang will extend laterally outward from the side of the mouth to deliver venom by making a slashing-stab motion. *Atractaspis* venom possesses a high percentage of low molecular weight toxins, but is devoid of cholinesterase and hydrolase activity [323]. The most significant toxins isolated from the venom of this family are the *sarafotoxins*, which are a cysteine-rich group of acidic polypeptides, capable of inducing cardiotoxic effects rapidly by the vasoconstriction of coronary vasculature [324]. As little as 2.5 μg of sarafotoxin, from *A. engaddensis* venom, in a mouse caused 100% fatality [323].

Envenomation from *Atractaspis* can be a serious event, and deaths, although not common, have occurred from bites by *A. irregularis* and *A. microlepidota* [325]. Symptoms of envenomation usually involve pain and swelling, with vomiting, abdominal pain, dyspnea, profuse salivation, and necrosis [326]. There is no specific antivenom, or antivenom that is known to provide paraspecific protection from the effects of envenomation, and treatment is supportive [323].

Colubridae

The colubrid snakes are highly diverse, comprising 290 genera representing 1700 snake species, and those species in the family that produce some form of venom or toxic secretions account for greater than 50% of all colubrid species [319]. The bites of a large number of colubrid snake species can cause varying degrees of toxicity, yet documentation of colubrid envenomation in humans has only been reported for approximately 50 species representing 30 genera [327], and depending on how toxicity is defined, there may be many more [315,328]. However, both colubrids that lack a specific fang structure (Aglyphous), and those with rear-fanged

structures (Opisthoglyphs), have been documented to cause severe envenomation, and even human fatalities have resulted from the bites of the rear-fanged South African *Dispholidus typus* (boomslang) [329], and *Thelotornis capensis* (twig snake) [330], the East Asian *Rhabdophis tigrinis* (yamakagashi), which lacks a venom-injecting mechanism [331], and the Argentine racer (*Phylodryas* sp.) [332].

Given the obvious powerful effects of these colubrid venoms, it is unfortunate that very little is known about their toxins or composition. Early studies of salivary secretions, or venoms, from colubrids, with or without enlarged postmaxillary teeth, revealed the presence of 7–10 proteins [333], while more recent electrophoretic venom analyses revealed 10–20 constituents, of 4–200 kDa molecular weight, with species-specific profiles, suggesting that the oral secretions of some colubrids are equally as complex as many front-fanged species of venomous snakes [334]. Enzymatic properties had not been associated with colubrid venom properties until recent investigations characterizing the venoms and oral secretions of a several colubrids, such as the keeled water snake (*Amphiesma stolata*), brown tree snake (*Boiga irregularis*), and false water cobra (*Hydrodynastes gigas*), showed the presence of proteolytic activity. Phosphodiesterase activity, although slight, exists in the western hognose snake (*Heterodon nasicus nasicus*) and wandering garter snake (*Thamnophis elegans vagrans*), acetylcholinesterase is present in the venom of the brown tree snake (*Boiga irregularis*), and moderate to high levels of phospholipase (PLA_2) activity are present in mangrove snakes (*Boiga dendrophila*) and Lyresnakes (*Trimorphodon biscutatus lambda*), respectively [334].

Neuromuscular activity effects of colubrid venoms on avian skeletal nerve muscle are exhibited via the inhibition of postsynaptic actions by venoms from the dog-toothed cat snake (*Boiga cynodon*), mangrove snake, and other *Boiga* spp. and the Egyptian cat snake (*Telescopus dhara*). Inhibition by *Boiga cynodon* venom was a reversible process, while contrastingly, the venom of *Trimorophodon biscutatus* demonstrated irreversible presynaptic neurotoxic activity [335]. Studies of Rufous beaked snake (*Rhamphiophis oxyrhyncus*), a relatively small South African colubrid of the subfamily psammophiine, venom revealed a high protein fraction with PLA_2 activity and in animal nerve muscle preparations showed postsynaptic neurotoxicity. The venom of this species also could induce hypotension followed by cardiovascular collapse in rodents, which would be effective in subduing its prey such as the Naked mole rat [336]. Collectively, these findings concerning the pharmacological and toxicological activities of colubrid venoms show their diversity, potentially high bioactive potency, and only begin to reveal the potential for their use in biomedical research. The composition of colubrid venoms not only confirms their similarity in make-up to more venomous noncolubrid species, which undoubtedly relates to their effectiveness in acquisition of a preferred prey, but also raises concern as to their potential for severe toxicity to humans in clinical medicine.

The bites of colubrid snakes demonstrate the extremes of toxic effects, with most bites resulting in signs and symptoms confined to the bitten area, and pronounced, life-threatening, coagulopathic effects in only a few [337]. Brief details of envenomation to humans by many species of colubrids are frequently all that is available, and it is impossible to know if the symptoms observed represent a severe bite or those associated with a small amount of venom ineffectively delivered [315]. Thus, many colubrid species may harbor quite toxic venoms, but in cases of envenomation, as with viper bites, the degree of envenomation is not always fully expressed, and the incidence of bites by some species so rare, that the true significance of colubrid venom toxicity in humans is unknown. However, it is believed that at least one species in nearly every colubrid family is capable of envenomation leading to severe medical complications in humans, including the potential for fatality (Bryan Grieg Fry, personal communication, 2005.)

Envenomations by the boomslang, twig snake, and red-necked keelback can be most severe, and are known to have caused severe coagualpathic complications resulting in a delayed onset of extensive and prolonged superficial hemorrhaging, that progressed to death [337]. Their bites do not induce local symptoms of envenomation, fang punctures do not bleed at the time of the bite, and their venoms are defibrinating, and activate prothrombin, making them procoagulant in effect, with anticoagulated blood resulting due to consumption [338–340]. Unfortunately, only a species-specific antivenom exists for treating boomslang envenomations, and despite similarities in blood coagulopathy effects observed with envenomation by other similar species such as *Thelotornis capensis*, there is no antivenom cross-reactivity [338]. As such, supportive care and replacement of blood components may be the only potentially effective therapies available [340].

Not as potentially deadly as the genera previously mentioned, but of interest because of its wide range across the North American continent, frequent exposure to humans, and general commonplaceness, is the genus *Thamnophis*, the aglyphic colubrid snakes commonly known as the garter snakes [341]. In addition to their possessing enlarged, grooved, post-maxillary teeth, the salivary secretions of the common garter snake (*Thamnophis sirtalis*) are elaborated from mandible glands, and serve as evidence of a venom delivery system [342]. Several cases of envenomation have resulted from bites by the garter snake species, *Thamnophis sirtalis* [343] and *Thamnophis elegans vagrans* [344]. The victims of these bites suffered local swelling, edema, hemorrhagic vesicles, and ecchymosis, to the extent that they required hospital admission [343,344]. Systemic symptoms did not occur, but the clinical presentation was likened to that observed with pit viper envenoming [344]. Thus, even colubrid species considered to be totally harmless are capable of causing some degree of toxinological insult.

Colubrid species will continue to be redefined toxinomically, and be of significant importance on the forefront of venom research, as the science of their venoms will play an important role in the understanding of venom evolution in the animal world, and in the laboratories of biomedical and pharmacotherapeutic research [332].

Elapidae

Elapid snakes rank high among animals in the toxin world and are predominantly found on four continents (Table 19.6). This family is represented by 63 genera that contain 272 species, and the cobra, coral snake, mamba, and taipan are names of familiarity and notoriety [319]. Ranging in size from inches to meters, they are present in an abundance of brilliant colors, and color patterns, such as the coral snakes of the *Micrurus* and *Micruroides* genera, and exhibit functional threatening anatomical adaptations, such as hooding in cobras (*Naja* spp.)—an image well known and respected by humans for thousands of years. The most noted of the elapids are the cobras, coral snakes, kraits, mambas, and the Australian elapids where the diversity of the family is greatest (Table 19.6) [345]. All elapids are venomous and possess proteroglyphous dentition [315].

The elapid venom delivery system consists of tubular or grooved front teeth that are usually located on the anterior of the maxilla, followed by conical solid teeth. In general, their fangs are shorter than vipers and the associated venom glands have a lesser storage capacity. Elapid venoms are considered to be primarily neurotoxic in effect as their venoms contain nonenzymatic proteins, some enzymatic proteins, and cytotoxic components [346]. The presynaptic neurotoxins are usually phopholipase A_2 (PLA_2) isoforms that either inhibit or induce transmitter release from the presynaptic myoneural junction [347] while the postsynaptic neurotoxins are either short-chain (60–62 amino acids) or long-chain (66–74 amino acids), with their tertiary structure maintained via four to five disulfide cross-linkages, and competitively block acetylcholine from binding to the receptor forming a nondepolarizing block [26]. Elapid PLA_2 toxins may induce pharmacological effects independent of their enzymatic activity, and independent of other venom components such as is the case with *notexin* from *Notechis* sp. (Tiger snake) venom [347], while other PLA_2s require complexing with other protein factors, forming subunits, to express their pharmacologic actions as in the case of *taipoxin* from *Oxyuranus* sp. (Taipan) venom [348]. The PLA_2 enzymes exist in a wide variety of forms, and each enzyme may be highly specific in its action, as is the case with beta-bungarotoxin from the venom of *Bungarus multicinctus* (many banded Krait) that acts via presynaptic effect on nicotinic acetylcholine transmission, but not on adrenergic transmission [349]. To add to the complexity of PLA_2 enzyme functions is the fact that any number of the PLA_2 isoforms may express identical pharmacological effects, but by different mechanisms. Thus, the generalizing of PLA_2 venom activities for all elapid species would be erroneous [31].

In contrast to neurotoxins, some elapid venoms induce hemotoxic effects by possessing procoagulant properties that enhance the conversion of prothrombin to thrombin. Australian elapid venoms possess toxins with this pharmacologic action. For example, *notecarin* from the Tiger snake (*Notechis scutatus scutatus*), *oscutarin* from the Taipan (*Oxyuranus scutellatus*), and *pseutarin* from the Brown snake (*Pseudonaja textilis*) are all potent procoagulant toxins [340]. These varied venom pharmacological properties make snakes of the family Elapidae some of the most dangerous snakes in the world. The more prominent elapid genera will be briefly discussed.

Cobras, genus *Naja* (including the genera *Boulengerina, Hemachatus*, and *Pseudohaje*), are elapids that can attain a relatively large size (3 m), making their ability to inject large quantities of venom a concern to humans. Their highly toxic venom is frequently used to paralyze other venomous snakes they seek for food [319]. In addition to the traditional envenoming with fangs by biting, some species of cobras (i.e., *N. mossambica, N. nigricollis*, and *N sputatrix*) can spit or spray venom from an orifice in the front of their luminal fangs, for a distance of 2–3 m. All cobras can dorsoventrally compress their necks to form a hood. The king cobra (*Ophiophagus hannah*) is not a true cobra but shares similar anatomy and venom properties to true colors, but a cytotoxic, L-amino acid oxidase (*hannatoxin*) has been isolated from their venom [350], and the king cobra is perhaps the world's largest venomous snake, attaining a length up to 5.7 m [351].

Cobra venoms contain cytotoxins and numerous PLA_2 isoforms. The cytotoxins are basic proteins that can account for up to 40% of the total venom protein, and are membrane-active toxins that alter membrane permeability [346]. The cobra PLA_2 toxins are of the group I type based on their structural similarity to pancreatic secreted PLA_2 and exhibit myotoxic effects [352]. Many of these toxins have been referred to as cardiotoxins and are the most abundant of cobra venom constituents. They are basic proteins that are quite similar in conformation to the neurotoxic components of cobra venom but produce primarily cardiotoxic effects [353].

The combined effects of cobra venom toxins following envenomation yield a painful bite with swelling, and neurological symptoms of respiratory distress and paralysis. Despite the common thought that cobra venoms are not tissue damaging, their cell membrane toxins frequently cause necrosis of the skin and subcutaneous tissues. In particular, the venom of African spitting cobras, *N. mossambica* and *N. nigricollis*, can cause significant tissue damage in addition to neurotoxicity [345]. However, bites by cobra species (*Naja naja philippinensis*) of the Philippines cause minimal tissue damage, and exhibit severe and rapidly developing neurotoxicity [354]. Spitting cobras also defensively eject their venom into the eyes of their predators, including humans, causing profound conjunctivitis, pain, photophobia, blepharitis, and corneal opacification. These oculotoxic effects are believed to be related to the free/unbound cardiotoxins in the venom [355]. Death following cobra envenomation can occur rapidly as a result of respiratory paralysis, which can develop in as little as 15 min. However, neurotoxic symptoms may also be delayed for several hours, causing a delay in seeking medical attention.

Antivenoms are available for treating envenomations by African and Asian species, and adequate dosing of the appropriate antivenom usually neutralizes the medically dangerous symptoms that threaten life [356]. Additionally, the anticholinesterase drugs, edrophonium and neostigmine have been

successfully used in treating Philippine cobra (*Naja naja philippinensis*) [354] and monocellate cobra (*Naja kaouthia)* [357] envenomations, respectively. The treatment of ocular exposure from the venom of spitting cobras has involved irrigation, and the topical application of heparin-tetracycline solution [358].

Coral snakes, genera *Micrurus* and *Micuroides*, are native to the southern United States, Mexico, Central and South America, reaching their greatest diversity near the equatorial belt, and are relatively abundant compared with their Asian relatives (*Calliophis* spp. and *Maticora* spp.). The genus Micrurus is represented with nearly 70 species, with wide range of red, black, white, or yellow, ringed color patterns, and many mimic nonvenomous species [315]. These elapids are considerably smaller than cobras (<50 cm–1.5 m), and their fangs are correspondingly smaller.

Coral snake venoms have pharmacological activities that are capable of inducing cardiotoxicity, hemorrhagic toxicity, myotoxicity, and neurotoxicity, with the majority of *Micrurus* species possessing PLA_2 enzymatic activity capable of exhibiting anticholinesterase and anticoagulant actions. [359]. Neurophysiological effects can result from irreversible neuromuscular blockade, reducing evoked acetylcholine (ACh) release, but increasing spontaneous ACh release [360].

Coral snake envenomation to humans gives rise to a bite with minimal pain, and symptoms of nausea and vomiting, dizziness, generalized weakness, drowsiness, euphoria, followed by muscle fasciculations, diaphoresis, bulbar paralysis, diplopia, and slurred speech. Complete paralysis with the loss of respiratory function can last for several days. The neurological symptoms may not be immediately evident and are often delayed in their presentation for or up to 12 h [361].

Treatment of corals snake envenomation is most effectively accomplished with vigilance of respiratory function, and the use of antivenoms. In the absence of appropriate antivenom, it is possible to maintain life by providing artificial respiration for sustained periods until spontaneous breathing returns [362]. Antivenoms for coral snake species in Central and South America are available and are effective treatment, but many victims in these countries suffer symptoms without relief as they do not have timely access to a medical facility [356].

Mambas, genus *Dendroaspis*, are large (up to 4 m), agile African snakes with extremely toxic venoms. They come in grayish and green color morphologies, the terrestrial-inhabiting black mamba (*Dendroaspis polylepis*) and the arboreal-inhabiting green mamba (*Dendroaspis angusticeps*), with three subspecies of green mamba. The black mamba is named not for its body color, but for the charcoal colored interior of its mouth [363]. They are proteroglyphic with enlarged maxillary front fangs, that are moderately moveable, with no teeth posterior to the fangs, and these are associated with a well-developed venom gland.

Mamba venoms contain *dendrotoxins*, toxins of 57–60 amino acid residues cross-linked with disulfide bridges. These are neurotoxic in effect and stimulate acetycholine release from neuromuscular junctions. They have minimal antiprotease activity, and selectively block certain subtypes of voltage-dependent K^+ channels in neurons, with green (alpha-dendrotoxin) and black (Toxin K) mamba venoms differing in the subtype of K^+ channels pharmacologically affected [364]. Thus, via their blocking action on K^+ channels, they facilitate transmitter release at peripheral synapses, thereby inducing nerve action potentials repetitively. In the central nervous system, dendrotoxin induces seizures when injected into animals [365]. The mamba toxin, *fasciculin*, is an anticholinesterase toxin that is a selective potent inhibitor of acetylcholinesterase. Not surprisingly, mamba venoms also contain cardiotoxins [366] and muscarinic toxins of several types that selectively act on different muscarinic acetycholine receptor subtypes [367]. Their venom is also high in hyaluronidase activity that enhances venom spread. These different toxins, with their respective pharmacologic actions, work synergistically and are likely the explanation for the pronounced cardiovascular problems in victims of mamba envenomation.

Mambas can store from 4 to 8 mL per venom gland/fang, and they will bite repeatedly injecting a significant quantity of venom. There may be only a tingling sensation at the site of the bite, and symptoms can appear rapidly (10 min) or be delayed. Symptoms are progressive, and commonly consist of unconsciousness, diaphoresis, flaccid paralysis, respiratory paralysis, sustained hypotension, and direct cardiotoxicity leading to death [368].

Treatment of mamba envenomation victims with polyvalent antivenom (South African Vaccine Producers, (Pty) Ltd., Sandspringham, South Africa) is effective, and the treatment of choice. Symptomatic and supportive treatment is important and should complement antivenom therapy, but not be in lieu of antivenom use [368].

Taipans are the quintessential venomous snakes of the elapid family, and are native to Australia and Papua New Guinea. These are sizable snakes (2–3.5 m), and similar to mambas in their alert, nervous behavior, being very aware of their environment, and any intruder. There are two forms, the coastal taipan (*Oxyuarnus scutellatus*), and the inland taipan (*Oxyuranus microlepidotus*), sometimes referred to as the Fierce snake, which is believed to be the most venomous terrestrial snake in the world. The inland taipan's average bite delivers enough of its potent venom to kill 62 humans (70 kg body wt.), and that of the coastal taipan 27 humans [363].

Taipan venom contains neurotoxins, a Ca^{2+} complex-specific channel blocker, and a prothrombin activator [369]. The neurotoxin, *taipoxin*, is a trimeric PLA_2 that inhibits the release of acetylcholine form the presynaptic cholinergic nerve terminal, and is perhaps the most clinically important toxin. Postsynaptic PLA neurotoxins have also been found to be present in taipan venom [370].

Envenomation by taipans to humans results in progressive neurotoxicity exhibited by ptosis, opthalmoplegia, trismus, and paralysis of bulbar, peripheral, and respiratory muscles [371]. A pronounced consumptive coagulopathy may also occur [371]. Antivenom is available and effective if given early after the bite, but is less efficacious in ameliorating

neurotoxic symptoms after they are present, and respiratory support may be required [372]. The single most important measure to assure survival of taipan envenomation is rapid presentation to a medical facility with life support equipment.

Viperidae

Vipers and pit vipers, like the elapids, are indigenous to four continents, and represent two subfamilies, comprising 30 genera that radiate to 230 species (Table 19.6). Their various unique anatomical structures and behaviors have given them a reputation as creatures to be *reckoned with*. The viperidae do have a prominent and well-developed venom delivery system, possessing the advanced solenoglyphic dentition interfaced with a highly flexible maxillary bone that is associated with a sizable venom gland. As such, regardless of venom toxicity, they are capable of effectively delivering a large quantity of venom deep into the tissues of their victims. The viperidae are subdivided to two subfamilies based on the presence or absence of infra-red sensory facial pits that are used in sensing and sizing-up their prey. Those without heat sensing pits (*Viperinae*) are Afro-Asian (i.e., Horned vipers, *Cerastes* spp.) and Eurasian genera (i.e., European adders, *Vipera* spp.) that are not found in the Americas or Australia. Viperids with the specialized heat-sensing capability (*Crotalinae*, pit vipers) are found in both the Americas (i.e., Rattlesnakes, *Crotalus* spp.) and Eurasia (i.e., Malayan pit viper, *Callocelasma rhodostoma*), but are absent in Africa and Australia (Table 19.6). Given their multi-continental and latitudinal distribution (69° N to 47° S), they inhabit arboreal, semi-aquatic, and terrestrial ecosystems from tropical to temperate climates [319]. The venoms of crotalinae and viperinae subfamilies, and their toxicological effects, are probably the most extensively studied of all snakes.

Viperidae venoms, although they possess some toxins similar to elapid toxins such as PLA_2s, differ from those of elapids in their clinical pharmacologic effects. Viperidae venoms are complex, with a variety of constituents that are chemically different, and each constituent may exhibit a different pharmacological activity (Figure 19.1). In contrast to neurological complications observed in most cases of elapid envenomation, viperidae venoms exhibit a wide variety of pharmacological mechanisms that primarily lead to venom-induced hemorrhagic effects. Exceptions to this basic concept are the neurological effects caused by venom of South American rattlesnake toxin, *crotoxin (Crotalus durissus*), and Mojave rattlesnake, *mojavetoxin* (*Crotalus scutulatus*) in certain regions of the southwestern United States. For example, Asian viperidae venoms, such as the Malayan pit viper's venom contains a serine protease glycoprotein that cleaves fibrinogen-A from fibrinogen, while the venom of the saw scale viper (*Echis carrinatus*) contains the prothrombin-activating zinc metaloprotein, *Ecarin*, and venom of the Russell's viper (*Daboia russelli*) has protease constituents that activate factor X in the clotting cascade [321]. Viperidae, both viperinae and crotalinae, venoms alter blood coagulation, cause myonecrosis, and possess numerous enzymatic proteins that can impact nearly every organ system [373]. Prothrombotic venom toxins of the viper *Bitis arietans* (Bitiscetin), and the pit-vipers *Bothrops jararaca* (Botrocetin) and *Trimeresurus albolabris* (Alboaggregin), contain C-type lectin and metalloproteinase-disintegrins that promote platelet aggregation. In contrast, similar toxins within these same C-type lectin and metalloproteinase-disintegrin families, from *Trimeresurus flavoviridus* (Flavocetin), *Bitis arietans* (Bitistatin), *Agkistrodon halys blomhoffi* (Mamushigin), and *Crotalus atrox* (Catrocollistatin), have been found to inhibit

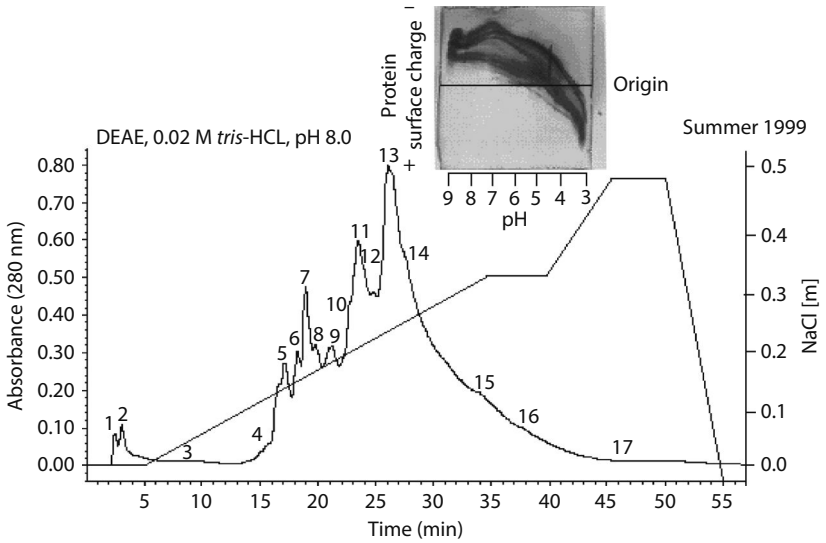

FIGURE 19.1 Profile of *Crotalus horridus* (timber rattlesnake) crude venom obtained by cation exchange high-performance liquid chromatography, illustrating the complexity of snake venom, as each peak represents a different venom fraction possessing its own pharmacologic activity. Inset: electrophoretic profile of venom showing separation of venom components based on pH, acidic or basic venom protein characteristics. (From John Perez, Natural Toxins Research Center, Kingsville, TX.)

platelet function, and demonstrate the commonality of some venom components across genera and across different continents [374]. Some crotalinae and viperinae (*Crotalus adamanteus, Crotalus ruber,* and *Agkistrodon halys blomhoffii*) venoms also contain phosphodiesterases [375]. The timber rattlesnake of the United States, one of the most widely distributed species of crotalinae, has a venom component, *crotalocytin* that causes rapid platelet aggregation [376].

Other venom toxins within the viperidae are the myotoxins, which are expressed as PLA_2 isoforms, with and without enzymatic activities, and in some pit vipers (*Bothrops asper*) these have been shown to change in expression depending on the age of the snake, with juveniles not expressing any PLA_2 isoforms. These are quantitatively important venom constituents that contribute to muscle necrosis and are triggered at the plasma membrane as a result of membrane destabilization with a loss of Ca^{2+} permeability [377]. Within the pit vipers, zinc metaloproteinases have been isolated from western diamondbacked rattlesnake, *Crotalus atrox* (*atrolysins*), and from eastern diamondbacked rattlesnake, *Crotalus adamanteus* (*adamalysins*), and differ from most venom proteases in that they all have hemorrhagic activity. These are known as the SVMPs (Snake venom metalloproteianases) [378]. L-amino acid oxidases are also common to most viperidae venoms and serve as an aid in prey digestion [321].

Envenomation by pit vipers or vipers usually results in complications with blood coagualation, tissue damage, and a redistribution of body fluids due to alterations in vascular permeability. The severity of envenomation can vary considerably depending on how much venom was injected with the bite, and where the bite was sustained. Large quantities delivered intravenously represent a worst-case scenario. Fortunately, most pit viper/viper bites are fairly superficial and large quantities of venom are not injected, and at times no venom is injected [379]. Pronounced pain is almost always present following bites, and edema and ecchymosis common.

Envenomations by pit vipers and vipers can result in profound hypotension, incoaguable blood due to venom anticoagulant effects, or as a result of consumption of blood coagulation components. Red cell membranes may rupture producing hemolysis, and delayed kidney damage. In general, victims may exhibit a DIC-like syndrome (disseminated intravascular coagulopathy). A few species of viperidae (*Crotalus scutulatus, Crotalus durissus terrificus* and *Vipera aspis*) have been studied for their potential therapeutic effects [380,381].

Treatment of viperidae envenomation usually involves the use of monovalent or polyvalent antivenoms, which are effective if administered within a reasonable time frame (Norris and Bush, 2001). Antivenoms are available for most prominent species of pit vipers and vipers [356]. Supportive care and wound care are essential in the treating of viperidae envenomation, and necrotic lesions may be slow to heal. Morbidity from viperidae envenomation frequently occurs when antivenom therapy is unavailable or an inadequate dose of antivenom has been administered [379].

Lizards

Heloderma, the only genus of lizards known to be dangerously venomous in the world, comprises only two species, the beaded lizard (*Heloderma horridum*) and the Gila monster (*Heloderma suspectum*), which are commonly referred to as escorpi'on. These two species further divide into five subspecies, *H. suspectum suspectum, H. s. cinctum, H. horridum horridum, H. h. exasperum,* and *H. h. alvarezi* [382]. The Gila monster is native to the extreme southwestern United States, and northwestern Mexico, while the range of the beaded lizard interfaces with that of *H. suspectum*, at its southern extreme in Mexico, but continues on down along the Pacific coast to Guatemala [315]. They are stout and solidly thick in the body, legs, and tail, and have a beady covering that is thoroughly, and irregularly, blotched with a pink/yellow pattern on a black/brown background. Interestingly, the forked tongue of *H. suspectum* is black, in contrast, to the pink forked tongue of the *H. horridum*. The Gila monster prefers an arid, rocky terrain, and will even dig burrows or use burrows of another animal, while the beaded lizard will inhabit forested areas, and is frequently observed in trees during the rainy season [383]. These lizards range in size from 35 to 70 cm, with the beaded lizard being the lengthier of the two.

Heloderma have short, slightly recurved, sharp, peg-like teeth that are grooved on both the anterior and posterior surfaces, with these grooved teeth anchored to the maxillary and mandible jaw bones. Although these lizards appear sluggish and somewhat uncoordinated when walking, as they appear to be waddling, they can rapidly flex their body and clamp their jaws around a victim. They will frequently chew once attached to a victim, and will not let go making them difficult to remove.

The delivery of venom by *Heloderma* stems from multilobed glands, lacking in musculature, that are located in the anterior portion of the lower jaw, on both sides, with each gland lobe independently having a duct that opens at the base of a tooth. When biting a victim, the muscle contraction of the jaw forces the venom from the ductal openings and channels it along tooth grooves, both upward and downward, with venom conduction via capillary action.

Venom chemistry studies of *Heloderma* have not been comprehensive, but limited studies show the venom of both species to be similar [384]. The venoms are known to possess enzymatic properties, as demonstrated by the presence of hyaluronidase, arginine hydrolase, phospholipase A_2 [385], and kallikrein-like enzymes [384,386]. One of the kallikrein-like enzymes capable of inducing hypotensive effects has been dubbed, *Helodermatine*. Additionally, an acidic protein neurotoxin, *gilatoxin*, a hydrolase toxin, *Horridum toxin*, and a hypothermic toxin, *Helothermine* (toxin from a *Heloderma* with a hypothermic effect), capable of significantly lowering body temperature in animals, have all been identified in beaded lizard venom [387,388]. *Heloderma suspectum* venom appears to be absent of hemolytic, hemorrhagic, and proteolytic properties [389], but vasoactive peptides and a vasodilating venom component have been isolated [387].

Heloderma have few enemies other than man, and it is humans who suffer the consequences of their powerful bite. Although claims and reports of death from *Heloderma* envenomation exist, their legitimacy has been greatly questioned [382]. Envenomation from *Heloderma* is quite painful, and much of the pain is a result of pure mechanical trauma injury. These lizards will hang on when they bite for extended periods of time without lessening their grip. Teeth may even be broken off when human victims are trying to remove the tightly attached lizard. Prominent symptoms, aside from intense pain, are edema, erythema, nausea and vomiting, dizziness, hypotension, leukocytosis, weakness, tachycardia, diaphoresis, and lymphangitis [390]. Arteriospasm, with severe pain, of an envenomated digit may also occur [391]. Hypotension is a major concern and, in some cases, may be related to venom-induced anaphylaxis involving swelling of the tongue [392]. Symptoms may persist for greater than 72 h, and wound care is critical, as broken teeth may need to be removed, and secondary infection may ensue. Treatment of envenomation consists of good supportive care, and may involve fluids, antihistamines, and corticosteroids, with careful cleaning of the wound and antibiotics [390].

Amphibia

In addition to the variety of freshwater and toxic marine animal life forms such as sedentary anemones, swimming catfish, or drifting jellyfish, and the numerous semi-aquatic and terrestrial toxic animal life forms such serpentine-swimming sea snakes, walking lizards, and crawling land snakes, nature has filled another ecological niche with, not only awkwardly walking, but *hopping* amphibians. They may live in water, on the land, or utilize both as adults, but usually have some stage of their life cycle overlapping between these two habitats. Amphibians, possessing toxic properties in their body or skin inhabit subtropical and tropical regions of the earth, but a few are also found in temperate regions. A few of the approximately 2600 amphibia are known in the realm of toxinology for their toxic skin secretions (Table 19.7) [393]. As a group, amphibians commonly are the prey food items of a variety of animal predators, such as birds, fish, snakes, and humans. Thus, given their limited modes of locomotion, making escape from hungry predators difficult, antipredation strategies that would ensure their continued survival often depend on their ability to fend off predators in toxinological ways, making them quite distasteful, or even poisonous. Thus, the ability of some amphibians to secrete skin toxins has been a phenomenon for which toxinological explanations have been sought and one of great interest to medicine and science.

Man's interest in amphibian skin secretions began over two millennia ago in China when powdered frog skins were used as heart stimulants and diuretics [89]. Today, the quest for knowledge concerning amphibian toxins and related biologically active compounds continues as evidenced by the continuing studies of one of the most widely distributed and potent natural toxins, tetrodotoxin (TTX). Present in a spectrum of unrelated taxa, such as bacteria, a flatworm, goby fish, octopus, and puffer fish, TTX is also prevalent in several amphibian genera and species and subspecies [394]. However, of the greater than 5000 amphibian species, categorized to 44 families [395], only 6 appear to contain TTX [396]. First isolated from the puffer fish of oceanic waters, and then from the terrestrial amphibian, the California newt (*Taricha torsa*), TTX continues to be discovered in new species [397].

Amphibian toxins are passively delivered, transdermally, when they are harassed, or actually being engulfed by predators. The skin of amphibians is a complex organ of interrelated biochemical, morphological, and physiological functions utilized for hydration, reproduction, respiration, thermoregulation, antifungal and antimicrobial protection, and antipredator defense and is essential to amphibian survival [398]. Toxins are released from specialized mucous-secreting, or granular, dermal glands distributed throughout the dorsal skin but, in some cases, are also present in specialized anatomical structures, like the bulbous parotoid gland, located in the post maxillary region of some salamanders and toads [89]. These are sites of synthesis for alkaloids, biogenic amines, peptides, proteins, and steroids [398]. The consequence of toxin release is basically a distasteful adverse reaction to predators, but in some instances, death to another creature may occur following ingestion of the toxin-containing amphibian.

Frog, newt, and toad skin secretions, from several species, representing several major amphibian genera, contain the potent, nonprotein neurotoxin, *TTX* [399]. Frogs of the

TABLE 19.7
Terrestrial Vertebrate Animals: Amphibians and Reptilia (Lizards)

Phylum	Class	Common Name		Genera (Toxin-Containing)
Chordata	Amphibia	Frogs, poison dart frogs		*Aetlopus, Brachycephalus, Colosthetus, Dendrobates, Epipedobates, Gastrophryne, Kaloula, Limnonectes, Mantella, Phylobates, Polypedates, Rana*
		Newts		*Cynops, Notophthalmus, Paramesotrition, Taricha, Triturus*
		Salamanders		*Salamandra*
		Toads		*Ansonia, Bombina, Bufo, Leptophryne, Pedostypes*
	Reptilia	Lizards	Beaded lizard Gila monster	*Heloderma*

genera *Brachycephalus, Colosthetus*, and *Polypedates* [397], toads of the genus *Atelopus* [399], and newts of the genera *Cynops* and *Taricha* [400] are all different amphibian genera containing at least one species that possesses TTX in its body. The origin of TTX in amphibians, and all other animals, has been a topic of great interest, and there is considerable evidence that TTX may be of dietary origin, associated with a symbiotic relationship with TTX-producing bacteria, and as such it is bioaccumulated [401]. However, recent studies in rough-skin newts (*Taricha grannulosa*) suggest that TTX has been produced by granular skin glands, in high concentrations, independent of bacteria symbiosis, alluding to the possibility of different TTX origins in different amphibian species [402]. A recently discovered TTX, 11-oxotetrodotoxin (11-oxoTTX), has been isolated from the skin extract of the Brazilian frog, *Brachycephalus ephippium*, and found to be four to five times more toxic than TTX itself, demonstrating possible evolutionary changes in TTX [403]. Further research with two other species of the *Brachycephalus* genus (*B. nodoterga* and *B. pernix*) has confirmed the presence of several new deoxy-TTX and nor-TTX analogues [397].

Toxicity of TTX results from its ability to specifically, and irreversibly block, voltage-gated, Na^+ ion channels, abolishing the action potential at very low concentrations (μg/kg) [404]. The result of this action is varying degrees of paralysis in animals, but some predators like the common garter snake (*Thamnophis sirtalis*) appear to be resistant to TTX toxicity [405]. TTX toxicity to humans results from ingestion of significant quantities of TTX. Human fatality, within 24 h of ingesting a rough-skin newt, that caused cardiopulmonary arrest, has been reported [406]. Although TTX is relatively prevalent in amphibians, there are also other significant toxins of great interest, and potency, present in amphibians.

Frogs and Toads

Frogs and toads of several genera (Table 19.7) from particular bufonid, dendrobatid, mantellid, and myobatrachid anuran lineages, have yielded over 500 lipophilic alkaloid compounds, representing 22 different structural classes [407]. The alkaloids represent several truly unique toxins that appear to be present in dermal secretions, not because of de novo synthesis or genetic factors, but as a result of the arthropod diet of certain frogs. Diet preference for myrmicine ants appears to be a primary source that leads to alkaloid sequestration in several brilliantly colored, Central and South American, dendrobatid poison dart frogs, and these ants have comprised up to 73% of stomach contents of *Dendrobates* spp. [408]. The frogs (poison dart frogs) best known for the more potent alkaloid toxins are those in the genera *Allobates, Dendrobates, Epidobates*, and *Phyllobates*. Toxic alkaloids present in these genera include the voltage-gated Na^+ channel activating *batrachitoxins* (BTXs), noncompetitive nicotinic receptor-gated channel blocking *historonicotoxins* (HTXs), cardiotoxic and myotoxic *pumiliotoxins* (PTXs) and allopumiliotoxins (aPTXs), and the nicotinic receptor agonist, epibatidine with its antinociceptive properties [409]. Additional skin alkaloid toxins include coccinellines, cyclopentaquinolizidines, decahydroquinolines, tricyclic gephrytoxins, indolizidines, piperidines, pseudophrynamines, and pyrrolidines [410]. On the other side of the ocean from these dendrobatid frogs are several varieties of frogs and toads from Thailand that have toxic skin secretions. The ranid frogs, *Rana raniceps*, *R. signata,* and *R. hosei*, along with the rhacophorid frog, *Polypedates leucomystax*, have skin extracts that cause locomotor difficulties and prostration when injected into mice, and a skin extract from *Limmonectes kuhli* contains pumiliotoxin alkaloids. Virtually nothing is known about potential toxic effects of these extracts to humans [411].

Toads of the Americas and Asia are also noted for their noxious secretions. The widely distributed marine toad (*Bufo marinus*), Amazonian (*Bufo aqua*), Asian (*Bufo gargrizans*), European (*Bufo vulgaris*), and North American, Colorado river toad (*Bufo alvarius*) are a few of over 200 toad species, all having paratid glands capable of producing biologically active compounds [412]. The biologically active substance serotonin is found in both humans and toads, but it is a 5-hydroxydimethyltryptamine, that is present in all toad species, and is known as *bufotenine*. When this compound is methylated it becomes bufotenidine, dehydrobufotenidine, and 5-methoxydimethyltryptamine (5-MeO-DMT), a potent bufotenine only known to exist in *Bufo alvarius* [413]. It is the 5-MeO-DMT that is believed to induce hallucinations in humans following licking of toads, or smoking their venom [414]. In addition to bioactive toad venom components there are the bufodienolides (bufogenins and bufotoxins), biosynthesized from cholesterol, that are highly toxic cardioactive steroids that most likely work in concert with epinephrine and norepinephrine, which are also present in toad venom [415].

Toxicity associated with toad venom is similar to that of digoxin [416], with inhibition of the sodium–potassium ATPase pump [417]. Small, companion animals have died as a result of toad ingestion [418]. Chan Su poisoning in humans who ingested dried toad venom as an aphrodisiac and people who *licked* or *mouthed* toads to get high have caused profound cardiotoxicity that has resulted in several fatalities [419]. Successful treatment of cardiotoxic effects has been accomplished with the use of digoxin-specific antibody Fab fragments [419]. Still further demonstrating the diversity of toad toxins has been the isolation of morphine-like toxins from *Bufo marinus* skin extract [420], and a sleep-inducing factor from the extract of the Indian toad, *Bufo melanostictus* [421], in which only males of this species possess a Bidder's organ, located *rhostorventrally* to the kidney, that produces a Bidder's organ-cardiotoxin capable of inducing cardioneuromuscular blockade in animals [422].

Newts and Salamanders

Newts are similar to salamanders in appearance except that their skin has a dryer, less clammy, or slimy feel to it. Toxic species are native to the east and west coasts of the United States and Japan. Their primary toxin is tetrodotoxin and has been discussed earlier.

Salamanders of European terrestrial forested habitats may be bright orange and black bicolored, or solid black, and have a wet sheen appearance to their bodies. They are of the genus *Salamandra*, the European fire salamander (*Salamandra salamandra terrestis*) and the Alpine salamander (*Salamandra atra atra*), and like their dendrobatid amphibian relatives, secrete toxic alkaloids from their skin. Samamandarine and samamanderone are the steroidal alkaloids of prominence, but cycloneosamandione, samamandinine, samanine, samamandaridine, and samandenone may also be present [423]. These are possibly cholesterol-derived steroidal alkaloids, and they are synthesized by the salamanders rather than taken up in the diet or some other form. Detailed pharmacologic actions of these salamander alkaloids are lacking but have been shown to have nerve-blocking activity both peripherally and centrally in the nervous system [424]. Although these toxins may serve as deterrents to predators, their effects in humans are unknown.

Marine Animals: Poisonous, Toxin Bioaccumulated

Poisonous marine animals do not synthesize the toxins they carry in their bodies and organ systems but rather accumulate the toxins from their diet or environment. Thus, the toxins are actually produced by other organisms that live in the surrounding marine environment. A variety of marine animals are seafoods common to the human diet that may potentially become toxin-bearing and cause severe risk to human health and life following their consumption (Table 19.8). For example, the most popularly known seafood poisoning, exhibited as neurotoxic symptoms and cardiopulmonary toxicity, and potentially human death, may result from eating puffer fish. Prepared in gourmet restaurants as a delicacy dish, *fugu*, if improperly prepared may lead to severe tetrodotoxin poisoning in the innocent diner [425].

Marine animals most commonly acquire these toxins from dinoflagellate (toxic microalgae) species such as *Alexandrim* spp., *Gymnodinium catenatum, Pyrodinium bahamense* (all associated with Paralytic shellfish poisoning and Red tides) [426], *Dinophysis* spp. (Diarrhetic shellfish poisoning) [427], *Nitzschia* spp. (Amnesic shellfish poisoning), and *Gymnodinium brevis* (Neurotoxic shellfish poisoning) [428]. Still other toxins, associated with different poisoning symptoms, also come from dinoflagellates, such as *Gabierdiscus toxicus* and *Ostreopsis lenticularis* (Ciguatera poisoning), that contain toxin-producing bacteria [429], while *Pfiesteria* spp. have caused considerable human sickness yet no toxin has been identified [430]. Not surprisingly, the presence of dinoflagellate toxins in marine animals will fluctuate in some species with seasonal climate changes that trigger algal blooms. Additionally, some toxins

TABLE 19.8
Marine Animals: Poisonous and Toxin-Containing

Phylum	Class	Common Names	Genera (Potentially Toxic)	Toxins
Arthropoda	Crustacea	Crabs	*Atergatis, Atergatopsis, Birgus Cancer, Carpilius, Demania, Eriphia, Lophozozymus, Platypodia, Zozymus*	Gonyautoxin Palytoxin Saxitoxin Tetrodotoxin
Chordata	Chondrichthyes	Sharks	*Carcharinus*	Carchatoxin
	Osteichthyes	Fugu (puffer fish)	*Arothon, Tetraodon*	Tetrodotoxin
	Scromboid fish	Anchovies	*Engraulis*	Ciguatoxin
		Barracuda	*Sphyraena*	Maitotoxin
		Bluefish, Bonito	*Pomatomus*, *Sarda*	Scaritoxin
		Grouper	*Epinephelus, Mycteroperca*	
		Herring, Mackerel	*Clupea, Decapterus,*	
		Mahi mahi	*Coryphaena*	
		Parrot fish	*Scarus, Ypscarus*	
		Red snapper	*Lutjanus*	
		Sardines	*Esulosa, Sardina, Sardinella, Sardinops*	
		Skipjacks	*Euthynnus*	
		Tuna	*Thunnis*	
Molusca	Bivalvia	Clams, cockles, mussels, oysters, scallops, shellfish	*Mytilus, Saxidomus, Seliqua*	Brevitoxin, gonyautoxin, pectenotoxin, saxitoxin, yessotoxin, dinophysistoxin Domoic acid, okadaic acid
	Gastropoda	Snails	*Babylonia, Charonia, Nassarius, Natica, Niotha, Oliva, Polinices, Tectus, Tutufa, Turbo, Zeuxis*	Surugatoxin, neosurugatoxin Saxitoxin, neosaxitoxin Gonyautoxin, tetrodotoxin Tetramine

Histamine

form, and accumulate, in food fish as a result of spoilage, such as the case in scromboid poisoning [431].

There are no simple antidotes for treating victims of marine food poisoning, and symptomatic and supportive care, including life-support, are essential for successful outcomes.

Animal Toxins and Poisons from Unusual Sources

The amazing thing about animal toxins is that in addition to being present in snakes, poison dart frogs, jellyfish, scorpions, spiders, and other likely *poisonous creatures*, there are toxins and poisons that show up in the most unlikely of creatures (Table 19.9). No one would ever think of poisonous birds from Papua New Guinea with toxins in their feathers to deter predators, and that the toxins are similar to those found in Central and South American poison dart frogs [432], nor would anyone think that the powerful Polar bear could be poisonous and cause serious vitamin A toxicity from ingestion of its liver as a human dietary food [30]. The gracefully swimming sea turtles, like many poisonous fish, may accumulate algal toxins from the oceans, and when their meat is consumed, cause death [89]. The littlest of mammals, the shrews, have salivary toxins capable of inducing hypotension and paralysis [433]. Even the most unusual of mammals, the egg-laying platypus, has a toxin secreting, crural venom gland, connected by a duct to an erectile keratinous spur, just above the webbed foot on each hind leg. The males only possess this venom apparatus, and if skin penetration to a predator occurs, it can cause severe pain, edema, and hyperalgesia [434].

In contrast, to these creatures, one might expect a vampire bat to have a toxin in its saliva that would alter blood coagulation, and such is the case with this flying mammal, as the anticoagulant *draculin* allows the free flow of blood from its dietary victims [435].

Toxins in the animal kingdom, whether used for defense against predation, or for prey acquisition, have been an evolutionary key to the long-term survival of toxin-containing, toxin-producing, and poisonous creatures. The animal phyla, classes, genera, and species that make up nature's living synthesis laboratories will continue to be the true Nobel chemists of the world despite human intellect.

BENEFICIAL USES OF NATURALLY OCCURRING TOXINS

So what does all this massive group of information on the diverse effects of multiple natural toxins come down to? Is there continuity to the available information? Can generalizations be made and extrapolated across species? What can be learned for the betterment of societal health and preventive and therapeutic medicine?

The varying potencies and clinical damage produced by the plants, algae, mushrooms and diverse representations of the animal kingdom clearly indicate the variety and intensity of toxicity resulting from these naturally occurring intoxications. The variety of body systems affected both in animals and in human beings compliments the wide identification of toxic chemical entities identified already from each natural vector. Correlating the presence of specific toxic compounds with their physiological effects allows researchers to consider developing new chemicals derived from these natural products to produce or modify drugs to influence physiological functionings. Such modifications could improve health, could block disease mechanisms, or could offer valuable insight into therapies and new modalities for therapeutic intervention. The abundant reports of individuals being bitten by snakes and treatment-specific antivenom followed by the course of the toxicity offer information on future improved therapies. A young male bitten by a king cobra and treated with massive doses of Thai King Cobra Antivenom within

TABLE 19.9
Animal Toxins and Poisons from Unusual Sources

Phylum	Class	Common Names	Genus	Toxins/Poison
Chordata	Aves	Rubbish birds		
		Blue-capped ifrita	*Ifria*	Batrachitoxin
		Hooded pitohui	*Pitohui*	Homobatrachotoxin
		Quail	*Coturnix*	Coniine, hyoscyamine
				Solanins
Mammalia		Platypus	*Ornithorhyncus*	Enzymatic proteins
				Hyaluronidase
		Polar bear	*Thalarctos*	Retinol (Vit. A)
		Shrew	*Blarina, Neomys*	Kallikrein
			Sorex, Solenodon	
		Vampire bat	*Desmodus*	Draculin
	Reptilia	Sea turtles		
		Green	*Chelonia*	Chelonitoxin
		Hawksbill	*Eretmochelys*	
		Loggerhead	*Caretta*	

an hour of envenomation, and the rapid uneventful recovery with no after effects, provides good clinical experience and insights into the value of such early and massive therapy [436]. This case and others document the efficacy of an efficient emergency medical system and the prompt utilization of intravenous dosing with specific volumes of antivenom.

Components of natural toxins may also be used to attenuate or reverse illnesses in humans, often on an initial empirical basis and later to more recognized efficacy in treating human functional disturbances. Snake venom derived Arginine-Glycine-Aspartic acid containing Disintegrins (Rhodostomin) has been shown to inhibit cell adhesion and particularly the adhesion of breast and prostate carcinoma cells to bone extracellular matrices. Rhodostomin also inhibited migration and invasion of the breast and prostate carcinoma, markedly inhibiting tumor growth and bone destruction. These disintegrins from snake venom strongly inhibit adhesion, migration and invasion of tumor cells as well as tumor growth of human breast cell cancer in bone; they thus have the potential to be developed as alternate therapy for the bone metastasis of cancer cells [437].

Snake venoms have also been used to provide more effective therapy of human and animal problems. Coagulopathy is a significant cause of morbidity and mortality in snake-bitten humans; yet probably prepared fractions of snake antivenoms are effective treatment, more so than standard factory placement therapy and heparin [438]. Understanding the mechanisms of action for such potent benefits serves to advance medical therapeutics and prevent human suffering. The disintegrins and other snake venom proteins have shown extreme promise in the treatment of a range of hemostatic disorders; for example, ancrod from the Malayan Pit Viper (*Calloselasma rhodostoma*) has been effectively used as an anticoagulant for therapeutic defibrination [439]. These and other fractions from naturally occurring toxins offer continuing mechanisms to study modes of action from these molecular structures to benefit human and animal health.

The diverse animal and plant sources for these potentially useful products highlight the numerous opportunities for identifying beneficial fractions from plants and animals that could have wide use in medicine. The recent observation that extra virgin olive oil contains a compound that provides the same pain-relieving effect as the anti-inflammatory compound ibuprofen. Since inflammation is believed to form the basis for a variety of chronic diseases, and current anti-inflammatory compounds have undesirable side effects, this discovery is just another example of the possibilities that chemicals in natural products may offer.

The opportunities are all around us in our plant and animal kingdom, and the experiences gained in nonhuman animals from their continuing exposure to plants and animals living in the wild offer instances of excitement when potent chemical effects are observed from such exposures. The comparative value of animal effects to naturally occurring toxins emphasizes the importance of applying such information to human medicine and to toxicology.

It is true that there is one medicine…and also one toxicology!

QUESTIONS

19.1 What are the differences in the chemical characteristics of a venom and a synthetic chemical?

19.2 What conditions affect the concentration and type of toxin in a poisonous plant? What conditions affect the concentration and type of toxin in the venom of a poisonous animal?

19.3 What body organs are commonly affected by poisonous plants? Give two plants that primarily affect each such organ.

19.4 Discuss the laboratory procedures used to detect the presence of toxins in plants.

19.5 What plant toxins are secreted in breast milk or the milk from lactating animals?

19.6 What are the common toxins that are generated by blue-green algae? What body systems do they each affect?

19.7 Why are blue-green algae of public health significance?

19.8 What are the clinical effects produced by mushroom consumption?

19.9 Through what mechanisms do animal toxins exert their harmful effects?

19.10 What are the five categories (classes) of toxic marine invertebrates?

19.11 Give three toxic marine animals in the Cnidaria group.

19.12 Discuss the potentially beneficial uses of the toxins found in plants and animals.

19.13 What are the five bony fish known to be venomous and hazardous to humans?

19.14 What are the effects of envenomation by sea snakes?

19.15 How might terrestrial invertebrate animals deliver their toxins?

19.16 What are the largest sized arachnoids? What are the oldest living toxic creatures?

19.17 In what parts of the world are scorpion bites serious health problems?

19.18 Give the common names of five different venomous spiders.

19.19 What is the chemical composition of centipede venom?

19.20 What are the common names of five venomous members of the *Insecta* group of animals?

19.21 What is unique about Australian caterpillars?

19.22 What dose of venom is injected by the sting of one bumblebee? What is the dose of venom injected by one hornet sting? What is the dose of venom injected by the sting of one killer bee?

19.23 Name and characterize five venomous reptiles.

19.24 What are the various types of dentition that venomous snakes have?

19.25 Describe the difference in venom activity between colubrid, elapid, and pit viper snakes.

19.26 Discuss the various treatments available for the bites of venomous snakes.

19.27 Name the two venomous lizards in the world and how they deliver their venoms.

19.28 How are amphibian toxins delivered to victims?
19.29 What is the potent nonprotein neurotoxin found in skin secretions from several amphibian genera?
19.30 Discuss the unique toxins in the dermal secretions of frogs and toads.
19.31 What commonly dispensed drug induces similar toxicity to that associated with toad toxicity?
19.32 What is the primary toxin elaborated by newts?
19.33 What are five marine animals that commonly acquire toxins from dinoflagellate species?
19.34 Discuss the animals that are not usually considered to carry toxins, but are capable of inducing poisoning in unsuspecting humans.

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Section III

Methods

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20 Humane Care and Use of Laboratory Animals in Toxicology Research

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CONTENTS

INTRODUCTION

Procedures for the use of animals in research are heavily driven by regulations, and regulations are heavily driven by public expectations. This chapter is intended to introduce the scientist to these regulations and expectations, as well as the scientific concepts that underlie the regulatory standards. The complexity of these requirements is such that scientists are not expected to be experts in this area. Laboratory animal science and medicine have become their own fields of study with professional associations, training programs, certifications, and even a Board specialization in veterinary medicine.[1] Scientists should cultivate cooperative relationships with the professionals responsible for laboratory animal care and welfare at their institution, or if such professionals do not exist, bring this expertise in-house or utilize available consultants. This can be a challenge, as animal welfare compliance can sometimes conflict with scientific freedom, which entails some risk of developing adversarial relationships between scientists and compliance personnel. It is important for both scientists and compliance staff to remember that each of their roles is critical to the success of the research mission and open communications are critical.

Public Perception of Animal Research

The selection of the best model for risk assessment of new drugs and chemicals is impacted not only by the scientific

merits of the model, but also by public attitudes toward the use of animal models in research. Public attitudes have affected the science of toxicology through subtle effects on model selection due to pressure on institutions from shareholders, public constituents, animal extremist protesters, and through the creation of government regulations and industry standards directly affecting the cost of conducting animal research. Regulations and the associated standards are discussed throughout this chapter to provide the basic framework for all procedures related to the humane care and use of laboratory animals.

Attitudes toward animals and their use in research vary throughout the world based on philosophy, culture, religion, gender, socioeconomic level, species of animal, and the intended research use.[2–5] However, there are several basic concepts underlying animal welfare regulations and standards in existence throughout the world. First, there is the concept that there is intrinsic value to life and in particular, sentient life. The second concept is that sentient animals have the capacity to experience pain or distress and try to avoid pain and distress wherever possible. The third concept is that research animals share common genetic and physiologic attributes, making them useful for biological modeling in humans as well as animal species. Thus, the use of animals in procedures, which have the potential for pain and distress or are terminal, is perceived to represent an ethical cost and should only be performed where there is scientific justification and no suitable alternative.[2,6]

Depending on how individuals value each of these concepts, a spectrum of attitudes can be displayed toward animal use.[4,7] One philosophy is often referred to as animal extremist or animal rights. This is the belief that the rights of animals are equal to those of humans, and the use of animals in research is unethical because animals cannot consent to the research procedures for study. People with these beliefs may express them through lifestyle choices related to the use of animals or animal products for food, fiber, clothing, work, companionship, or sporting use. Others become involved politically in an attempt to influence public policy or corporations, while some protest through media, writing campaigns, and/or publically. Few go to extremes and may harass or induce violence toward people working at research institutions, their customers and investors, infiltrate research operations for the purposes of generating publicity or regulatory investigations, causing property damage and theft, or the release of animals.[4,8]

Animal rights activities have profound effects on the use of animals in research by driving the development of a regulatory infrastructure for the care and use of animals in research. There have been positive effects from this infrastructure as it has led to significant learning about the specific contributions animal models make versus nonanimal models in safety assessment research and the control of variables when using animal models in research. However, there have been negative effects as well. The cost of research and the time required for planning and conduct of research have increased, and most importantly, a significant gap has appeared in communication between biomedical researchers and the public regarding the importance of animal models in the development of medical treatments as well as chemicals for our lifestyle and civilization. Attitudes have continued to support limiting animal research as well as limiting the species of animals available for use. Western Europe, in particular, has seen significant restriction in animal use over the last decade.[2,8] As animal research can be conducted in other countries, it has not affected the overall progress of research, but if this trend encompasses the globe, profound impacts on the progress of biological sciences could occur. On a practical level, institutions using animals need to screen employees for animal extremist activities and maintain internal and external security to protect from incursions. It is also important for all staff to be trained to understand the need for animals in research, but also understand the approval process justifying the use of animals and the extensive care provided to them.

The opposite of animal rights extremism is a lack of value for animal life or recognition of the ability of animals to experience pain or distress.[9] This attitude is somewhat perplexing from a scientific standpoint, as in order for animals to be good models of human biology, they must share similar anatomical, physiological, and neurological capabilities that would logically result in their ability to experience pain or distress in the same way that humans do. Like animal extremism, not valuing animal life can also result in problems within research institutions if it leads to resistance to regulatory compliance. When expressed openly, it may be viewed as insensitive and perpetuates the traditional stereotype of science being cold and uncaring. This can polarize the attitudes of the public as they may react to one extreme viewpoint by adopting the other extreme viewpoint. Staff may require sensitivity training to understand and respect the diversity of viewpoints regarding animals and the importance of complying with both the spirit of animal welfare regulations and the letter of them.

Fortunately, these extreme views are relatively rare. The majority of individuals recognize that there are ethical tradeoffs with many of the decisions humans make. In general, as long as researchers demonstrate they are paying attention to basic principles, such as respect for life, minimizing pain or distress, and using animals only when scientifically justified, the public supports the use of animals in research.[4,7,10] It is important that the public can see that these basic principles are being employed. It is critical for biomedical research institutions and individual researchers to maintain open communications with their communities and constituencies so that the importance of the research performed and the ethical standards that are used to determine when and how animal models are used is also clear. Institutions need to maintain this communication internally as well as externally as scientists and nonscientific staff may also have questions or concern about animal use and need the same information to be comfortable with animal use at their institution. In fact, institutional employees may be one of the most important audiences for this message. If a staff member becomes dissatisfied with an aspect of animal use and does not feel that

they can address it internally, they become a risk for passing information on to an animal extremist group, the media, or a government agency in an attempt to get their issue addressed. Communication with the public creates certain risks of confronting the attitudes of extremists as does talking with the media, who may not have a moral agenda but will modify the tone of their report to maximize the sale of their product. Therefore, external communication must be done carefully. Institutions commonly have policies restricting communications with the media or nonemployees and may have designated leaders and/or trained public relations staff to manage these communications. Outreach programs for schools and the community are also common. Successful programs often have well-prepared presentations, clear messages, and experienced and engaging speakers who know how to present biomedical research in a positive light and are able to respond to questions posed by the public. There are professional organizations that assist research institutions with this task (Table 20.1) and scientists or institutions can become involved with and support these organizations and their missions.

Scientific Justification for Animal Use and Alternatives

To address both public concerns about the use of animals in research and meet compliance needs, one must scientifically

TABLE 20.1
Selected Professional Organizations That Support Public Communications on the Use of Animals in Research

Organization	Selected Resources
American Association for Laboratory Animal Science (AALAS)	Animal Research FAQs: http://www.aalas.org/association/animal_research_faqs.aspx AALAS Foundation Education Resources: http://aalasfoundation.org/public_outreach.html www.aalas.org Kids 4 Research Education Resources: http://www.kids4research.org/
Americans for Medical Progress (AMP)	Animal research benefits and FAQs Posters, videos, speaking points card http://www.amprogress.org/
American Physiologic Society (APS)	Animal research, finding cures, saving lives: http://www.animalresearchcures.org/treatedwell.htm Animal research position statements: http://www.the-aps.org/mm/SciencePolicy/Animal-Research
Federation of American Societies for Experimental Biology (FASEB)	Educational resources: http://www.faseb.org/Policy-and-Government-Affairs/Science-Policy-Issues/Animals-in-Research-and-Education/Teaching-Advocacy-Material.aspx Animal rights extremism: http://www.faseb.org/Policy-and-Government-Affairs/Science-Policy-Issues/Animals-in-Research-and-Education/Animal-Rights-Extremism.aspx
Foundation for Biomedical Research (FBR)	Educational resources Media resources Speaker education www.fbresearch.org
National Research Council	Science, medicine, and animals: teacher's guide: http://www.nap.edu/catalog.php?record_id=11564 Science, medicine, and animals: student brochure: http://www.nap.edu/catalog.php?record_id=10089
National Association for Biomedical Research (NABR)	Benefits of Biomedical Research Regulatory oversight Animal welfare Animal activism Animal law Webinars www.nabr.org
NIH—OLAW	Why are animals used in NIH research? How does the NIH ensure their welfare? http://grants.nih.gov/grants/policy/air/general_public.htm
Society of Toxicology (SOT)	Animals in research public policy statement: http://www.toxicology.org/ms/air6.asp Educational resources: http://www.toxicology.org/teachers/teachers.as; http://www.toxicology.org/kids/kids.asp
Speaking of Research	Speaker education and resources: http://speakingofresearch.com/
States United for Biomedical Research (SUBR)	Educational resources: http://www.statesforbiomed.org/content/educators

justify when and how animal models are used in the process of developing drugs and chemicals, and explore alternatives to animal use. In order to scientifically justify animal use in research, scientists must document a search for *alternative* models.[11–13] Alternative models include not only a choice between animal and nonanimal models but also alternatives to procedures in animals causing more than momentary or slight pain or distress. This concept, first described in a book by Russell and Burch in 1959, is categorized as the *3 Rs*: *replacement*, *reduction*, and *refinement*.[12,14,15]

Replacement alternatives include the use of nonanimal in vitro and in silico models. Clearly, a nonanimal model cannot experience pain or distress. However, replacement alternatives can also involve the use of less sentient animals, as it is believed by some that less sentient animals have a lower ability to experience pain or distress.[6] Therefore, invertebrate models are preferable since they have a less-developed central nervous system (CNS) in comparison to a vertebrate. Furthermore, the use of a *lower* vertebrate such as a fish or amphibian is also considered preferable to the use of a bird or mammal, and the use of a lower mammal such as a rodent is considered preferable to the use of a higher mammal such as a dog or nonhuman primate. This concept has been questioned based on both biological and ethical grounds since there are some invertebrates like cephalopods with advanced CNSs, and the relative level of sentience of different vertebrates is difficult to measure among different taxa that evolved parallel behaviors.[16,17] In addition, perception of sentience or other *moral value* is affected by religious and cultural norms, as well as personal experience.[6] For example, dogs may be perceived as having higher value than nonhuman primates by some individuals, despite a large body of literature suggesting sentience is higher in nonhuman primates. In the Western world, rodents are often viewed as vermin, and therefore of lower value, but behavioral evaluation of mice and rats shows a high level of cognition and capacity to experience pain or distress.[18]

One potential replacement alternative is the use of human volunteers. This is not classically considered a replacement alternative, and is not consistent with the idea of using a less sentient organism for research. However, humans can freely choose whether to participate in the research procedures and can be educated on the potential for pain or distress from the procedure. The results of the research also directly benefit the humans; therefore, they directly bear any risk from the research for their own benefit. While it could be argued that this alone should drive the use of humans rather than animals for research, regardless of a human's willingness to volunteer, most individuals and cultures have determined that the involuntary use of humans is considered more unethical than the involuntary use of animals, as reflected by laws and religious mores. There is also a scientific case arguing against the routine use of humans for the development of new drugs and medical devices: It is much harder to control variability in human populations due to genetics, lifestyle, and compliance with research procedures. Therefore, the sensitivity of a human bioassay is inherently lower than a well-defined and controlled animal assay, assuming there is good relevance of the animal assay to the human condition.[19]

The appropriate use of replacement alternatives is already incorporated into regulatory guidelines for submission of data for new drug or medical device registration,[20,21] but it is also driven by scientific and economic considerations. Not only does animal use tend to be more expensive compared to nonanimal models, it is generally more difficult to control experimental variables. Nonanimal models are very useful for high-throughput screening of test articles, particularly where there are well-defined mechanisms of disease or toxicity that can be detected by the assays.[22,23] However, because not all of these mechanisms are known, initial screening assays must be supplemented by live animal assays. Live animal data are required prior to gaining approval for testing in humans because the risks associated with new therapies are often greater than most humans will volunteer to take. In addition, scientists will typically choose a rodent model over a nonrodent model due to the lower cost to buy, care for, and use rodents and the ability to control genetic and environmental variables in many rodent models. Regulatory guidelines also require the use of two species of animals prior to human studies, one of which must be a nonrodent due to the higher level of relevance of nonrodent models for certain biological systems.[24]

Reduction alternatives are those that minimize the number of animals used. It is important to evaluate reduction alternatives against the bigger picture of a research program, as simply reducing the number of animals on an experiment can have negative effects for the study including nonmeaningful data, the need for additional animals to be added later, or the need to repeat or conduct additional studies. Therefore, when designing studies to produce valid data, statistical methods are very useful and can help define the appropriate number of animals needed for valid results. For this reason, the animal use review process will typically ask whether statistical methods have been incorporated into the study design.[11–13,15] In some cases, statistics are not utilized for study design, as some assays driven by regulatory requirements mandate a certain number of animals,[24] usually based on industry experience with the assay. When regulatory requirements are not driving the study design, studies should be designed to provide appropriate statistical power to detect biologically relevant differences among groups for the specific endpoints measured. If the statistical difference in mean response considered biologically relevant and level of variability in response among individuals on the study is not known, the use of pilot studies to generate preliminary and/or comparison data should be considered. Pilot studies are also useful for other reasons, as they can help to troubleshoot practical aspects of procedures and can also be used to evaluate refinement alternatives as discussed later.[12]

A final concept in application of reduction alternatives is to remember that the overall goal of alternatives is to reduce pain or distress, not just reduce the number of animals used. In this sense, subjecting the smallest number of animals possible to pain or distress is desirable. Early studies in the

development process typically use fewer animals than those later in the process, and they are not necessarily conducted according to strict regulatory standards that may limit the ability to alter experimental design or procedures in response to the condition of the animals. Therefore, if it is necessary to conduct an experiment resulting in significant pain, distress, or even death of the animal, it is better to perform those studies in the early stages of drug development, and communicate the need for these studies to employees and animal welfare compliance bodies at the institution who may react negatively to these events. By employing methods that ensure significant toxicity is clearly defined and readily measured, subsequent studies using greater numbers of animals can be designed with more humane endpoints, and the overall level of pain or distress involved with the development program will be minimized.

Refinement alternatives reduce the potential for pain or distress of animals that are on study. To justify that a refinement cannot be used, there needs to be scientific evidence that it will interfere with the goals of a study.[11–13] As time has passed since the initial development of animal welfare regulations and standards, expectations regarding compliance have also evolved. At one time, ethical review bodies accepted a justification for an exception if there was a theoretical risk that a refinement procedure would interfere with the goals of a study. However, as evidence has accumulated to show what types of procedures actually do interfere with research goals, and the conditions under which they can do so, the expectation has evolved that exceptions to these procedures be supported by data rather than theory. This is the purpose of the requirement for a literature review for alternatives and with the current availability of electronic databases and publications, the ease of performing this review is greater than it was only a few years ago. However, refinement data may not be available in the literature. Outside of publications specifically focused on alternative models, most publications emphasize the data derived from a model rather than animal welfare aspects of the model. This means that in order to scientifically evaluate whether a particular refinement will interfere with study goals, the scientist may need to generate the data themselves.[12,15]

Collection of data on experimental refinements is not traditionally considered a business goal for research institutions and scientists, therefore, appropriate allocation of time and resources are not always available for this purpose. However, from a legal standpoint, it is a scientist's responsibility to demonstrate that alternatives are not available and institutions have a responsibility to support all aspects of their research program.[11,12] Therefore, strategies must be developed to obtain these data with minimal impact on resources. Pilot studies are often useful for this purpose. Pilot studies are often run in the course of development of new models and methods, and in the early developmental stages for new drugs and devices. Additional data may be collected from these studies for assessment of animal welfare endpoints or to assess the impact of animal welfare procedures on research endpoints.[25] This type of approach may also be applied when training new personnel on standardized models. Ethical review bodies should be willing to approve the use of positive control articles along with these activities, while setting the animal welfare procedure as the independent variable to determine its impact on study data. Likewise, the effects of procedures intended to improve animal welfare can be assessed through assessment of measures of well-being, such as animal behaviors, activity, physiologic state, and endocrine homeostasis that have previously been shown to be associated with pain or distress.[12,26]

Institutional Responsibilities for an Animal Care Program

The myriad requirements for the humane use of animals in research often necessitate the development of an institutional infrastructure and employment of staff whose jobs are dedicated to meeting these requirements. The exact requirements for an animal care program not only vary by country but also by the particular regulatory or oversight body. There can be some flexibility in program requirements, allowing institutions to design a program to best meet their needs; however, most programs are very similar and have several similar characteristics to their structure and the roles and responsibilities of staff. The following sections define the basic structure of an animal care and use program, including management, compliance, veterinary care, research staff, husbandry, and facility management.

Management

Institutions are responsible for ensuring the availability of adequate resources to meet animal welfare requirements, and typically a specific individual at a relatively senior management level is assigned this responsibility. In the United States, this person is termed the institutional official (IO) for the animal care program. There are specific legal requirements for the IO. The IO must be the chief executive officer (CEO) of a company, or she or he may be another leader who can legally commit resources to the animal care program and represent the institution when making commitments to outside agencies or oversight organizations.[11–13] It is also the responsibility of the managers in all departments or business units at the institution for implementing aspects of the animal care program within their areas, not only at the direction of the IO but also in regard to basic animal ethics and welfare practices.

Compliance Oversight

In the United States, emphasis is placed on self-monitoring for compliance with regulatory and animal welfare standards. Therefore, the organizational level and structure of the compliance group, its resources and authority, and strong evidence it can and is doing its job will be a focus of regulatory or other oversight organizations' audits of an animal care program. In the United States, it is the Institutional Animal Care and Use Committee (IACUC) that is responsible for compliance processes such as the review and approval of individual research activities, establishing policies and procedures,

monitoring research activities for compliance, inspecting facilities, investigation concerns about animal welfare, creating corrective and preventative action plans where there are deficiencies, advising management and the IO on resources needed to manage the animal care program, and reporting on the status of the animal care program to regulatory and oversight organizations.[11–13] At some institutions and under some regulatory systems, there is separation between evaluation of animal use for ethical considerations and review of the scientific justification for the work being conducted.

IACUCs are designed to provide diverse inputs on research proposals, as balancing scientific and ethical needs is often a matter of judgment, and it is important to ensure as much bias of judgment as possible is eliminated. The majority of the membership of an IACUC should be scientific staff.[12] An ideal IACUC membership would include representatives from all scientific areas at the institution to provide appropriate scientific expertise when reviewing research proposals. U.S. regulations also require membership to include at least one veterinarian, one nonscientific member, and one person who is not affiliated with the institution to represent the perspective of the general public.[11–13] Controlling for overrepresentation by a single business area at the institution is also required and needed to prevent conflicts of interest when reviewing and approving proposals.[11]

Veterinary Care

Animal care programs are required to have one or more veterinarians with training or experience working with research animals.[11–13] If an institution is not large enough to require a full-time Attending Veterinarian, they may use a consultant on a part-time basis as long as there is regular interaction between the veterinarian and the facility staff. Professional board certification is provided by the American College of Laboratory Animal Medicine (ACLAM) and the European College of Laboratory Animal Medicine (ECLAM). In the United States, generally, it is legal for any veterinarian to serve as the Attending Veterinarian, even if they are not licensed in the state where they work. Research animals are owned by the institution and veterinary care provided to them does not constitute the *practice* of veterinary medicine as regulated by the state. However, unlicensed veterinarians will be limited in the activities they can perform, such as signing health certificates for interstate shipping of animals, and access to prescription drugs and controlled substances, unless they are able to operate under a researcher's license.

Institutions may also employ veterinary technicians or nurses to support the veterinarian. Veterinary technicians may also be certified or licensed by the state or they may be personnel with appropriate training and experience. Professional organizations like the American Association for Laboratory Animal Science (AALAS) provide training and certification for technicians to provide medical care. Veterinary technicians are also not constrained by state veterinary practice acts when they work in a research institution and are able to diagnose and treat animals, and perform surgery without direct oversight by a veterinarian. Thus, there can be operational efficiency by employing several veterinary technicians who are supervised by a veterinarian. It is important to note, however, that animal welfare regulations and standards require evidence of oversight by a veterinarian for all veterinary care provided, for example, through the use of veterinarian-approved standard operating procedures, or receiving veterinary approval for treatment plans.[12]

Research Staff

There are often several levels of research staff in an institution including senior, junior, and technical personnel. The senior scientific staff can be Principal Investigators or study directors, and are primarily responsible for the design of studies, oversight and decision making, interpretation of data, and writing a report or publication on the data. These staff members normally have training in independent research gained through an MS or PhD program, or they may have gained research experience by working in the field for a period of time. Junior-level scientists in training or who have an undergraduate degree may also be employed, but people in this role usually do not perform research independently. Junior scientists often work under the supervision of a senior scientist and perform some of the same roles. They may be more involved with the daily conduct of the study and also perform study procedures, particularly at a smaller laboratory. The final level of research staff is the technical personnel who perform the study procedures. Large institutions may have a dedicated professional technical staff to perform these activities, while smaller laboratories may have relatively few dedicated technicians working for each scientist, with much work conducted by the junior scientific staff or trainees.

Different levels of research staff may require different training and qualifications to perform their jobs and meet animal welfare requirements. Staff involved in study design, decision making, and data interpretation need to be trained on approval requirements for research using animals, on scientific justification for animal use and alternatives, on institutional policies regarding procedures that may or may not be used, or the guidelines under which they can be used, and on humane endpoints. Personnel performing actual study procedures need to know all of these topics as well, but only at a general level of understanding. It is most critical for staff who perform study procedures to be trained and competent to handle animals, perform technical procedures for test article administration, surgery, anesthesia, analgesia, biological sample collection, recognition of pain or distress, and procedures for humane euthanasia.[11–13] Organizations like AALAS provide training and certification for these procedures, but it is common that institutions develop their own training programs to meet the specific needs of the research they perform.

Animal Husbandry

Institutions must also provide daily care to animals in the way of monitoring, provision of feed and water, cleaning, and sanitation.[11–13] Larger institutions often have a centralized service to provide animal husbandry and may charge a

per diem or flat cost to cover these overhead costs. Smaller laboratories may delegate husbandry tasks to the technical research personnel. Training requirements for husbandry personnel are increasing compared with the needs in the past. Although husbandry personnel may not need advanced scientific training to perform their jobs, they must be aware of animal welfare requirements and have technical training for the procedures they perform. The specific nature of animal welfare standards necessitates having personnel who can attend to the details of animal care as well as ensure that they are not adversely impacting the research being conducted. Animal facilities are becoming increasingly more automated and computerized, but attention to animal care and knowledge of animal welfare is still a very important aspect of husbandry. AALAS has training programs for animal husbandry procedure and animal facility management.

Facility Management

Animal facilities have a number of specialized features that are discussed later in this chapter, such as advance heating, ventilation, and air conditioning (HVAC), and containment of hazardous agents. These features necessitate having a dedicated and skilled engineering and maintenance staff who can properly design facilities that meet animal welfare requirements and keep them operating.[12] It may not be practical for a small facility to maintain a full-time maintenance staff, in which case it is critical for outside service providers to be available and appropriately qualified or experienced in the needs of research animal facilities. As expertise in research animal facility management is not always easy to find, experts from related fields, such as the agriculture industry, may need to be consulted.

ANIMAL WELFARE REGULATIONS AND STANDARDS

Resources for animal welfare standards and regulations are summarized in Table 20.2. The emphasis of this chapter is on standards for the United States. The reader should consult regulations and standards for each country in which they work, but the general concepts discussed in this chapter are relevant internationally. Europe has consolidated standards through the European Union,[27] but each country still has its own authority for enforcement with specific procedures.

United States Department of Agriculture

The United States Department of Agriculture (USDA) has authority to regulate animals used in research through the Animal Welfare Act of 1966 and as amended in 1970, 1976,

TABLE 20.2
Selected Agencies and Organizations for Research Animal Use Regulations and Standards

Country	Agency or Organization	Regulation or Standard/Web Address
Global	Association for the Assessment and Accreditation of Laboratory Animal Care, International	*Guide for the Care and Use of Laboratory Animals*: http://www.nap.edu/catalog.php?record_id=12910
		Guide for the Care and Use of Agricultural Animals in Agricultural Research: http://www.fass.org/docs/agguide3rd/Ag_Guide_3rd_ed.pdf
		Reference Resources List: http://www.aaalac.org/accreditation/resources.cfm
		Position Statements: http://www.aaalac.org/index.cfm
United States	OLAW, NIH	PHS Policy on Humane Care and Use of Laboratory Animals
		Guide for the Care and Use of Laboratory Animals
		http://grants.nih.gov/grants/olaw/olaw.htm
	USDA, APHIS	Animal Welfare Act
		CFR 9, Animal Welfare
		Policy Manual
		Inspectors Training Manuals
		http://www.aphis.usda.gov/animal_welfare/index.shtml
Canada	Canadian Council on Animal Care	http://www.ccac.ca
Europe	European Union	ETS 123: http://conventions.coe.int/Treaty/en/Treaties/html/123.htm
		Appendix A: http://conventions.coe.int/Treaty/EN/Treaties/PDF/123-Arev.pdf
		Directive 2010/63/EU: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:En:PDF
		Directive 86/609/EEC: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31986L0609:EN:HTML
Other countries		Refer to links at the following sites:
		AAALAC Int.: http://www.aaalac.org/resources/internationalregs.cfm
		FRAME: http://www.frame.org.uk/page.php?pg_id=154
		Michigan State University Animal Legal and Historical Center: http://www.animallaw.info/nonus/

1985, and 1990. The act is implemented through the Animal Care section of the Animal and Plant Health Inspection Service (APHIS) division of USDA. The USDA animal welfare regulations cover warm-blooded animals used in research but exclude rats of the genus *Rattus*, mice of the genus *Mus*, birds bred for research, and livestock used in research related to food or fiber production. If livestock are used for biomedical research, they are regulated by the USDA. These regulations also cover the breeding of these species for research, as well as some non-research-related activities, such as commercial breeding, transportation, brokering, and exhibition of animals. Institutions performing these activities must be registered or licensed with the USDA and are inspected at least annually by veterinary inspectors from Animal Care–APHIS–USDA for compliance with the regulations. Inspectors can issue citations leading to warning letters, fines, or even suspension of an institution's research registration.[11]

The USDA animal welfare regulations cover many of the same areas as other regulations that are discussed in the section on standards. However, there are some unique aspects to the regulations, such as composition of the IACUC, record keeping, cage space, cleaning and sanitation, and reporting requirements of research institutions. Many of the USDA regulations are very specific, establishing engineering standards for animal welfare requiring relatively rigid compliance. In addition to these regulations, there are a number of administrative *policies* created by the USDA and published on their website that represent interpretation of sections of the regulations that are not specific. Another useful document on the Animal Care website to help research institutions in understanding USDA regulatory requirements is the inspectors' manual that is used as a training guide by Animal Care personnel.

Public Health Service

The U.S. Public Health Service (PHS) has authority for animal research under the Health Research Extension Act of 1985 that created the PHS Policy on the Humane Care and Use of Laboratory Animals. This policy covers all vertebrate animals used in research funded by the PHS (e.g., National Institutes of Health or NIH), and institutions must have an Assurance of Compliance on file with the Office of Laboratory Animal Welfare (OLAW) within the NIH to be eligible for this funding.[13] The PHS Policy primarily details administrative requirements for compliance and refers to two other documents for standards of compliance. The first is the *U.S. Government Principles for the Use of Animals in Research*. This document lists nine basic concepts for research animal use. The second document is the *Guide for the Care and Use of Laboratory Animals*—a more detailed document of standards to be discussed later.[12] Both of these documents establish performance standards for animal use rather than the engineering standard approach reflected in the USDA regulations. Also unlike USDA, OLAW does not routinely inspect research facilities. Instead, there is significant reliance on self-reporting of gaps in compliance with the policy and associated standards. However, if there is continuing or severe noncompliance, OLAW may form a team to inspect an institution and, if the inspection results in findings, may withhold funding for research.

Good Laboratory Practices

The good laboratory practice (GLP) standards created by the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) primarily establish requirements for the planning, conduct, and reporting of research results used to support registration of drugs, vaccines, medical devices, and chemicals. However, the GLPs impact animal research in several ways. First, for many bioassays, the animal is the test system and all aspects of the care and use of the animals must be defined and documented to ensure data integrity.[21] In many cases, procedures that ensure data integrity are also procedures that support the welfare of the animals and typically this does not result in any conflicts between the different regulating bodies. However, specific compliance procedures and compliance philosophy between GLP inspectors, and animal welfare inspectors or auditors can be different. It may seem that GLP inspectors are more detail oriented and *stricter* than animal welfare auditors, but the primary difference is in the details that are relevant to each set of regulations and standards.

The second area of impact for the GLPs on animal welfare standards is with authority for making decisions. Animal welfare standards focus on the role of the IACUC and the veterinary staff as the primary authorities for decision making around study designs and treatment for animals on study, while the GLPs establish the study director as the single point of control for conduct of a study. Legally, there is not a conflict between these seemingly disparate requirements. The USDA, OLAW, and FDA have established a Memorandum of Understanding between the agencies that they will support the compliance missions of one another.[28] To make this arrangement work in practice, it is important to understand where the responsibilities of these three groups lie. The study director is the point of control for study conduct; however, the study design, procedures, and endpoint must first be approved by the IACUC, and any scientific justifications provided by the study director to deviate from animal welfare standards or regulations need to be evaluated to ensure that they are supported by data. The veterinarian has authority for decision making regarding veterinary care when study animals are experiencing pain or distress that has not been approved by the IACUC. It is the veterinarian's responsibility to interpret what constitutes pain or distress, to discuss with the study director whether there are treatment options that will not interfere with the goals of the study and if there are not, to recommend euthanasia. The study director is responsible for following the veterinarian's recommendations, or applying to the IACUC for a change in the scope of the study. The complexity of this arrangement makes good documentation very important, which is why the IACUC requires a written

description of the goals, procedures, potential for pain or distress, and detailed criteria for veterinary care or euthanasia in order to approve a study.

U.S. Government Research Institutions

Intramural research performed by the federal government using animals as well as research funded by these agencies is regulated in much the same way as with public and private institutions. Federal agencies such as the Department of Defense,[29] the Veterans Administration,[30] the NIH,[31] the National Aeronautics and Space Administration,[32] and the National Science Foundation[33] all have policies and procedures for the humane care and use of research animals but, in all cases, point to the *U.S. Government Principles*, the *Guide for the Care and Use of Laboratory Animals*, and the USDA animal welfare regulations for their standards.

State Regulations

Many U.S. states specifically exempt legitimate research from animal cruelty or humane laws and from Veterinary Practice Acts. However, not all do and facility managers and laboratory animal veterinarians should consult with local laws. Some state regulations are relevant even at research institutions. Requirements designed to control animal diseases requiring certain disease testing, vaccinations, and reporting are normally applicable, as are pharmacy and controlled substances regulations.

AAALAC International

The Association for the Accreditation and Assessment of Laboratory Animal Care, International (AAALAC Int.) is an accreditation organization for research institutions using animals that has evolved from its organization in 1965 as a U.S.-based organization promoting animal care standards to its current status as an international organization providing accreditation of animal care programs. AAALAC Int. is generally recognized as being the industry gold standard even though there is no regulatory requirement for an institution to be accredited. Accreditation, however, may be required by agency or institutional policies, may engender different requirements for periodic inspection or auditing by regulatory agencies or sponsor institutions, and may be perceived as a competitive advantage for commercial institutions or for researchers seeking grants. Institutions pay an annual fee for the cost of accreditation, including administrative fees and site visits. Sites are visited or audited every 3 years.

AAALAC Int. uses the *Guide* as its primary standards document, but also holds compliance with applicable law and regulations as a basic requirement for meeting accreditation standards. AAALAC Int. also maintains a list of policies on its website clarifying the agency's interpretation of certain aspects of the *Guide* and other standards. The AAALAC Int. accreditation requires a written description of the animal care and use program be submitted, followed by an on-site visit from a team of experienced site visitors who are industry peers. The site visit is followed by an exit interview where the visitors present a summary of their observations and the recommendations they will make to the AAALAC Int. Council on Accreditation. The council meets three times a year and the final outcome of the site visit is not determined until after this meeting. Institutions may achieve full accreditation with or without suggestions for improvement of the program. Institutions may also receive Conditional Accreditation, where there are certain mandatory deficiencies to be addressed to achieve full accreditation. It may be possible for AAALAC Int. to determine conditions have been met through a written plan from the institution, or it may be necessary for a follow-up visit to the institution. AAALAC Int. may also withhold accreditation if there are significant gaps between standards and conditions at the institution, or if the council doubts the plan of correction from the institution is likely to succeed in addressing deficiencies.

AAALAC Int. site visitors come from a diverse background, which allows for a strenuous peer review process for accreditation. However, this diversity can also be a challenge for institutions, since AAALAC Int. site visitors bring their collective experiences with them when they evaluate a program and compare what they are seeing with best practices throughout the industry. This creates a high bar for the institution's program and also creates somewhat of a moving target for meeting accreditation standards, due to inherent variability among different site visitors. Subsequent site visits can result in suggestions for improvement in areas previously found to be acceptable even without changes in regulations or standards. In the author's experience, institutions should be open in their communication with AAALAC Int. site visitors to clarify expectations and share ideas on how to achieve those expectations. Sometimes a site visitor's comments are based on a limited view of a situation or comments may be misinterpreted by the institution. These situations can be clarified with additional communication at the time of, or following the visit and the process of clarification can be extremely beneficial for establishing a quality animal care program. By contrast, if issues are not addressed but are commented on and require repeat visits, they are likely to move from a suggestion for improvement to a deficiency, a mandatory requirement for maintenance of accreditation. Addressing a comment by AAALAC Int. Council does not necessarily mean significant changes need to be made to the program. It means that the idea will be objectively evaluated for its applicability to the program. If changes are not made, there is an expectation that the performance standard prompting the original comment or suggestion be fully met by one method or another.

For additional information, refer to www.aaalac.org.

Guide for the Care and Use of Laboratory Animals

The Guide, as it is commonly termed, is a publication of the Institute of Laboratory Animal Research (ILAR) of the National Research Council that provides a comprehensive summary of standards for the care and use of vertebrate

animals used in research.[12] *The Guide*, now in its eighth edition, was originally published by the NIH in 1963 and was revised most recently in 2011. It is written by a committee representing the laboratory animal medicine and biomedical research communities and each revision has incorporated the most current ideas and research on methods for ensuring optimal animal welfare and minimization of variables affecting animal models. As previously indicated, *the Guide* is the standard reference for all regulatory agencies, except the USDA, and is also the standard guideline document for AAALAC Int. Even USDA refers to *the Guide* within their policies to establish standards for procedures not covered by their regulations. The most recent revision of *the Guide* incorporates standards and language to make it more applicable internationally,[12] which reflects the globalization of the field of biomedical research and commerce.

The Guide is written primarily as a set of principles that are supported by citations from the peer-reviewed literature. As such it establishes performance standards for animal welfare that allow institutions a certain amount of flexibility in establishing procedures to meet these standards. As would be expected, the regulated community has evolved a certain *standard of practice* with common policies and procedures, and there is a certain amount of peer pressure from regulatory and oversight organizations to follow these procedures. However, if researchers and institutions are able and willing to evaluate their procedures using the performance standards outlined in *the Guide*, IACUCs may approve modifications to standard procedures that are considered compliant.[12] This is very unlike the USDA animal welfare regulations that tend to create much more rigid standards. There are some standards in *the Guide* that are equally inflexible. The verbiage of the document uses the term *must* to indicate items that are considered mandatory for a good animal care program, whereas the terms *should* and *may* precede most of the standards.[12] It is important for institutions to pay attention to these terms as the nature of the term to a large extent dictates how the issues will be viewed during an inspection or site visit.

Agricultural Guide

The Guide for the Care and Use of Agricultural Animals in Agricultural Research is published by the Federation of Animal Science Societies and is used by most regulatory and oversight organizations to establish standards for common agricultural research involving food or fiber production animal.[34] The *Ag Guide*, as it is normally referred to, may also be referenced when using agricultural animals for biomedical research in agricultural settings. Like the ILAR *Guide*, the *Ag Guide* is also written to describe performance standards, and the information within it can be applied to an animal care program in much the same way as that in *the Guide*.

Animal Transportation

There are a variety of regulations to consider when transporting animals or animal tissues or blood domestically and internationally. *The Guide*, the *Ag Guide*, and the USDA animal welfare regulations all define transportation standards.[11–13] These standards include size and construction of shipping containers, environmental conditions, and care and monitoring of animals during transport. For an international shipment, the International Air Transport Association (IATA) Live Animal Regulations document establishes guidelines for commercial carriers.[35]

Regulation of transportation of nonhuman primates, as well as tissues or blood from these animals for international shipment, is performed by the U.S. Fish and Wildlife Service through the Convention on International Trade in Endangered Species (CITES), an international treaty to prevent illegal trade of endangered species. CITES requires any international shipments of endangered animals, blood, or tissues be accompanied by a CITES permit number.[36] Animal importers typically provide the permit number with animals when they are purchased. If a research institution ships these animals or samples from these animals internationally, a new permit must be obtained, but the original permit information is required to get the new permit. Therefore, institutions need to maintain records on this information and also need to plan ahead prior to shipping samples internationally, as CITES permits take several weeks or more to obtain.

The Centers for Disease Control (CDC) regulates quarantine requirements for the importation of nonhuman primates for the purpose of prevention of introduction of diseases, such as tuberculosis, monkey pox, and filoviruses (e.g., Ebola, Marburg). Quarantine facilities must be approved through the CDC and must meet their requirements for isolation housing, monitoring, and testing.[37]

AVMA Panel on Euthanasia

The American Veterinary Medical Association (AVMA) publishes a set of recommendations on humane methods of euthanasia widely cited by animal welfare regulations and the *Guide*. Methods for euthanasia must not cause pain or distress to animals and must provide a rapid and peaceful death. Methods are classified as *approved*, *conditional*, and *unacceptable*. Approved methods are recommended for routine use, while unacceptable methods should never be used. Conditional methods require scientific justification for use, as pain or distress to the animals may occur if performed improperly, or they may be unaesthetic, and their use is discouraged due to impact on personnel and other animals that may be exposed to the procedure.[38]

Professional Organizations

Many professional associations have established their own position statements or standards for the humane care and use of animals (Table 20.3). For example, the American Society of Mammalogists (ASM) has recommendations for field euthanasia of wildlife not found elsewhere in the literature. In addition, position statements by organizations such as the AVMA, AALAS, and ACLAM provide professional

TABLE 20.3
Selected Organizations with Humane Animal Use Guidelines

Organization	Documents
Academy of Surgical Research (ASR)	Certification study guide: http://www.surgicalresearch.org/downloads/ASR_Certification_Study_Guide.pdf
American Association for Laboratory Animal Science (AALAS)	Position statements: • Humane care and use of laboratory animals • Alleviating pain and distress • The scientific basis for regulation of animal care and use • Performance-based criteria as the basis for determining laboratory animal housing standards • Standards for assessing the quality of laboratory rodents • Health care for genetically altered rodents • Infrastructural support for animal-based research • Use of animals in precollege education • Animal rights terrorism http://www.aalas.org/association/position_statements.aspx
American College of Laboratory Animal Medicine (ACLAM)	Position statements: • Adequate veterinary care • Animal experimentation • Animal use in research, testing and teaching • Assessment and management of pain in rodents and rabbits • Guidance document on evaluation of laboratory animal care standards • Guidance document on adequate rodent cage sanitation and sterilization • Pain and distress • Rodent surgery • The three Rs • Training and education in use of animals • Value of AAALAC accreditation • Veterinary medical records • Academics and research committee report • Report on the ACLAM task force on rodent euthanasia August 2005 http://www.aclam.org/education-and-training/position-statements-and-reports
American College of Toxicology	Position statement on the use of animals in toxicology: http://www.actox.org/StragegicObjectiveOnAnimalUse/
American Medical Association (AMA)	H-460.985 Support for Use of Animals in Teaching, Product Safety Testing and Research: http://www.ama-assn.org
American Physiologic Society (APS)	Resource book for the design of animal exercise protocols: http://www.the-aps.org/mm/SciencePolicy/Animal-Research/Publications/Animal-Exercise-Protocols.aspx Animal Research Position Statements: http://www.the-aps.org/mm/SciencePolicy/Animal-Research
AVMA	Policies: http://www.avma.org/issues/animal_welfare/policies.asp • Safety testing • USDA/APHIS animal welfare program • Use of animals in precollege education • Use of animals in research, testing, and education • Use of random-source dogs and cats for research, testing, and education Guidelines on euthanasia: http://www.avma.org/issues/animal_welfare/euthanasia.pdf
OLAW—NIH	Enrichment resources, experimental techniques, safety: http://grants.nih.gov/grants/olaw/request_publications.htm Training videos: http://grants.nih.gov/grants/olaw/TrainingVideos.htm
American Society of Mammalogists (ASM)	Guidelines for the use of wild mammals in research: http://www.mammalsociety.org/uploads/Sikes%20et%20al%202011.pdf
The Federation of American Societies for Experimental Biology (FASEB)	Statement of principles for the use of animals in research and education: http://www.faseb.org/Policy-and-Government-Affairs/Science-Policy-Issues/Animals-in-Research-and-Education/Statement-of-Principles.aspx
Society for Neuroscience (SFN)	Statement on the use of animals in biomedical research: http://www.sfn.org/index.aspx?pagename=gpa_AnimalsinResearch_GlobalStatement
Society of Toxicology (SOT)	Guiding principles in the use of animals in toxicology: http://www.toxicology.org/ms/air6.asp
Universities Federation for Animal Welfare (UFAW)	UFAW and animal welfare: http://www.ufaw.org.uk/animal-welfare.php

expectations for laboratory animal veterinarians and research institutions to follow. While most professional association standards closely parallel standards within the *Guide* or the *U.S. Government Principles*, these additional standards can aid and impact the interpretation of *Guide* or government regulations and standards.

Occupational Health and Safety

Historically, worker protection agencies like the Occupational Safety and Health Administration (OSHA) did not specifically address occupational hazard of using animals for research. Therefore, animal welfare regulatory and oversight organizations also established standards for personnel safety. While OSHA currently addresses these issues, OLAW and AAALAC Int. monitor facilities to ensure that the programs are compliant.[12,13] Occupational hazards of research involving animals include risk of allergies, bites, scratches and other injuries, zoonotic diseases, or biological agents and chemical or radiation exposure from test materials administered to the animals.[1] The *Guide for Occupational Health and Safety in Animal Research Facilities* is a good resource for details on methods for assessing risk and preventing exposure to hazards.[39] Institutions may manage these programs through a separate health and safety group or the IACUC may assume these responsibilities. In addition to the previously mentioned resources, for research proposals using infectious agents, toxins, and recombinant DNA, approval is needed from a Biosafety Committee.

Diseases that personnel can contract from research animals are termed *zoonotic*. A list of selected zoonotic diseases is provided in Table 20.4. Animals should be tested for common zoonotic diseases and these should be excluded from the facility whenever possible. If it is not possible to exclude a zoonotic disease, protection should be provided through the use of protective clothing, shoe covers, and other personal protective equipment (PPE), such as gloves, safety glasses, goggles, face shields, masks, and respirators. However, some diseases may require additional procedures following exposure to an animal with the disease. For example, if a staff member is bitten, is scratched, receives a puncture from a needle contaminated with macaque body fluids, or receives a splash of body fluids onto a mucous membrane from a macaque nonhuman primate or from a biological sample collected from a macaque, the person and the animal need to be tested for Cercopithecine herpesvirus-I (herpes B virus).[39] If the animal is still alive when the exposure occurs, blood must be collected from the animal and the mucous membranes swabbed and examined for evidence of infection. The animal may be anesthetized for collections. However, if the animal has been euthanized and the exposure occurs from a biological sample (blood, urine, etc.) from the animal, an additional aliquot of blood may be needed for testing. Some institutions bank a serum sample from each animal for this purpose. Other exposure prevention programs can include vaccinations against tetanus and even rabies.[39]

Another safety issue is laboratory animal allergy. Allergies to animals typically result from exposure to urinary proteins, hair, and dander. However, allergies to other substances in the research environment such as latex, antibiotics, and even feed and bedding products may also be risks. The seriousness of allergies can range from minor to life threatening. Therefore, personnel with a history of allergies should disclose this to the occupational health provider so appropriate prevention measures can be taken, such as additional PPE, regular monitoring, or assignment to tasks with lower risk of exposure.[12,39]

Other common occupational health and safety programs include hazard identification, hazard labeling requirements,

TABLE 20.4
Selected Zoonotic Diseases in Laboratory Animals[39]

Species	Diseases/Organisms
Rodents and rabbits	Bubonic plague, cryptosporidiosis, dermatophytosis, dwarf tapeworm, giardiasis, Hanta virus, leptospirosis, lymphocytic choriomeningitis, rat bite fever, salmonellosis, sporotrichosis, yersiniosis
Dogs and cats	Brucellosis, campylobacteriosis, cat scratch fever, cryptosporidiosis, dermatophytosis, giardiasis, listeriosis, nematodiasis (larval migrans), rabies, salmonellosis, sporotrichosis, toxoplasmosis, yersiniosis
Nonhuman primates	Amebiasis, balantidiasis, campylobacteriosis, Cercopithecine herpesvirus-I (B virus), cryptosporidiosis, giardiasis, nematodiasis, rabies, salmonellosis, shigellosis, simian immunodeficiency virus, tuberculosis, viral hepatitis (A, B, C), yersiniosis
Swine	Balantidiasis, brucellosis, campylobacteriosis, cryptosporidiosis, influenza, leptospirosis, listeriosis, salmonellosis, yersiniosis
Sheep and goats	Brucellosis, campylobacteriosis, contagious ecthyma, cryptosporidiosis, dermatophytosis, leptospirosis, listeriosis, Q fever, sporotrichosis, yersiniosis
Cattle	Brucellosis, campylobacteriosis, cryptosporidiosis, dermatophytosis, leptospirosis, listeriosis, salmonellosis, sporotrichosis, yersiniosis
Amphibians and fish	Mycobacteriosis
Birds	Influenza, Newcastle disease, psittacosis, histoplasmosis, salmonellosis, yersiniosis
Reptiles	Salmonellosis, yersiniosis

Source: Institute for Laboratory Animal Research, National Research Council, *Occupational Health and Safety in the Care and Use of Research Animals*, National Academies Press, Washington, DC, 1997.

material safety data sheet distribution and training, ergonomic programs, slip, trip and fall prevention, and management of eyewash stations and safety showers.[12,39]

Associated with personnel safety is environmental safety. Research facilities create a variety of environmental hazards to address when planning and conducting research. Myriad local, state, and federal regulations impact waste management and disposal through regulations for solid waste streams, chemical waste, sewage disposal, or incineration. In addition, facility construction and management are affected by building and fire code requirements. Animal facility managers need to become familiar with these requirements or engage experts in environmental management.

Emergency Management and Business Continuity

It is a requirement within the animal welfare standards to have a plan for responding to and recovering from emergencies.[12] These plans need to address the ways in which animal husbandry and research activities will be accomplished and veterinary care provided in the event of interruptions of power or water, staffing shortages, or interruptions in supply chain due to equipment failures, severe weather, natural disasters, fires, criminal activity, civil unrest, or epidemic disease. There is emphasis on monitoring and prevention, staff training, drills, tabletop exercises, emergency staffing plans, emergency power supply, reserve sources of feed and water, and plans for emergency care, evacuation, or euthanasia of animals to prevent suffering.[12,40]

COMPLIANCE PROCEDURES

IACUC and Ethical Review Boards

As previously discussed, the IACUC or other ethical review board must review and approve all animal use activities before they occur. There are some useful strategies for managing IACUC review and approval in a drug development environment. First, it is important to understand the difference in approach between animal welfare regulatory and oversight organizations and GLP compliance agencies. As described previously, animal welfare compliance requires the input of multiple parties (scientists, veterinarians, and the IACUC), and, therefore, can require more time than is needed for approval of a study protocol or study amendments. The second major difference is that animal welfare compliance focuses on the animals and not on the integrity of and ability to reconstruct study data as with GLP compliance.

It is critical for institutions to plan for the time required for animal welfare review of proposals, particularly in a toxicology facility where studies may need to be started quickly to meet drug development timelines, and where scientists are accustomed to being the single point of control for all other aspects of study planning and conduct. The general process for review is relatively standardized between oversight bodies within the United States. International regulations may vary in their details, but there are a number of common themes.[41] If the proposed study has the potential to result in pain or distress for the animal, a veterinarian must be consulted on methods to prevent or relieve pain or distress. Concurrent with this review, or subsequent to it, the full IACUC must be given the opportunity to review the proposal at a convened meeting of the committee, where it can be approved by a majority of the quorum of the committee, or alternatively the proposal can be distributed to committee members, and after a period of time established by the committee (if no member has requested review by the entire committee), review and approval can be delegated to an individual committee member.[11–13,40] Standard review times vary by the institution, but a minimum of a week should be expected, and in some institutions, reviews can take months. IACUCs do have the ability to expedite reviews, but because review still requires the entire committee to be involved, expediting protocol reviews is extremely disruptive and its use should be limited to extremely urgent situations.

The focus on animal welfare versus study data manifests itself in a slightly different way. Animal welfare review requires justification for the use of the animal model, the value of the research to society, animal numbers, potential for pain or distress, and the plan for preventing or relieving pain or distress.[11–13,40] As this is an ethical review and not a scientific review, not all reviewers of these proposals have a scientific background. Scientists need to limit their use of jargon and abbreviations, or define them in the text. They cannot assume that the reader understands the inherent benefits of the specific molecule being tested, the limitations of alternative systems, or how a particular study is conducted. It is not usually necessary to provide a high level of detail in the justifications, just to walk the reviewers through these issues in a clear and logical way, addressing the specific questions in the protocol, and referring to literature where appropriate.

The other way in which the focus on the animal is different from the focus on the study is in the level of detail required for review of study procedures. Because IACUC review is looking for impact on the animals, some of the procedural details important for a study protocol are not as important for the IACUC. In fact, many institutions allow a proposal to reference approved policies or guidelines giving ranges for parameters such as dose or blood volumes, fasting times, approved anesthetic or analgesic drugs, and even entire standard operating procedures. The IACUC still needs to understand all proposed procedures to be performed on animals and the timeline for when they will be performed, but if the details of the procedure are within the scope of standards the IACUC has reviewed and approved as being appropriate and incorporating all relevant refinements, additional detail may not be required. This allows institutions to start the more lengthy IACUC review process before all of the study details have been finalized and the protocol signed. As long as any final changes to the study do not diverge from the ranges in the IACUC proposal, additional IACUC review should not be needed. Some institutions have taken this approach to another level by maintaining *blanket* IACUC approvals for standardized study designs and procedures and running multiple individual studies under a single IACUC approval. This can work

well if studies are highly standardized, but there is risk of noncompliance if the IACUC approval is too generic to adequately address the goals of the study, the potential for pain or distress, the justifications for alternatives, and the plan to prevent or relieve pain or distress in the animals.

IACUC review can result in one of three outcomes: (1) approval of the study and procedures, (2) modifications to the proposal may be requested in order to obtain approval, and (3) the IACUC may reject a proposal.[11–13,40] In a well-functioning animal care program, rejection of a proposal is rare. However, in the case the proposal is rejected, the IACUC can work with the scientist to make appropriate modifications to the proposal so that it can be approved. In many cases, changes to the proposed procedures are not needed, but further clarification on justifications, the availability of alternatives, or a plan for veterinary care or euthanasia may be required to secure approval. Likewise, it is uncommon for proposals to be approved without any questions or requests for clarifications for the IACUC to make an appropriate decision. Because ethical review involves application of judgment and opinion and because society's views on animal use continually evolve, it is to be expected questions will arise on particular issues, even if they were not previously questioned.

Personnel Training and Qualifications

As with GLP regulations, animal welfare regulations and standards require documentation of training and qualifications for personnel performing study procedures on animals. In addition, training is required on the content and scope of animal welfare regulations and standards, on IACUC review procedures, on appropriate searches for and use of alternatives, and on veterinary care and humane euthanasia methods.[11–13,40] Facilities in compliance with GLP requirements typically do not have problems meeting animal welfare compliance requirements. The IACUC must simply assure itself that the appropriate training program and documentation are in place and overall responsibility can be delegated to other organizational units already managing these functions. In individual proposals for animal use, the scientist submitting the proposal may reference the institutional training program without providing further detail.

In institutions that do not have the GLP infrastructure, if there is not a comparable program for managing training, the IACUC may have to establish its own procedures. Typically, this will involve establishing training and certification requirements for specific procedures that will be performed, establishing a process for certifying trainers or trainees, or establishing an internal training program to provide appropriate didactic and hands-on training. Training records must be maintained, and the IACUC needs to have a way to link the procedures to be performed by individual staff with documentation of their training when they review an animal use proposal. Training programs can take many forms and further information on establishing a training program is available from other sources.[42,43] Training on animal care and use procedures is also available through professional associations and commercial providers.

Training and qualifications also need to be assessed and maintained for personnel from outside of an institution who provide contract or consulting services, or participate in collaborative research. Institutions may choose to enroll these consulting staff in the institution's training program or accept proof of prior training or qualifications, such as a curriculum vitae.[12]

Inspection, Program Review, and Postapproval Monitoring

IACUCs are required to perform a review of the animal care program and facility inspections at least twice a year.[11–13,40] The program review is a formal process that goes through all of the regulatory requirements and checks for institutional compliance and changes in the program. Inspections need to be performed in all areas of the facility where live animals are taken, as well as direct support areas, such as cage wash and equipment storage areas. Any gaps between regulatory requirements or standards and actual procedures or conditions within the facility need to be identified, and a plan for correction established. Any deficiencies need to be classified as minor or significant, based on whether they represent a direct risk to animal health or welfare, are serious, or are repeat findings. The IACUC submits a report of the results of the semiannual program review and inspections to the IO, along with any recommendations for program improvements or needed resources to ensure compliance.

In addition to semiannual review and inspections, IACUCs are expected to perform postapproval monitoring of studies to ensure compliance with approved procedures.[11–13,40] This monitoring can include review of protocols, data, and visits to the areas of animal use to observe procedures. Noncompliant items are also identified and classified according to impact. If there are serious, ongoing deficiencies identified through inspections, postapproval monitoring, or program review that have not been corrected, IACUCs have the authority to suspend specific research activities or the animal use privileges for a particular scientist.

Animal Welfare Concerns

IACUCs are also required to investigate any reports of concern about animal welfare or noncompliance reported by institutional staff, including those reported anonymously. Staff are protected under federal law from any reprisals by the institution resulting from reporting concerns.[11–13,40]

Reporting

Institutions are required to submit annual reports to regulatory agencies and accreditation organizations in order to provide information on animal usage and any significant changes to the program. There are additional reporting requirements specified by each agency or organization. These typically include requirements for reporting changes in key personnel such as the IO, the Attending Veterinarian,

or the IACUC chair; the addition of new facilities; significant events resulting in animal loss, pain, or distress; or suspension of approved activities by the IACUC. Reporting typically must be performed within a specific amount of time following the identification of an incident. Reports to regulatory agencies can become available to the public through Freedom of Information Act (FOIA) requests.

Documentation

Certain records must be maintained to meet animal welfare compliance requirements. Records typically must be retained for at least 3 years after the final disposition of the animals. Required animal care program records include all IACUC records such as protocols, review and approval documentation, meeting minutes, investigations, inspections, and program reviews and reports. In addition, records of animal receipt, veterinary care, animal disposition, facility monitoring and maintenance, animal husbandry, and room and cage sanitation are also required.[11–13,40] While electronic records are acceptable, records need to be readily available during an inspection, which means an effective business continuity plan for an electronic records system is required.

FACILITIES

Design and Construction

Animal facilities or vivariums need to be designed and built with considerations for access, workflow, and maintenance. Facility access needs to be managed for purposes of security, prevention of animal escape, and to minimize the risk of exposure of personnel to animal diseases, allergens, or hazardous agents used in experiments.[1,11,12,44] Historically, separation between animal areas and office space was not prioritized and many older facilities do not lend themselves to this requirement. Ideally, the vivarium would be completely separated from office or public areas and from other operational areas requiring access by multiple departments, such as laboratories, shipping and receiving, storage, and building maintenance. In addition, laboratories processing animal blood or tissues or preparing test articles for administration to animals should be immediately adjacent to the vivarium to limit the transport of these items through public areas.[1]

For enhanced security, a vivarium should be locked or secured using electronic control mechanisms so only people working in the area and have been properly trained in safety procedures and standards for humane care and use of animals are authorized for entry. The entry should have a place for changing clothing, such as a locker room, or space for donning required PPEs such as laboratory coats, shoe covers, or gloves. There should be appropriate areas for research activities such as procedure rooms, monitoring rooms, surgical areas, necropsy, or imaging within the perimeter of the vivarium so the animals are not transported through public areas to other laboratories.[1,12] It is highly recommended to have dedicated procedural space rather than trying to perform research procedures in animal rooms. Other than basic examinations, dosing, and sample collection, it is not appropriate to perform procedures within an animal room that may cause stress to the animals or to animals observing the procedures. In addition, research equipment maintained in the animal room makes it difficult to maintain the room in a sanitary manner and can contribute to the presence of endemic diseases in animal facilities.[11,12] It can also result in competition for space between different research groups and with husbandry personnel, potentially adversely affecting quality and efficiency.

There may need to be secondary containment within a vivarium to contain hazards associated with particular studies or species of animals.[1,39,44] Nonhuman primates are typically separated from other species of animals because of the extra safety procedures required to work with them. Having a dedicated area with a separate entrance and area for changing clothing or PPE is highly advised. This is required for working with biohazardous agents, and similar separation is also typically required for working with radioisotopes and chemical hazards. It may be necessary to separate studies or animals because of the impact of noise, or because of different environmental or housing conditions required. Surgical facilities also require separation of activities generating possible microbial contamination (animal preparation and recovery) from aseptic areas, including surgery personnel and instrument preparation areas and the operating room itself.[11,12]

Consideration should be given to the amount of flexibility required within a vivarium. It is typically less expensive to design and construct space if the intended use of that space is known and will not change. However, as research programs change and the animals and study types used also change with them, the additional cost of building flexibility into the design of the facility may be less expensive and long term rather than having to completely retrofit an area to accommodate different requirements. Some of these requirements are discussed in the sections on Environmental Conditions, Caging, and Husbandry for different models.

Requirements for regular cleaning and sanitation of animal facilities result in a need for design and construction considerations to support these activities. Particularly when working with larger animals, facilities need to be constructed out of durable materials that can withstand physical damage from heavy equipment and frequent washing with water and disinfectants, and require drains or a method for regular removal of waste. Small animal housing areas may not require as much water washing but still need to be constructed of sanitizable materials and be resistant to physical damage. Other strategies for preventing damage to facilities include having corridors and doorways that are wide enough to allow easy movement of equipment, metal or plastic guards on walls and doors to protect areas commonly struck by caging, and automatic door openers to facilitate movement through corridors.[1,11,12]

Traditional vivarium construction is with concrete block and surfaces are sealed with durable material such as epoxy paint, ceramic, or even metal.[12,45–47] Because of the extremely

high costs of this type of construction and difficulty of modification when facility needs change, new ideas are evolving that utilize nonporous materials like polymers that can be easily set up and modified or repaired with the idea that long-term durability is not as important as flexibility. This concept has been extended to facility infrastructure and supplies of water, power, and supply, and exhaust air may be provided through flexible, semidisposable plastics rather than traditional metal piping and conduits.

Another design issue for a vivarium is the way in which sanitation and waste removal will be performed. Animal cages can be sanitized within an animal room, but this must be done without causing stress or harm to the animals from noise, temperature extremes, or chemical exposure. It is common for daily cleaning and general floor and wall sanitation to be performed with animals in the room, but for the more thorough cleaning of caging and room surfaces, animals are either removed from the room, or clean caging is brought in, animals transferred from soiled caging, and the soiled caging brought to a central area for washing. There are alternative methods for this process that do not necessarily require a centralized washing area but that are scalable for large operations. These can include automated waste removal systems and disposable caging.[12]

Cage washing can be performed by hand using water and disinfectants, but this is very laborious for a large facility. Automated cage washers that use chemicals and hot, pressurized water are an industry standard. Areas for cage washing must be large enough to manage the workload, have high ventilation rates to remove heat and humidity, have supplies of steam or hot water and large drains, and be resistant to the water and chemicals used in the area.[12,46] They are typically divided into a dirty side and clean side. On the dirty side, animal waste may be removed and cages pretreated with chemicals to remove scale. Local area ventilation may be needed to minimize worker's exposure to chemicals, animal allergens, and microorganisms and separate spaces needed to stage soiled cages and to perform preparation activities. On the clean side, there may need to be sufficient space to reassemble caging components, test for complete sanitation, and maintain facility records. Following sanitation, cages may need to be stored before being put into use. Adequate storage space is very important as part of facility design.[12] In the author's experience, this is an area where institutions often cut corners in an attempt to reduce construction costs, but the ongoing labor costs associated with inadequate storage space and the regular need to move equipment around will outweigh the initial savings.

Animal facilities also need to be designed and constructed with redundancy in mind. In the event of equipment failure, there may need to be backup systems for power, ventilation, water supply, and capacity to clean and sanitize if a cage washer unit is inoperable.[12,40]

Because of all of these requirements, the vivarium is often one of the more expensive areas in a research facility. Careful planning is critical. It is important to resist the urge to cut costs by designing inadequate support areas for research procedures, sanitation, and storage as this can cost more money in the long run. Operational efficiency is often overlooked in the design of research facilities but should be considered as labor costs are typically a large expense in any institution's budget and they are ongoing costs, whereas construction costs are eventually depreciated.

Environmental Control and Monitoring

An animal's environment can have significant effects on its physiologic state and thus can represent an important experimental variable. It is important for scientists to understand potential impacts of environmental variables on their study so they can appropriately control and monitor them. In general, the animal species used for toxicology are more tolerant of a range of environmental conditions than are many animals in their natural habitat. They have been selected as models because of their similarity to humans, who are themselves a very tolerant species, and have been further adapted to laboratory conditions through selective breeding.

Lighting

Light can be an extremely important variable for many animal species living in natural environments. It allows them to change their behaviors and physiologic state to adapt to seasonal changes in the weather.[12] In laboratory species, the impact of light is often low because of the species used and the ways in which lighting is typically managed. However, its effects can be profound and it is important to understand when this is the case so it is not overlooked as a variable.[1]

Most research institutions eliminate windows from indoor animal facilities to allow for complete control of light. This also is desirable from the standpoint of security. It is common to set facility lighting to match the convenience of research staff. This means a diurnal cycle with 12–14 h of light and 10–12 h of dark. This cycle mimics a normal spring or summer season for animals that display seasonal variations based on light cycles. This is normally the season in which animals are reproductively and metabolically active. This is the physiologic state that is often desired for a model of the human condition because we are continuously reproductively and metabolically active. Where light cycle can become important is when trying to stimulate a specific physiologic condition that may not occur within this paradigm. For example, breeding performance of some animals is significantly enhanced by extending the light phase of the cycle. Likewise, anestrous conditions can be stimulated with shorter light cycles when it is desirable to minimize hormonal stimuli. Light cycle affects many behaviors and should be considered for neurobiological studies.

Diurnal effects also must be considered. Rodents, which represent the majority of animals used in toxicological testing, are nocturnal species. The daytime light cycle established for the convenience of human staff catches them in the time they are normally inactive behaviorally and metabolically. When monitoring many behaviors, including reproduction, activity,

and food and water consumption, it is important to include dark phase monitoring.[48] This may need to be done without having personnel present as turning on lights during the dark phase will interrupt these behaviors, can affect data interpretation, and can reset the animals' circadian rhythms.[49] It is important for staff to record when they have entered a room, particularly during the dark cycle, so that any data anomalies can be cross-referenced with the presence of personnel and a temporary interruption in the dark cycle. The way in which lighting is turned on and off can have significant, but relatively short-lived effects on some species. Increases in heart rate have been shown to occur when lights are abruptly turned on in nonhuman primate rooms compared to a gradual increase in lighting, simulating a *sunrise*.[50] The presence of light at night can introduce study variability such as decreasing blood pressure,[50] cytokine-induced acceleration of tumor growth,[51] reperfusion arrhythmias,[52] and alterations in adrenal activty.[53]

The characteristics of the light provided can also impact animals. The *Guide* specifies a range of light intensities to allow sufficient light for staff to adequately observe the animals and perform their functions, while minimizing the impact of excessive light. In general, high light intensity has been shown to affect animals more than low light intensity.[12,54–58] Albino strains of animals typically used in research are susceptible to retinal degeneration from excessive light levels.[1] They should not be housed directly under room lights without some type of shelter in or over the cage.[12] There are scant data to show that laboratory animals are adversely affected by low light, although reproduction can be affected in rodents.[1,59] Variability in light levels within a room has not been shown to be an important variable in nonhuman primates.[60] However, because of the extreme variation in light levels that can occur in rodent rooms, it is not uncommon to rotate the position of animals on a rack and even the location of the rack in the room during the course of a toxicology study.[1]

Light spectrum is another area that does not seem to be as important to animals as is sometimes hypothesized. Typically, incandescent or standard fluorescent lights may be used interchangeably for most research applications. Simulated sunlight, sometimes termed *full-spectrum light*, has effects on some nonhuman primates, including changes in affiliative, aggressive, and reproductive behaviors and cortisol.[61]

Ventilation

Facility ventilation rates are specified by animal welfare regulations and the *Guide* and are designed to remove waste gases (CO_2 and ammonia) and control humidity and temperature in the animal's microenvironment.[11,12] Excessive ammonia can cause direct irritation and damage to the respiratory epithelium and increases the risk of respiratory infection.[1] The recommended rate of ventilation is very high compared to normal office or laboratory ventilation rates, with 10–15 room air changes per hour being standard for animal housing areas. In addition, primarily fresh air should be used rather than recirculated air, thus requiring preheating or cooling and adjustment of humidity from outside air, adding considerably to the cost of operating a vivarium. There has been some success with alternative ventilation methods to reduce overall facility airflow needs. Air can be recirculated within a room at up to 50% of the supply if it is appropriately filtered or conditioned to remove waste products and prevent the spread of microorganisms.[12] Modifications to the configuration of supply and exhaust, and caging configurations can increase the efficiency of the airflow at removing waste products and decrease overall airflow needs. Rather than relying on a set rate of airflow, sensors can be installed to monitor for critical parameters of air quality and adjust the airflow to meet animals' needs. Individually ventilated cages, ventilated racks, and isolators are also used to focus ventilation to the areas of greatest need while reducing the overall volume of air being managed.[1,12] Typically, environmental conditions are monitored at the room level and not the cage level. Some cage designs limit airflow into the cage, allowing the room conditions to be adequate but not the cage conditions. It is also possible to provide too much ventilation at the cage level, resulting in noise and thermal stress on the animal, which can adversely affect reproduction and animal health.[12]

Air balance is another aspect of ventilation. Room air can be adjusted so the pressure within the room is higher (positive) than in the adjacent corridor to keep airborne particles, odors, or microorganisms from passively flowing into the room. Positive pressure is typically used for the maintenance of immunodeficient or pathogen-free animals and surgical suites. Negative airflow is the opposite (the pressure in the room is lower than in the adjacent corridor). It is utilized to contain airborne particles and organisms within a room and is useful for general animal housing and biocontainment.[12] Corridors can also be adjusted to provide the same type of biocontainment or bioexclusion resulting in somewhat complicated ventilation systems within a vivarium. To maintain the pressure balance, room and corridor doors need to be kept closed when not in use. Air supply and exhaust may also be filtered to contain or exclude particles and microorganisms.[39]

Ventilation should be monitored regularly.[12] Automated monitoring systems can do this continuously. Otherwise, it is common for a daily assessment to be made by personnel in each room to verify that air balance is correct (through installed manometers or the simple measurement of directional airflow when the door is opened) and the room ventilation is normal (through a general feeling of *stuffiness*, presence of odors, or noise associated with ventilation systems). Airflow and balance should be monitored formally using qualified equipment. Annual ventilation monitoring is common in research facilities.

Temperature

Temperature is directly impacted by humidity and ventilation, necessitating coordination of monitoring and control of all of these conditions. Temperature ranges to prevent hypothermia, hyperthermia, or thermal stress (excessive energy expenditure associated with thermoregulation) are listed in the *Guide* and the animal welfare regulations.[11,12] Like air

balance, if room temperatures are not monitored continuously through automated building systems, they should be monitored at least daily using a thermometer.

For indoor housing, acceptable temperature ranges from the low 60s (°F) to the low 80s (°F).[11,12] In general, smaller animals require and can tolerate higher temperatures and larger animals lower temperatures. In particular, neonates and mice have been shown to require temperatures near the higher end of recommended temperature ranges.[12] Some species such as rabbits and sheep tend to do better at the lower end of the temperature ranges independent of their relative body size. By contrast, swine and nonhuman primates tend to prefer warmer temperatures compared to animals of similar size. This is likely due to inherent physiologic needs stemming from their evolution prior to being raised in captivity.

When managing the diverse temperature requirements for animals in an animal facility, it is important to remember personnel also can experience thermal stress. This is particularly problematic with higher temperature requirements, as personnel may be required to wear PPE that is fluid resistant, inhibiting ventilation. Therefore, an appropriate balance must be made between personnel and animal requirements.

Providing animals with a level of control for other than environmental temperature can be a useful strategy for managing temperature needs in a facility with diverse requirements, and also provides enrichment for the animals. This is most often done by providing nesting or shelter material to animals requiring higher temperatures.[12] For outdoor facilities, the use of fans or water mists is useful for giving animals the ability to cool themselves when ambient temperatures are high.[34]

Animals that are ill or experiencing anesthetic or test article effects that affect thermoregulation may require supplemental heat provided through heat lamps, water blankets or bottles, or heated air units. It is critical to monitor animals directly when these devices are used to prevent burns or hyperthermia. In addition, if animals get wet during cage cleaning or if there are leakages from the water supply, they should be dried or transferred to dry housing with bedding to prevent hypothermia.

High temperatures can also be a problem in research facilities. The heat load produced by animals housed in confinement can cause room temperatures to rise rapidly if there is failure of the ventilation system. This can result in large-scale mortality in animal facilities and is one of the reasons that environmental monitoring, automated alarms, and redundant or backup systems are so critical. Less commonly, hyperthermia can also be seen in animals that are anesthetized if excessive thermal support is provided. Swine can carry a gene making them susceptible to malignant hyperthermia and this can be a problem in susceptible breeds of animals during anesthesia, and if animals are stressed from study or husbandry procedures.

Humidity

Humidity should be maintained in a range of 30%–70% in animal rooms and below 50% in feed storage rooms.[12] Excessive humidity can lead to mold and it exacerbates the effects of high temperatures and ammonia and can affect topical drug absorption.[1] Humidity extremes can also be a risk factor for respiratory diseases[1,62] and *ringtail* in rodents, a type of ischemic necrosis that occurs on the tail.[1,12] Humidity can also be monitored by automatic monitoring systems. Continuous monitoring is less critical for humidity than for temperature and ventilation function, because humidity extremes do not affect animals as quickly as these other factors, but it should be recorded at least daily. Humidity can be monitored at the room level using inexpensive hygrometers. For more precise measurement, such as calibration checks of automated systems, a manual sling hygrometer is commonly used on a periodic basis.

Noise and Vibration

Interpreting the impact of noise in animal facilities is complex. Concerns are hypothesized with loud noises, with certain sound frequencies, particularly those in the ultrasonic range, and with the effects of noise from loud or agonistic species.[1,12] It can be difficult to control noises in animal facilities. Loud noises can be generated by equipment, use of water for cleaning, animals becoming excited when people enter the room or feed them, alarm testing, and ventilation systems. The impact of these noises may be overstated by some, as animals can acclimate to noise.[63] Noise exposure above 85 dB is consistently shown to have adverse effects, such as changes in behaviors, blood pressure, heart rate, gastrointestinal microvascular inflammation, and even seizures.[1,12,64–66] Providing exercise may improve the animals' ability to cope with noise stress.[67] It is important to protect studies involving behavior, measurement of stress hormones, and cardiovascular physiology from excessive noise, or at least to manage facility activities around the study schedule. Otherwise, the primary need is to control noises above the OSHA limit of 90 dB associated with hearing loss in humans.[12,39]

The impact of ultrasonic noise is poorly understood. Rodents can hear into the ultrasonic range and specific frequencies have been associated with stress vocalizations,[12] so it has been hypothesized that something emitting this type of noise will elicit a stress response in rodents.[1] There are currently no solid data to support this hypothesis, but an investigation of the presence of ultrasonic noise may be indicated if there are unexpected findings on studies for which no other explanation can be found.

The only requirement for noise monitoring in animal facilities is for the protection of personnel. Noise should be monitored in high noise areas such as dog and swine animal rooms, and cage wash to determine if hearing protection is required for personnel.[39] However, as more is learned about the impact of noise on animals, particularly ultrasonic noise, requirements for additional monitoring may evolve.

Vibrations in the animal facility have also been shown to be important under some circumstances, but as with noise the critical variables have not been established.[1,12] It is recommended that vibration sources be minimized through

appropriate maintenance of equipment and minimizing animals' exposure to construction and maintenance activities.

Vermin

Unwanted animals and insects need to be excluded from animal facilities to prevent transmission of disease, and damage to food supplies and facility structure.[11,12] Exclusion should be based primarily on physical methods. Doors, walls, ceilings, and floors should not have openings to allow vermin to enter. Drains should have traps installed. Facilities need to be kept clean to eliminate a source of food and a place for vermin to hide. Feed needs to be stored off of the floor and away from walls, and once feed bags are opened, it should be kept in sealed containers. Even with these precautions, vermin may still enter the facility. Vermin monitoring programs should be in place to detect these incursions so appropriate action may be taken. Monitoring traps should be checked regularly, particularly those designed to catch mammals so these animals may be humanely euthanized. When vermin are discovered, in addition to developing a plan to eliminate them, it is important to make an assessment of the risk vermin present to the vivarium. One approach is to test vermin for the presence of diseases to determine the risk of these infecting animal colonies. This information must be interpreted in light of the location the vermin were discovered and the mode of transmission of the particular disease. This approach is most useful with mammalian vermin. With insects, an accurate identification of the species of insect provides more information on the risk of them serving as a disease vector. In the author's experience, insects identified in animal facilities are typically agricultural pests or scavengers. Their presence may reflect a problem with facility exclusion or cleanliness but not a direct risk for disease transmission.

Microbial Control and Sanitation

Control of microorganisms is another important aspect of environmental control.[11,12] It is important to understand the scientific basis for microbial control as it is often misinterpreted with the emphasis placed on creating a sterile environment for the animals. Maintaining a sterile environment is not the goal for microbial control in a toxicology facility, as germ-free or gnotobiotic animals are not normally used. The goal is to maintain an environment that is not conducive to maintenance of pathogens. The primary method of excluding these pathogens is to prevent them from entering, through good control over animal sources and basic PPE requirements. Periodic cleaning and sanitation essentially *break the cycle* of infection in the event an organism does get by this first level of defense. It is not even necessary to completely eliminate all exposure to these organisms, only to keep the number of organisms below the threshold for infection in animals. A sterile environment is typically not provided for an entire toxicology facility because it is not practical with large animals, and even for small animals, it is quite expensive to provide sterile water, food, caging, and air and maintain sterility when performing study procedures. A sterile environment also does not represent a normal physiologic state for animals that are modeling the future application of the test articles in patient populations. On the other hand, there are circumstances under which gnotobiotic and axenic or *germ-free* animals may be used, requiring the maintenance of a higher level of microbial control. Gnotobiotic animals (those having specific microorganisms associated with them or *defined flora* animals) represent a slightly more normal model but still do not represent the real world, and true *germ-free* or axenic animals with no microflora have differences in their immunologic function, gastrointestinal physiology, and behavior compared to normal animals.[68–70] Typically, these types of animals are only used for specialized models in the areas of immunology or infectious disease research; however, there are circumstances under which they may be used in toxicology, and in those cases, it is important to maintain a higher level of microbial control.

The typical level of microbiological control in a toxicology facility is a specific pathogen-free (SPF) health status. This term is often misunderstood to mean animals are free of all pathogens or even free of all microbes. In fact, the term simply means the animals have been tested and shown to be free of a certain organism or set of organisms that the user defines. In Europe, there are standardized recommendations for organisms that should be excluded,[71] and in the United States, commercial vendors tend to exclude a similar battery of organisms. However, it is important to understand that these animals still have commensal microflora and typically also some opportunistic pathogens, that is, organisms that may act as commensals under most circumstances but can become pathogenic under certain conditions that are often poorly understood. Maintenance of SPF animals can be somewhat less stringent than gnotobiotic or axenic animals. By specifying the organisms that will be excluded and understanding the biology of those organisms, a facility can design bioexclusion procedures that specifically exclude the organisms of concern and are more practical or cost-effective than full containment procedures. The relative level of the exclusion of different organisms and effort required to exclude them can vary greatly. To exclude rodent-specific organisms, a facility can maintain a basic level of biosecurity by controlling the source of animals to those that have been screened for the excluded diseases and implement basic PPE, pest control, and sanitation procedures to prevent these diseases from being brought in from the outside. Sterilized food, bedding, water, and caging are typically not required as long as these items are also produced or managed in a way to prevent rodent contamination and receive some basic treatment. In order to control pathogens that can be carried by humans or opportunistic microflora, housing and management conditions may need to be implemented that are equivalent to those needed to maintain gnotobiotic or axenic animals. Scientists and facility managers should look carefully at the organisms they are excluding to ensure that the effort being put into it is equivalent to the value gained. Many of the organisms not specific to rodents only cause disease under conditions of significant immune suppression.[72] To evaluate test articles intended to cause immune suppression, a high

level of microbial control may be warranted, as opportunistic infections are to be expected. However, it may be valid to control infections through the use of antimicrobials rather than environmental control, as this is the same condition under which drugs will be used clinically. For test articles where immune suppression is an unintended effect secondary to toxicity, the appearance of opportunistic infections may be interpreted to represent the maximum tolerated dose (MTD) for the test article and identifying that may in fact be a key goal for the study.

As discussed in the section on facility design and construction, the use of easily sanitized materials greatly facilitates microbial control of the environment. Regular sanitation must be performed. Daily to weekly sanitation of floors and work surfaces is standard in the industry. Animal caging with bedding should normally be sanitized weekly, while caging without bedding must be sanitized at least every 2 weeks.[11,12] Walls and ceilings should be cleaned and sanitized as needed. For *wet* rooms such as those used for larger animals, sanitation of all room surfaces is typically performed every 2 weeks along with cage and equipment sanitation. For *dry* rooms such as those used for rodents and rabbits, full room sanitation may be performed less frequently. Monthly to quarterly frequencies are commonly used in the industry. This may only involve a simple wipe-down of the surfaces with a disinfectant. Full room sanitation using more effective methods may only be performed between room uses, or at annual intervals.

Drinking water systems also require sanitation as microorganisms can grow in the water and form a biofilm on the surfaces of the watering systems.[12] These organisms are not typically pathogenic,[73] but biofilm can provide an environment for pathogens to live in, and the presence of biofilm is representative of the level of sanitation for drinking water lines. There are a variety of methods for maintaining microbial quality of watering systems. If completely sterile water must be provided, this may be done using presterilized water or hydration gel in sterile pouches or bottles or chlorinated or acidified water to control microbial growth.[12,74,75] There are also ultraviolet (UV) treatment systems to eliminate microbes within a recirculating water system; however, it is important to properly maintain the UV lights for these systems as they do lose efficacy over time.[76]

For immunocompetent animals, sterile water is not necessary. Normally, the goal is to minimize microbial levels in water and periodically remove any potential pathogens. A periodic cleaning or sanitation process is appropriate for this purpose. If water bottles are used, they are replaced and sanitized on a weekly basis.[12] For automatic water systems, a common method for cleaning is to flush a large volume of water through the water delivery lines in a room and cage rack.[12] This may be done between one and three times a week, as needed to maintain water quality at a desired standard. When cage racks are sanitized at 2-week intervals, waterlines should also be sanitized at this time. A concentrated disinfectant like chlorine may be run through the lines but it is critical this be rinsed thoroughly before use.[12] Some cage washer units are designed to connect a high-temperature waterline to the cage rack waterline to provide sanitation; however, some facilities have success without this type of treatment, just allowing the waterlines to be heated in the cage washing process. Purging residual water from the lines with compressed air eliminates any residual waterborne microorganisms and waterlines can stay within microbiological standards this way while they are being stored.

In order to assure the goal of sanitation is being met, a monitoring program must be in place.[12] The gold standard for microbiological monitoring is testing for microorganisms directly through culturing a sample from the item being monitored, quantitating the growth of microorganisms, and identifying the particular organisms that grew to determine if they are pathogens. Simple quantitation is normally done through a plate count or replicate organism detection and counting (RODAC) plate that monitors for total number of microorganisms present. Unless the goal of the sanitation is sterilization, a low number of organisms may be acceptable as most of these organisms should be nonpathogenic. It is not practical to try to identify all of the organisms found, so a common strategy is to use a selective growth and identification medium to identify pathogens, such as coliform bacteria. Normally, there should be no coliforms in samples from properly sanitized equipment or water systems.

Because of the time and cost associated with microbiological testing, this has historically only been performed at periodic intervals (monthly or quarterly), and more frequent monitoring is performed on the process or equipment to create the conditions that result in effective sanitation. An example of this is monitoring the temperature of the rinse water in an automatic cage washer to ensure that it is high enough for the expected contact time to inactive vegetative microorganisms. A common standard for this is for the rinse water to reach 180°F, as this will achieve sanitation after only a few seconds of contact. Lower temperatures can be equally effective with longer contact times.[11,12,77,78] Another method would be to monitor the concentration of the disinfectant used. This type of monitoring is often performed at least daily, if not every time the sanitation procedure is performed. With the development of newer technologies for microbiological monitoring, it is practical to perform microbial monitoring more frequently. An example of this is the use of a swab-based ATPase assay employing a fluorescent marker that can be read in a handheld unit. This provides rapid, quantitative data that have been shown to correlate with microbial count data.[12,79,80] There are specialized swabs allowing sampling for specific types of microorganisms, such as coliforms. As the methods for monitoring directly, rapidly, and inexpensively for microorganisms become more accepted within the industry, monitoring sanitation conditions (e.g., temperature or chemical concentration) may become less important. However, at this time many animal welfare compliance auditors still expect to see this type of monitoring performed regularly, even if the more relevant microbiological monitoring is being performed at the same interval.

CAGING

Animal cages (often referred to as *primary enclosures*) must meet a variety of requirements, some of which are conflicting. They need to provide enough usable space to meet the animals' basic physiologic and behavioral needs. They also need to provide an appropriate microenvironment, be easily sanitized, durable, prevent animal escape or injury, allow observation of the animals, and allow research functions to be performed.[11,12] An important trend in animal welfare regulatory and oversight standards is the expectation animals will be housed socially unless there is scientific justification for individual housing.[11,12] This creates some additional requirements for animal housing including being able to separate animals when required, ensuring adequate space for feeding to prevent food competition, and space for animals to obtain privacy when they choose. The space required for multiple animals in a single housing enclosure does not necessarily increase linearly with the number and size of the animals.[12] Because caging is very expensive, it can be tempting to cut corners to cut costs when designing and purchasing caging, but in the author's opinion, the long-term labor savings and the improved study success justify investment in quality design and construction of caging.

Cage space and design standards for most animal species are listed in the USDA animal welfare regulations and in the *Guide*.[11,12] For species not listed, the requirement is to meet a performance standard where the animal is able to perform species-typical behaviors and show no evidence of stress, such as abnormal behaviors, failure to maintain body weight or normal growth, abnormalities in hormone levels or balance, or abnormalities in the complete blood count or immune function. This performance standard is applied to all animals, such that even if a cage is meeting regulatory standards, if the animal is not healthy, it is not compliant. European cage space standards tend to emphasize greater amounts of space and more structure in the cage to allow climbing, perching, nesting, and shelter.[27]

While a wide variety of cage designs are available, there are some standard designs commonly used. Large animal cages tend to be constructed of stainless steel with slated or mesh floors and walls, allowing easy cleaning and good ability to observe animals. Cages for species that climb, such as nonhuman primates, cats, and ferrets, need to be enclosed on the top, but for other species with limited ability to jump or climb, they may be open on the top. Climbing species also may have elevated platforms built into the cage for the animal to perch. Nonhuman primates in particular benefit more from the vertical space in a cage rather than the floor space.[12] Cages are adapted for social housing by having a door or panel that opens between adjacent cages to allow animals to be paired when desired and separated when needed. Some designs allow multiple units to be connected and opened so any number of animals may be maintained in a group. These cages may be free standing on the floor, as with a kennel run, livestock pen, or group primate housing, or also may be mounted on wheels (castors) to allow easy movement for cleaning and room reconfiguration.

Historically, multitiered systems were wall mounted, but this is less common now, as it makes it more difficult to clean rooms and to reconfigure rooms when study needs change. Multitiered caging is still commonly used in the United States, but no longer meets European standards for primate caging[27] and is not favored for dogs. Tiered caging increases room capacity compared to single-tier caging or freestanding kennels, but kennels can be easier for personnel to use and perform daily cleaning. With nonhuman primates, tiered caging has been shown to affect the dominance hierarchy established among animals in different cages within the same room,[60] leading some experts to believe it is not optimal for animal welfare. One challenge with multitiered caging is the risk of injury to animals if they jump out of an upper cage and to personnel who must lift animals in and out of these cages. This can be managed through the use of team lifting, lifting devices, ramps, and weight limits for animals placed in upper cages.

The type of slatted or mesh flooring used in large animal housing can be important. Hoofed animals are susceptible to slipping on mesh floors and injuring themselves.[1] There needs to be enough grip to prevent this from happening. For all species, the space between flooring bars needs to be designed so feet or limbs cannot slip between them and become injured or entrapped. If a large animal pen is used for different species, interchangeable floors may be needed as the mesh size may need to be different for each species. Dogs can be susceptible to the development of interdigital cysts if the flooring is improper.[81] The exact cause of this is not known, but the lowest risk for these lesions seems to be associated with a mesh that is extruded rather than woven, is rubberized, and does not allow pooling of water or accumulation of feces.

Large animals can also be maintained on absorbent bedding if it is cleaned and replaced regularly, as it presents the least risk of foot or limb lesions. However, the logistics of managing the amount of bedding needed for large animal housing results in absorbent bedding being relatively uncommon in a typical laboratory environment. Farm-type facilities, however, often use bedding and it can be useful for certain study types where contact with moisture is undesirable or improved footing is needed for the animals.

Rodent caging has historically been constructed similarly to large animal caging. A common system is a metal unit with solid walls and a wire mesh floor that slides into a multitiered rack. A solid shelf or pan under the cages may contain bedding material or absorbent paper to collect waste. This type of caging is very durable, requires relatively low labor input for cleaning, and provides good ventilation for animals. However, the use of wire-floor caging is discouraged by animal welfare standards in the United States and Europe.[12,27] The concern with wire-floor caging is that animals can develop ulceration on the sole of their feet when housed on wire for prolonged periods of time.[12] This may be related to pressure, as it seems to be more common in larger animals. Aside from the risk of foot lesions, wire-floor caging does not allow rodents to display species-typical behaviors of

burrowing and nesting[12] and may increase energy requirements for thermoregulation.[12,82] Wire-floor caging does not lend itself to studies requiring containment of experimental hazards or exclusion of microorganisms.

Newer caging systems for rodents utilize a solid bottom with a layer of bedding to absorb waste. The cages are typically constructed of polycarbonate or other plastics to allow for visualization of the animals that are durable enough to sustain repeated high-temperature washing and chemicals. Cages are kept on multitiered racks as with the wire-floor cages. In some systems, the cages also slide into runners underneath the rack shelf, which serves as the top to the cage, while other caging types have perforated or wire tops and are simply set on a rack shelf. An advantage of the slide-in caging for toxicology studies is there is less labor involved with daily examination and dosing of animals. A cage can be slid out partway, the animal removed, procedures performed, the animal replaced, and the cage slid back in. For cages with a top, there are several extra steps involved, as it is necessary to place the cage onto a separate work surface, and remove and replace the cage top. The advantage of the latter system is that individual cages may be removed from the room and taken elsewhere as a contained unit, while with slide-in caging systems, the rack is needed to keep the animal contained in the cage. Facilities readily work around this by having a supply of lids for these cages that can be used when it is desired to remove individual cages from the rack. If cages are set on the top of a rack shelf rather than slid underneath, it may be necessary to avoid the use of the top shelf on a rack or provide some type of shield between the top cages and room lights to prevent excessive light exposure to these animals.

Besides the benefits to animal health and welfare, solid-bottom cages also allow for containment of hazards and exclusion of microorganisms. Solid lids with high-efficiency filters can be used to keep chemicals and particles in or out of the cage while still allowing ventilation. These lids do reduce overall ventilation,[12] so systems have been designed to push or pull air through the cages by the use of fans built into the racks or connections to building ventilation systems. This type of housing is more expensive to purchase and operate than more conventional caging designs, but can be very useful for certain types of research. It is important to remember when using this type of containment caging that once the cage is opened, the barrier between the cage and the room environment is broken. Typically, these cages should only be opened in a biosafety cabinet or other containment system to prevent contamination.

Social housing for rodents is typically managed through establishing pairs or groups in the standard cages. This can work well, but is a challenge for species or individuals that are highly territorial. The industry has not addressed this issue well, and in the future rodent housing systems may adopt some of the design features used in large animal cages, such as separate compartments or platforms to help manage social housing with territorial species.

Rabbit housing methods somewhat straddle the housing used for large animals and rodents. It is common to house rabbits in metal cages with slat or mesh floors with waste pans underneath. However, rabbits are also susceptible to foot lesions from mesh,[12] and do well on bedding. The logistics of bedding for rabbits is similar to that of large animals and many facilities housing rabbits for a long term will use mesh flooring but are careful to manage the diet of the animals to prevent them from becoming obese.[12] Facilities have adapted large animal cages or pens for rabbit housing, which can work very well, particularly when trying to house them socially.

ANIMAL MODELS

The use of animals in toxicological research dates back to the second and fourth centuries BC when Aristotle and Erasistratus performed experiments on living animals. Documentation of the use of dogs and nonhuman primates in animal experimentation can be dated to the second century AD when Galen conducted animal experimentation, specifically anatomical dissections, on living animals (vivisection) since dissection of human cadavers was outlawed according to Roman law.[83]

While animal testing has been conducted throughout recorded history, the use of animals was primarily experimental in nature: fundamental biological research, acquiring knowledge, or in training or teaching exercises. The conduct of animal studies for predicting the safety prior to testing in man was not required for new drugs prior to marketing until 1938 when the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 301–392) was passed as a result of the sulfanilamide deaths. The proof of efficacy requirement was not required until the Kefauver–Harris Amendment was introduced in 1962 following the thalidomide tragedy.

While animal models used in research include, but are not limited to, pharmaceutical testing, toxicology testing, efficacy testing, surgical research, and pathophysiological research, the majority of the animals used in research are used for pharmaceutical and toxicology testing, specifically with regard to the nonrodent species.[84] With few exceptions, the regulatory guidelines (U.S. as well as international) require that toxicology studies be conducted in one rodent and one nonrodent species for support of pharmaceutical development. When choosing the nonclinical species, the appropriate model should be selected such that the response desired is most predictive of the human response, whether it is toxicity or efficacy being evaluated. Animal models may be exploratory, explanatory, or predictive in nature.[85] For the purposes of this section of the chapter, models of a predictive nature will be focused on since the majority of preclinical models in research are used in this capacity. This section is general in nature and will cover only an overview of the use of preclinical models used in research. A number of resources are available for selection of the appropriate animal models for specific disease states.[86–88]

The animal model(s) selected for use in safety assessment for support of clinical trials should be one that is the most predictive of human toxicity. In the absence of specific data on species relevance, the most sensitive species should be

selected based on prior experience with the compound class. The means by which the species are selected are dependent on the agent being developed and generally fall into one of two categories: small molecules and biopharmaceuticals (large molecules). For small molecules, the standard means to select the nonclinical species is through the use of in vitro metabolic profiling. Liver microsomes from a full spectrum of species (mouse, rat, dog, nonhuman primate, and human) are incubated with the test material and a profile of metabolic products is obtained. The profile from human is compared to the profile of the other species and the rodent and nonrodent species to be used for the nonclinical studies is based on the species that best covers the human metabolic profile. For most small molecules, history has shown this to be the rat and dog; however, it is not uncommon for one of these *standard* species to have a profile different than human and, therefore, the mouse or nonhuman primate may need to be considered. Minipig is also a species that can be included in the metabolic profiling as another option for the nonrodent species.[89] Should the metabolism data not provide a compelling case in support of a particular species, then attributes such as receptor expression or bioavailability can be used in support for or against a particular species. For biopharmaceuticals, regulatory agencies require the nonclinical studies to be conducted in the *most relevant* species, which is based on the pharmacology of the test material. Species selection for biopharmaceuticals is supported through the use of in vitro assays that demonstrate the ability of the test material to elicit a pharmacological effect in cells from a particular species. Most biopharmaceuticals are designed to be specific for humans; therefore, they may not exert their pharmacology in lower-order species such as the mouse and rat. In these cases, it is possible that the nonhuman primate may be used as the only species for the nonclinical studies.

Rats and Mice

In toxicological research, the two most commonly used rodent species are rats (*Rattus norvegicus*) and mice (*Mus musculus*), which were among the first species to be domesticated for scientific evaluation purposes. In fact, toxicity testing has historically often been conducted in both of these species for many test compounds, from initial range-finding studies to 2-year carcinogenicity studies.

Over a long and fruitful scientific history of nearly a century, the rat has generally become a species of choice in toxicological research because of its metabolic similarities to humans, its small size, generally friendly behavior (albeit with occasional individual animal exceptions), short gestation period (approximately 22 days), large litter size (average of 12), lack of emetic response, and fairly short life span (24–30 months). In addition, its use as a primary research species over the course of many years has led to a very robust historical control database for numerous biological parameters/endpoints, both in-life and pathologically, which further enhances its use as a predictor of human response to a compound. The Wistar Institute in Philadelphia, PA, was a prominent player when early researchers diligently worked to establish breeding programs to minimize animal variability and maintain optimal/desirable animal characteristics for scientific purposes.[90] Thus, many of the rat strains that are commonly used in the industry today (which include the Hannover-Wistar, Sprague-Dawley, and Long-Evans) can be traced back to that Wistar pedigree, while others were developed via a different lineage platform (such as the Fischer-344, which was developed for cancer research by the Crocker Institute of Columbia University in New York, NY).[90] The development of SPF rats, as well as the fine-tuning of animal husbandry practices over the course of time, has generally eliminated most of the disease outbreaks that may have potentially introduced variables into a study. However, the breeding process has typically produced some differences among the various strains that the toxicologist should be aware of and duly consider when selecting a particular strain for a toxicology program, rather than automatically defaulting to the standard or favorite strain utilized by a particular contract research organization (CRO) or internal laboratory that has been chosen to conduct the studies. Historical control data have shown that these physiological differences can affect how a particular rat strain reacts to a compound in terms of toxicity (strain-specific differences). As examples, the increased propensity for spontaneous renal disease that has been observed in the SD rat compared to the CD rat[91] may limit the utility of this particular strain for evaluating nephrotoxic compounds; the Fischer-344 rat is historically prone to higher incidences of interstitial cell tumors in the testes and lower incidences of female mammary gland fibroadenoma[90–93] than other strains; and the Hannover-Wistar rat has historically exhibited higher incidences of adenoma pars distalis of the pituitary gland[90–93] compared to other strains. Such considerations are especially important today, where continuing advances in breeding have made available various additional inbred and outbred strains that may be more geared to certain specific models of disease, or development of certain carcinoma types. In the modern research environment, even a casual perusal of any major animal vendor catalog or website offers myriad choices of rat models to consider, in terms of standard outbred strains such as CD, Long-Evans, SD, Wistar, and Hannover-Wistar rats, and standard inbred strains such as Brown Norway, Copenhagen, Fischer-344, and Lewis rats, along with various disease/translational models (such as immunodeficient, renal, cardiovascular, or oncology). Vendor customer service departments or websites often provide such detailed historical control data for customers to request or directly download. Therefore, the indication and mechanism of action of the particular compound being studied might also be a major driver in identification and selection of the optimal strain to use, with the caveat that newer and less frequently used strains do not necessarily have the robust historical control background of more commonly used strains. Such historical control data are often invaluable in assessing and interpreting incidence and severity across dose groups in a study from a histopathological perspective. Furthermore, the overall compilation and availability of such data over

decades also allow for a level of informational assessment of random genetic drift.[94] A number of what may be considered seminal works nicely address the specifics of the toxicologic pathology of the rat[95–97] in greater depth and informational detail, as this particular topic is one of the most abundant in terms of available literature for the toxicologist to reference.

Strain-specific differences can also be particularly important for carcinogenicity studies in terms of optimal survival levels over the course of a 2-year study period, for which valuable information can also be gleaned from historical control databases. In fact, Europe maintains the Registry of Industrial Toxicology Animal-data (RITA) Database, which is a database founded in 1988 by the Fraunhofer Institute of Toxicology and 13 pharmaceutical and chemical companies from Germany and Switzerland, and which companies from all over Europe now participate in by providing carcinogenicity study data for this computerized and standardized database.[98] Data from sources such as RITA in Europe, as well as the National Toxicology Program (NTP) in the United States, have allowed for comparisons of various strains, most notably the Hannover-Wistar rat (generally favored in Europe) and the Fischer-344 rat (with which the NTP has a long history), and the Charles River Laboratories SD rats (most commonly used in the United States), and Harlan SD rats (more often utilized in Europe). These geographical differences in use of strains, the robust data collected, and a trend toward decreased longevity in the CD and SD rats over time, suggest that the SD rat may eventually lose favorability due to its high background tumor rate, larger size, and lower survival rate, when compared to the Hannover-Wistar rat. The Hannover-Wistar is a leaner rat that exhibits a higher survival rate and a generally lower incidence of background tumors.[99] Its potentially increased future use over other strains, most especially if it becomes as favored in the United States as it is in Europe, would benefit the quality of carcinogenicity assessments in terms of minimizing the early termination of 2-year studies (or particular groups within a study) due to low survival, which impacts optimal statistical power for tumor analysis.

The utilization of the mouse for scientific research has an even longer history than that of the rat, as it spans several hundred years, including pioneering early efforts in the evaluation of animal reproduction, blood circulation, and respiration physiology. As with the rat, the mouse has also become a species of choice due to the many advantages it offers for toxicological research, which include its small size, general ease of handling, short gestation period (19–21 days), short natural life span (ranging from 1.3 to 3 years among varied strains), being more economical to purchase, house, and care for, and the availability of robust historical control data. However, the small size that makes the mouse more cost effective and less wasteful of test article also results in a less hardy animal that has more susceptibility to unexpected environmental deviations or to dehydration resulting from an automatic watering system issue/failure, while also limiting the blood sample volume that can be collected as well as urine output (although mouse urine is more concentrated).

A large number of the inbred and outbred strains of mice being used in research today are classified as Swiss strains, which trace back to an original noninbred stock from Lausanne, Switzerland, from which a set of animals was imported to the United States for cancer research by Dr. Clara Lynch of the Rockefeller Institute in 1926.[100,101] However, as with rats, the breeding process has typically produced some differences among the various strains that the toxicologist should be aware of and duly consider when selecting a particular strain for a toxicology program. The normal or typical body weight of various strains at different ages is one such variable to consider, in addition to the typical strain-specific differences shown in histopathological historical control data. Growth curves maintained by vendors such as Charles River Laboratories have shown that by 56 days of age (8 weeks), which is generally considered maturity, outbred strains generally tend to be larger than inbred strains, with the CD-1 outbred strain reaching the highest mean weight when compared with other outbred strains (such as CF-1 and CFW), various inbred strains (such as C3H, C57BU6, and BALB/c), and the hybrid B6C3F1 strain. Of these six particular strains, the C57BU6 and BALB/c strains are generally the smallest at 56 days of age. Some strains of mice, such as nude or athymic strains, are more sensitive to tumor development than heterozygous strains, and thus may exhibit higher incidences of tumors, as well as shorter latency periods. In addition, among numerous strains of mice, susceptibility to spontaneous lung tumors can exhibit wide variability, a situation where the potential chemical inducibility of a compound in this regard would suggest the use of a particular strain over another.[102] As an additional example, the hybrid B6C3F1 mouse exhibits higher spontaneous neoplastic lesions of the liver (hepatocellular adenoma and carcinoma) and the pituitary gland (adenoma) when compared to the CD-1 outbred mouse.[90]

There are a large and proliferating number of strains of mice available to choose from today (beyond inbred, outbred, hybrid, or immunodeficient), which now also include the advent of various genetically engineered mouse models such as the p53+/– knockout mouse (one or both of the alleles of a gene of interest are knocked out), the Tg.AC transgenic mouse (foreign genetic material is inserted into the genome and retains functionality), and the rasH2 transgenic mouse (human genetic material is inserted so that it carries a human oncogene), which offer even more choices for consideration by the toxicologist. While the p53+/– knockout mouse and the rasH2 transgenic mouse can typically be dosed orally (which is the most common route of administration in carcinogenicity studies), the Tg.AC transgenic mouse is typically dosed topically (skin paint study), which also is a decision factor to consider. The advantages of these genetically engineered mouse models are that a 2-year carcinogenicity study in mice can be reduced to a 6-month study that requires lower exposures, these studies require lesser numbers of animals due to their shorter duration, they may help minimize strain differences evident among other mouse strains, and could even be targeted to a specific mechanism of tumor formation.

Although these mice have specific genetic alterations critical to tumorigenesis, these alterations are in and of themselves insufficient to produce tumors before the end of a 6-month duration, thus exposure of these models to a carcinogenic compound would be expected to result in a rapid induction of compound-specific tumors. The particular three genetically engineered mouse models mentioned earlier have progressed past validation studies and have been utilized as part of submission packages in the United States and/or other countries and may indeed be the wave of the future as potential replacements for the 2-year mouse carcinogenicity study[103,104] as more historical control data are accumulated over the course of time and more such studies are commonly and successfully submitted to regulatory authorities going forward. In fact, the rasH2 transgenic mouse may now be taking the lead as the favored option for such studies, as anecdotal information suggests that approximately 20% of mouse carcinogenicity studies for new drug application in the United States now utilize transgenic models, with most of these typically using the rasH2 model, rather than the other two.

Dogs

The dog (*Canis familiaris*) serves as an experimental model in a variety of areas and disease states, including aging, cardiovascular disease, dermatologic disorders, endocrinologic disorders, hematologic disorders, immunologic diseases, musculoskeletal diseases, neurological disorders, ophthalmological disorders, radiation injury, and gene therapy.[105] The majority of dogs used in research are purpose-bred beagles for toxicology safety testing to support subsequent clinical trials; however, other breeds, including large hounds and mixed breed dogs, have been utilized for specific indications/observations in efficacy evaluations (see Table 20.5). The beagle was likely chosen for use originally due to their relatively small size, mild temperament, and ease of handling while still allowing for repeated sample collections/evaluations. These qualities allow for the repeated collection of samples or data from individual animals with a low risk of harm to the dog or the researcher, thus reducing the need for large number of animals. For example, repeated blood collection can be obtained from a single beagle for clinical pathology monitoring and toxicokinetic evaluations without compromising the health of the dog, resulting in the elimination of satellite groups or an increased number of animals that would be required for smaller species or breeds. The beagle was also made popular as a laboratory model in post–World War II research of biological effects following radiation exposure. In this research, important factors used for model selection included a long life span, genetic uniformity, physiological similarity to humans, economy of procurement and maintenance, availability, and ease of husbandry in a laboratory environment.[106] Because of the long history with the laboratory beagle, a robust historical database has been established in many laboratories, making this breed a logical choice for safety testing. Less than 1% of all animals used in research are dogs, and they represent approximately 7% of the USDA-covered species used in research in the United States in 2007 according to the latest available Animal Care Annual Report of Activities published by the USDA.

Nonhuman Primates

There are over 230 different species of nonhuman primates, of which only about 30 species are used in biomedical and behavioral research. There are three suborders of nonhuman primates: the Prosimii, the Tarsioidea, and the Anthropoidea (see Table 20.6). The nonhuman primates used in biomedical and behavioral research belong to the suborder Anthropoidea, so for the purpose of this discussion, only this suborder will be discussed. The suborder Anthropoidea is divided into two infraorders; the Platyrrhini or new-world monkeys (NWM)

TABLE 20.5
Examples of Canine Models Used in Research

Research Emphasis	Breed	References
Dystrophin-deficient muscular dystrophy	Golden Retriever, German Shorthaired Pointers	[305–307]
Becker muscular dystrophy	Japanese Spitz	[307]
Congenital myotonia	Chow Chow	[308]
Glycogenosis type II (Pompe's disease)	Lapland Dog	[309]
Glycogenosis type III	German Shepherd	[307]
Waardenburg's syndrome	Dalmatian	[310]
Cancer—mast cell tumors	Boston Terrier	[311]
Narcolepsy	Doberman Pinschers	[312]
Hereditary kidney cancer	German Shepherd	[313]
Non-Hodgkin's lymphoma	Golden Retrievers	[314]
Osteosarcoma	Great Dane, Wolfhound, Mastiffs, and Bernese Mountain Dogs	[315]
Aging and Alzheimer's disease	Beagle	[316]
Endocrinopathies	German Shepherd	[317]
Osteoarthritis—ACL transection	Mongrel, Foxhound, Beagle	[318]
Osteoarthritis—meniscectomy	Mongrel, Greyhound	[318]

TABLE 20.6
Taxonomy of Primates

Suborder	Infraorder	Superfamily	Family	Subfamily
Prosimii (prosimians)	Lemuriformes	Lemuroidea (lemurs)	Cheirogaleidae	
			Lemuridae	Lemurinae
			Lepilemurinae	
			Indriidae	
			Daubentoniidae	
	Lorisiformes	Lorisoidea	Lorisidae	Lorisinae
Tarsiiformes		Tarsioidea	Tarsiidae	
Anthropoidea (anthropoids)	Platyrrhini	Ceboidea (NWM)	Cebidae	Cebinae
				Aotinae
				Atelinae
				Alouattinae
				Pithecinae
				Callimiconinae
			Callitrichidae	
	Catarrhini	Cercopithecoidea (OWM)	Cercopithecidae	Cercopithecinae
				Colobinae
		Hominoidea (apes and humans)	Hylobatidae	Hylobatinae
			Pongidae	Ponginae
			Hominidae	Homininae

Source: Martin, R., Classification of primates, in: Jones, S., Martin, R., and Pilbeam, D. (eds.), *The Cambridge Encyclopedia of Human Evolution*, Cambridge University Press, Cambridge, U.K., 1992, pp. 20–23.

and the Catarrhini or old-world monkeys (OWM). Less than 1% of all animals used in research are nonhuman primates and they represent approximately 6.8% of the USDA-covered species used in research in the United States in 2007 according to the latest available Animal Care Annual Report of Activities published by the USDA.

Nonhuman primates serve as one of the best animal models in predicting human responses with regard to safety and efficacy due to the genetic similarities between humans and the nonhuman primates. These genetic similarities are reflected in anatomical, behavioral, developmental, physiological, endocrinological, and reproductive aspects. Ninety-eight percent of human DNA is found in the genes of chimpanzees, 92% in OWM, and 85% in NWM.[107] As a result of these similarities, the nonhuman primate has played a critical role in biomedical safety, efficacy, and behavioral research (see Table 20.7). These similarities make the nonhuman primate an ideal model for research in AIDS; vaccine development of various diseases; Parkinson's disease; Alzheimer's and other cognitive diseases; reproductive, fertility, and endocrine research; hepatitis; neurologic disorders; behavioral and cognitive disorders; genetic disorders; and xenotransplantation.

Old-World Monkeys

OWM (from Africa and Asia) generally used in research are part of the Cercopithecidae family and include the macaques (cynomolgus and rhesus) from Asia and the baboon (olive and yellow baboons), guenon (African green monkey), and mangabey (sooty mangabeys) of Africa. In addition to the nonhuman primates from the Cercopithecidae family, the chimpanzee (Pongidae family) is also used, although in only a few research settings. The chimpanzee is the only great ape used in biomedical research; however, the use of the chimpanzee in research is being limited to those that meet specific criteria established to assess the use of the chimpanzee in biomedical and behavioral.[108] Ninety percent of nonhuman primate toxicology studies are conducted in macaques (usually cynomolgus or rhesus).[109]

New-World Monkeys

NWM (from Central and South America) generally used in research are part of the Callitrichidae (common marmoset, cotton-top tamarin, and mustached tamarin) and the Cebidae (squirrel, owl, and capuchins monkeys).

Rabbits

Today's domestic rabbits used in research were derived from the European rabbit (*Oryctolagus cuniculus*). Rabbits are a desirable research model due to their manageable size, generally docile nature, and breeding characteristics. The earliest records of captive rabbits date back to third century BC. Classic irritation models such as the domesticated New Zealand white (NZW) breed are still in use today despite the in vitro alternatives. Irritation assessments play an important role in worker safety assessment, labeling, and to some extent for the evaluation of dermal products as they provide comparisons for predicting the concentrations that may be tolerated by humans primarily due to accidental exposure.

TABLE 20.7
Examples of Nonhuman Primate Models Used in Research

Research Emphasis	Species	References
Malaria vaccine development, Creutzfeldt–Jakob disease, biomechanics of labor and delivery, pelvic organ prolapse, and emphysema	Squirrel monkey	[320]
Behavioral, developmental, and reproductive biology, virology, and immunology	Macaques	[321]
Intrauterine, reproductive, and surgical research	Olive baboon	[322]
Epilepsy	Yellow baboon	[323]
Parkinson's disease	African green monkey	[324]
Alzheimer's disease	African green monkey	[325]
AIDS	Sooty mangabeys	[326]
Hepatitis C	Chimpanzee	[327]
Parkinson's disease	Marmoset	[328]
Colitis and colonic carcinoma	Cotton-top tamarin	[329]
Colitis cystica profunda, myocardial fibrosis, and membranoproliferative glomerulonephritis	Moustached tamarins	[330]
Choroidal neovascularization	Squirrel monkey	[331]
Viral diseases and oncologic studies	Owl	[332]
Memory	Capuchins	[333]

Other common uses for the rabbit in research include vaginal, penile, and rectal irritation and/or toxicity studies.[110,111]

In addition to an irritation model, rabbits are used in the production of antibodies, biomedical device testing, pyrogenicity testing, and for developmental (teratology) testing of pharmaceutical compounds. Rabbits are sensitive to teratogens and can provide predictive information relevant to humans. For example, rabbits have a similar developmental response to thalidomide as humans. Rabbits are also an acceptable model for pharmaceutical drugs targeted for ocular delivery such as glaucoma. In cases of melanin-binding compounds, the New Zealand red or Dutch-Belted breeds provide a pigmented alternative to the NZW.[110,111]

Guinea Pigs

Today's domestic guinea pig (*Cavia porcellus* or cavy) is a rodent derived from wild guinea pigs found in South America. The first reports of domestic guinea pigs occurred around 1530. Guinea pigs have structural and physiologic features that make them acceptable models for human correlation. For example, both guinea pigs and humans have a need for vitamin C, have similar immunological sensitization reactions, and a susceptibility to tuberculosis. Tuberculosis was discovered in 1882 using guinea pigs and this has been a useful model in the study of infectious diseases due to similarities to the human immune system. They are docile animals, relatively small in size, inexpensive, and easy to maintain in a research environment.[110,112]

Historically, guinea pigs have been used for skin and delayed-contact hypersensitivity testing. The two most common assays include the Buehler and Magnusson–Kligman test (or maximization test). These assays are still in use although the mouse local lymph node assay is now accepted by the regulatory bodies (FDA, EPA, and the Organisation for Economic Cooperation and Development [OECD]) to provide sensitization information. The general safety test is another common use for these animals. They are a common model for auditory research due to the anatomy and accessibility of the ear. One of the first species to be used for allergic testing, they are still a popular model for this arena. They also have been used as a research model for diseases affecting the airways such as asthma and chronic obstructive pulmonary disease (COPD).[112]

Hamsters

Hamsters arrived late (1930s) to the research arena compared to their rodent relatives. The species most commonly used in research is the Syrian or Golden hamster (*Mesocricetus auratus*). Although they are easily housed and inexpensive, they are much harder to handle and more likely to bite if given sufficient opportunity. Hamsters have a cheek pouch, which provides a good repository for compounds needing mucosal irritation assessment.

Syrian hamsters are also a desirable tumor induction model for carcinogenetic assessments as they are more prone to develop certain types of tumors than mice. For example, induced pancreatic tumors in Syrian hamsters are both morphologically and biologically similar to pancreatic ductal adenocarcinoma in humans. Tumor formation in the cheek pouch addresses another cancer target, oral squamous cell carcinoma. The progression of the cheek pouch tumor formation occurs in a predictable fashion, which allows for various stages of human treatments to be assessed (e.g., early warning stage). Another common use for this model includes the research targeted in metabolic diseases such as diabetes and atherosclerosis.

A common use of Chinese hamsters is in the genetic toxicology arena. One of the attributes that makes these animals so valuable is its large chromosomes and low chromosome number. Chinese hamster's bone marrow has been used for cytogenetic testing, while Chinese hamster's ovary cells are used to identify compounds that may induce chromosomal damage by employing the chromosomal aberration or sister chromatid exchange test.[110]

Minipigs

All domesticated pigs originated from the European boar (*Sus scrofa*). Early records indicate that domestication took place around 9000 years ago. Miniature pigs (including the micro pig) developed as purpose-bred animals as they are a more desirable research model when compared to a farm pig due to the reduced requirements for housing, feeding, and, most importantly, the amount of test compound needed for dosing. In addition, the smaller size made them easier to handle for testing purposes.

The minipig (Göttingen Minipig or Hanford Miniature Swine) has been used for years as the standard nonrodent preclinical model in the development of dermal products due to their nonpigmented skin, sparse hair growth, and similar anatomy to human skin. The minipig also has similar characteristics to humans in their gastrointestinal (GI) tract, cardiovascular system, and urogenital system, making them a desirable nonrodent model in the development of therapeutics delivered by routes other than dermal. GI tract similarities include the stomach and small intestine anatomy (similar pH changes and transit time), and similar gastric cell types. They are an excellent cardiovascular model due to the similarities in the structure of the heart and great vessels, and blood flow in the coronary arterial system is almost identical to that of humans. The anatomy of the kidney is more like that of humans than other common research model and renal cytochrome enzymes have similar activity to their human counterparts.[89] The urinary bladder is also very similar to that of humans and the minipig urinary tract provides a good model to evaluate urinary incontinence and renal hypertension.

In addition to the Göttingen minipig and the Hanford™, other common breeds include the miniature swine, Sinclair™ miniature swine, Yucatan, and micro-Yucatan™ miniature swine.[113] A comparison of the body weight growth of each of the different breeds of minipigs is shown in Figure 20.1.

Minipigs have several distinct advantages over both the dog and the primate in preclinical research. For example, they tolerate nonsteroidal anti-inflammatory drug (NSAID) and antihypertensive drug better than dogs and they are not as prone to exhibit vomitus or emesis as seen with dogs.[114] They are generally friendlier than primates and, thus, are easier to handle and dose. In addition, they are often the nonrodent model of choice among European laboratories due to existing ethical concerns over the use of dogs and primates in those countries.

Nontraditional Species

While the more common animal models discussed earlier are most often used in toxicology studies, a nontraditional animal model may be more appropriate as a predictor of toxicity to humans due to specific anatomical, physiological, pharmacological, or metabolic characteristics. In addition, toxicity data from nontraditional, environmentally relevant test species are used and required to support environmental assessments, safety assessments of chemical products, and the safety of environmental species from the release of treated wastewater effluents. The goal of employing such nontraditional animal models would be to most closely emulate the potential human response to a particular pharmaceutical therapy that targets a certain disease state, or as the surrogate to evaluate against environmental toxicity concerns. The major drawbacks in using nontraditional animal models as a toxicity test species are related to acquisition and husbandry issues, background

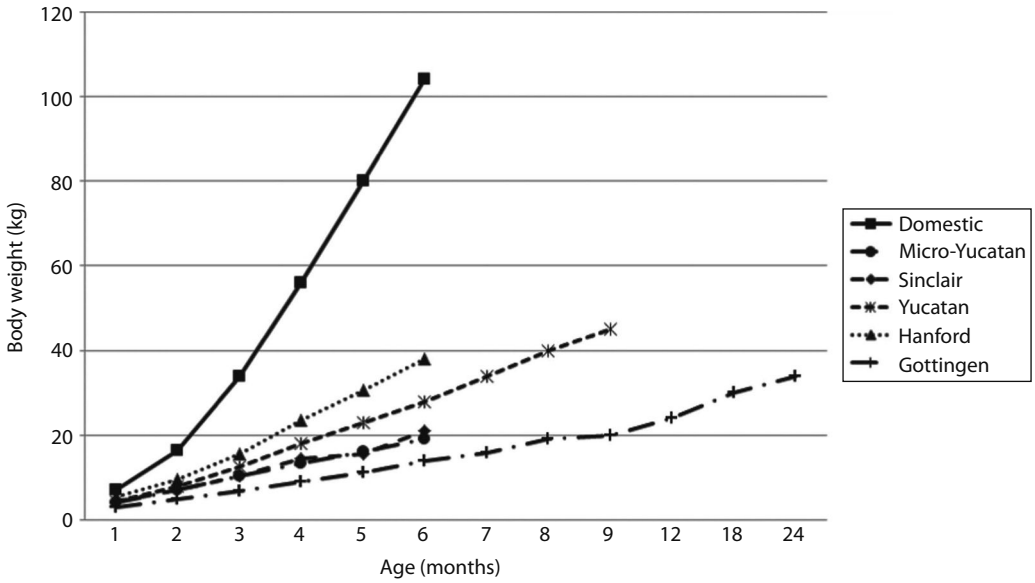

FIGURE 20.1 Comparative growth between domestic (farm) swine and miniature swine breeds. (Adapted from *Growth Chart Comparison*, [Chart on the Internet], Sinclair Bio-Resources, Cited June 28, 2012. Available from http://www.sinclairbioresources.com/. *The Gottingen Minipig Growth Curve*, [Chart on the Internet], Marshall Bio-Resources, Reference Data Guide, 2012, Cited June 28, 2012. Available from http://www.marshallbio.com/.)

TABLE 20.8
Nontraditional Animal Models Commonly Employed in Assessing Human Safety to Experimental Pharmaceutical Therapeutics

Research Emphasis	Species	References
Auditory structure and function	Chinchilla (*C. lanigera*)	[85,115,116]
Emetic response, diseases of the respiratory system (e.g., human influenza, SARS, H1N1, cystic fibrosis, and lung cancer)	Ferret (*M. putorius furo*)	[85,117–119]
HBV and associated hepatitis and hepatocellular carcinoma	Woodchuck (*M. monax*)	[85,120,121]
Leprosy and reproductive studies	Nine-banded armadillo (*D. novemcinctus*)	[122,123]
Congenital and hereditary disease, carcinogenesis, infectious disease, inflammation and wound healing, immunological disease, metabolic disease, endocrine disease, nutritional disease, psychological and behavioral abnormalities, and toxicity and poisoning	Zebrafish (*D. rerio*)	[124–126]
Environmental assessments for new human pharmaceutical agents or animal health products	Rainbow trout (*O. mykiss*)	[127,128]
	Daphnids (*D. magna*)	
	Algae (*S. capricornutum*)	
Safety assessment for chemical substances	Sediment/soil organisms and microorganisms	[129]
	Aquatic and terrestrial plants, amphibians, fish, daphnids, insects, and birds	
Regulatory permits for wastewater	Fathead minnow (*P. promelas*)	[130]
	Daphnid (*C. dubia*)	

knowledge on disease processes in these species, lack of robust historical control data, and the degree of concordance with human pharmacology. Table 20.8 lists the more common of these models. Additional aspects of these animals are described in the following sections.

Chinchilla

The chinchilla (*Chinchilla lanigera*) is a rodent native originally to South America that resembles a squirrel in size and appearance. It has been valued for its fur since the late nineteenth century and is a popular pet species.[115] As a toxicity test species, it has been widely used as an animal model for auditory experiments and in other studies in which the structure and function of the ear are of interest. This is due to its ear structure (cochlea) being similar in size and structure to the human cochlea, and an auditory sensitivity that is also similar to that of humans.[85,116]

Ferret

The ferret (*Mustela putorius furo*) is a small carnivore native to Europe that has a long slender body with predatory instincts, and is a popular pet species. It has similar oropharyngeal, gastric, and lung physiology as humans, and large lungs with a capacity about three times as predicted for their body size.[116,117] As a toxicity test species, it has been used to study emetic response in humans over the last three decades,[118] as well as respiratory diseases such as human influenza, severe acute respiratory syndrome (SARS), H1N1 (swine flu virus), cystic fibrosis, and lung cancer.[119] It has also been used in studies for nutrition research, gastrointestinal disease, cardiovascular disease, virus-induced neoplasms, bacterial infections, and viral diseases.[85,118]

Woodchuck

The eastern woodchuck (*Marmota monax*) or groundhog is native to North America, is a member of the rodent order, and can weigh up to about 30 lbs. As a toxicity test species, it has gained wide acceptance in the study of antiviral agents and immunotherapies, since it is prone to a naturally occurring woodchuck hepatitis virus (WHV) infection that is closely related to the human hepatitis B virus (HBV).[85] Woodchucks infected with WHV develop progressively severe hepatitis and hepatocellular carcinoma and are, thus, valuable animal models for pathogenesis and hepatocarcinogenesis. The response of the woodchuck to certain nucleoside analogs (i.e., fialuridine [FIAU]) appears to be identical to humans.[120] Further understanding was gained from their use after the unanticipated and delayed FIAU liver toxicity observed in a 1993 human clinical trial in which 5 out of 15 patients died. Although it has a low reproductive capacity, breeding colonies have been established and maintained in animal facilities allowing for controlled experiments and significant potential for molecular virology and immunological studies, and nonclinical drug safety assessment in antiviral, immunotherapy, or combination drug therapy.[121]

Armadillo

The nine-banded armadillo (*Dasypus novemcinctus*) is an insectivore found in North, Central, and South America. It is one of the few known nonhuman animals that can contract leprosy due to a low body temperature that favors the growth of leprosy-causing bacterium (*Mycobacterium leprae*). The bacterium cannot be cultured in artificial mediums and the armadillo is useful in propagation of the bacterium and as a primary test species for studying potential therapeutic agents

and vaccines.[122] The armadillo is also useful in reproductive research in which its reproductive system produces polyembryony or genetically identical twin eggs, that later separate into four eggs.[123] The group of four young are genetically identical and are useful in experiments requiring consistent biological and genetic makeup in the test species.

Zebrafish

The zebrafish (*Danio rerio*) is native to the streams of the southeastern Himalayan region.[124] They are popular tropical aquarium fish that can be raised in abundant numbers, and have advantages over traditional mammalian models such as small size, simple husbandry requirements, high fecundity, and transparent embryos, yet maintain many similar vertebrate phylogenetic features to humans.[125] With the use of genetic screening in the 1990s, the zebrafish became a mainstream model in developmental biology that led to its use as a model for human disease and drug discovery.[126] These disease models include congenital and hereditary disease, carcinogenesis, infectious disease, inflammation and wound healing, immunological disease, metabolic disease, endocrine disease, nutritional disease, psychological and behavioral abnormalities, and toxicity and poisoning.[126] The use of zebrafish as disease models also led to its primacy as a whole-animal model for screening lead therapeutic candidates in drug discovery.[126] Increased research in the area of zebrafish genetics is likely to lead to even greater understanding and further uses of this animal model in toxicology, pharmacology, and developmental biology.

Models for Environmental Toxicology

Environmental toxicity testing is a necessary consideration of the statutory risk assessment for drugs, chemicals, and wastewater.[127–130] Should toxicity data be needed to support an environmental assessment for a new human pharmaceutical therapy or animal health product, typical toxicity test species depend on the predicted environmental fate of the drug, but might include aquatic species such as fish (e.g., rainbow trout—*Oncorhynchus mykiss*), invertebrates (e.g., *Daphnia magna*), and plants (e.g., *Selenastrum capricornutum*), or terrestrial species such as microorganisms, earthworms, and plants. The rainbow trout is a representative cold water fish species of the Salmonidae family that is sensitive to many toxicants. The invertebrate, *D. magna*, is a daphnid or water flea that reproduces by cyclic parthenogenesis. The green alga, *S. capricornutum*, is a single-celled, crescent-shaped component of phytoplankton whose sensitivity to toxicants is measured by growth inhibition. Assessing the safety of chemical substances also depends on their predicted movement and fate in the aquatic and/or terrestrial environment, but typical ecotoxicity test species include sediment/soil organisms and microorganisms, aquatic and terrestrial plants, amphibians, fish, daphnids, insects, and birds. The safe release of wastewater from treatment facilities requires regulatory toxicity testing to support compliance for a given permit. In general, aquatic test species include fish (e.g., fathead minnows—*Pimephales promelas*) and invertebrates (*Ceriodaphnia dubia*). The fathead minnow is a warmwater fish species of the cyprinid family in which males guard and tend the eggs with the use of a fatty pad found on the dorsal side of the head region. The daphnid or water flea, *C. dubia*, is a sensitive test species to toxicants that reproduces by cyclic parthenogenesis.

Genetics

Maintaining internal breeding colonies is not recommended unless it is required for the purposes of the study or there is no alternative source of animals, such as with specialized transgenic strains. However, even in these cases, it can be advantageous to outsource to commercial vendors who specialize in transgenic and common purpose model colony breeding and maintenance. Outbred stock colony maintenance can be particularly complex and time consuming. Special breeding protocols must be in place to avoid genetic bottlenecking of the colony while also avoiding overproduction of animals. For information on breeding colony management, the reader is referred to published resources.[131,132]

Even when using commercial vendors for animals, there are certain aspects of genetics that are important for scientists to understand. The genetic makeup or genotype of an animal is a primary determinant of how it will respond to experimental conditions. The two aspects of the genotype are the presence of specific genes or alleles and the relative heterogeneity of these alleles within a population.

Animals can be classified as either *inbred* or *outbred* based on their genetic heterogeneity. A truly inbred animal is one that has at least 98.7% concordance at all genetic loci among all individuals in a population. Typically, the only fully inbred animals used in research are rodents. Inbred strains of rodents have been developed through identification of a desired phenotype of genotype, and then performing brother–sister mating for 20 or more generations.[132] A risk when developing inbred animals is that a phenotype will be found that causes significant health problems or is lethal when maximum homogeneity is achieved at all genetic loci. However, inbred strains of rodents are useful when there is a specific phenotype of animal desired and relatively little variation desired among individual animals. Even with fully inbred animals, there are some differences in phenotype among individuals due to ongoing mutations[132] and due to epigenetic and environmental factors affecting development.[133,134]

In general, inbred strains of animals are not used as commonly in toxicological research as they are in discovery research. The inbred state does not represent the norm in clinical populations, so these animals are more useful for modeling specific disease conditions than in screening for toxicity within a population. However, if specific mechanisms of toxicity are known, inbred models can be useful for screening test articles for these types of toxicity as they are potentially more sensitive than an outbred model due to lower individual animal variability.[135,136] There is the potential to use fewer animals to obtain data with lower exposure levels and shorter study duration.

Institutions should verify that vendors are monitoring inbred strains to ensure genetic homogeneity is maintained. This can be done through phenotypic monitoring of key physiologic characteristics of the strain, biochemical monitoring for key isoenzyme characteristic of the strain, or genetic monitoring for specific DNA sequences using restriction fragment length polymorphism (RFLP) or single-nucleotide polymorphism (SNP) methods.[1,12,137,138] If research institutions perform their own breeding, inbred animals are easier to maintain than outbred animals, as only a small population of breeding animals is required. Some effort must be made to prevent genetic drift of the strain from the original animals as it only takes a few generations for subline divergence to develop, particularly if there is any type of selective pressure placed when selecting breeding stock to maintain the line. This can be achieved through selecting a common parent cage to maintain a full-sib-mated inbred colony every five generations.

Outbred animals are those with heterogeneity at multiple genetic loci. This is generally thought to better represent a clinical population, although this conclusion assumes that the genotype and phenotype of the model are relevant to the clinical population for which the animals are serving as the test system.[135] The term outbred is a relative term as all populations of recognized breeds or stocks of animals have some homogeneity that imbues them with their breed characteristics. This is expressed as an inbreeding coefficient that is calculated from the breeding history or pedigree of the population.[132] When evaluating vendor animals, a review of the breeding system and the method for tracking the pedigree should be performed. Phenotypic characteristics should also be evaluated to monitor and characterize an outbred population. Until recently, there was not a good method for monitoring the genotype of outbred animals because of the large number of genetic loci to be evaluated. Methods for monitoring heterogeneity through SNP analysis have now been developed for this purpose, and are useful for understanding differences between animals of the same breed or stock from different sources.[138]

If institutions need to maintain breeding colonies of outbred animals, they must plan for relatively large populations. In addition to starting with a large genetic pool of animals (25–50 breeding pairs of unrelated animals is recommended), care must be taken to prevent inbreeding when selecting animals to serve as future breeders to maintain the colony. Rotational or sectional breeding schemes have been developed for this purpose.[132] It is also important to select breeders randomly from animals that express normal phenotypic characteristics, as any type of selective pressure will result in changes in allelic frequency that rapidly spread through the population (S. Wildt, Harlan Laboratories, personal communication).

Preventative Medicine

Preventative medicine is particularly important with research animal colonies. The presence of disease creates an experimental variable, and treatment for disease can also be a variable. In addition, whenever large numbers of animals are maintained in close proximity, if a disease enters the facility it can spread quickly throughout colonies. Thus, it is very important to prevent disease from occurring in the first place. Institutions need to evaluate the risk of exposure of animals to specific diseases and the consequences of the disease if it infects the animal colony. Strategies for preventing diseases can include purchasing animals free of the organism, vaccinating, or administering anti-infective agents or chemoprophylactic drugs. Preventative medicine programs for different species are published in the veterinary literature. However, it is important to understand the reservoir of the disease and mode of transmission, as many diseases relevant for animals in nonresearch settings are not relevant to animals maintained indoors in closed colonies. This is particularly true for diseases transmitted by insects and wildlife, and parasitic agents with complex life cycles.

Animals may need to be housed under quarantine conditions when they arrive at the research facility until they are tested and shown to be free of diseases, after which they may be housed with other animals in the facility.[12] However, if animals are purchased from trusted vendors who regularly perform testing for common pathogens, they may be brought in without a quarantine requirement. Even if animals have been tested for pathogens by the vendor, it is common practice to test large animals for intestinal parasites after they have arrived in the facility. Rodents and nonhuman primates may be tested for a variety of viral and bacterial diseases and animals may be administered antiparasitic or antibiotic drugs to eliminate common organisms. Vaccines, on the other hand, are not commonly used in research institutions since animals are usually vaccinated against important diseases by the vendor and revaccination is not usually necessary for animals in a closed research colony. One exception is that dogs and cats are commonly vaccinated against rabies in the research environment. The risk of exposure to rabies is negligible in laboratory-housed animals, but most municipalities require evaluating animals for rabies following a bite if they have not been vaccinated, and the only definitive test for rabies requires submission of a brain sample.[139]

Ongoing testing of nonhuman primates for tuberculosis is standard within the industry. The risk of acquiring new infections while housed in the facility is low, but tuberculosis can often be subclinical in nonhuman primates and animals may be anergic to the organism, which results in a false-negative test result. Therefore, repeated testing has evolved as a strategy for ensuring the disease is excluded from the facility.[140]

Rodent colonies are tested for a number of organisms through the use of sentinel animals. Sentinel testing should be performed at least quarterly, or more often if the risk of disease introduction is high due to multiple sources for animals or the presence of breeding colonies that can support endemic infections. Sentinels are typically provided with soiled bedding from other animal cages in the room every time waste is removed from them. These sentinels may be extra animals purchased when the study was initially set up

so new animals are not introduced into an occupied room, reducing the risk of introducing organisms into the room. However, as sentinels age they are more likely to produce false-positive results due to immunologic cross-reactivity with the assay.[71,141] For long-term studies, the use of new, younger animals for each monitoring interval may be used if care is taken to ensure that the animals introduced into the room are themselves free of disease.

Another aspect of preventative medicine is monitoring animals for problems that develop as they age or are used in multiple studies. Animals may require dental care, trimming of nails or hooves, physical examinations, and clinical pathology evaluations to ensure they are healthy and suitable for study use. This is also important for animals with chronic, indwelling catheters or instrumentation. In addition to the maintenance of instrumentation itself, it can be beneficial to periodically perform a physical examination and selected clinical pathology to assess for the presence of subclinical infection or organ damage.[142]

Husbandry

Feed

Diet has the potential to be an important variable to toxicology studies; however, it typically is not due to the commercial availability of properly formulated, well-controlled diets. These diets are formulated to meet the nutritional requirements for each species, have defined nutritional levels, and can be certified by the manufacturer to be free of contaminants. Facilities typically rely on manufacturer certification and analysis to ensure diet quality, however, contaminations can and do occur in diet production and may not be detected by the periodic analysis performed. Therefore, detecting these incidents is heavily dependent on manufacturer quality control procedures. These procedures should be carefully reviewed when qualifying diet vendors.

Typical dietary contaminants can include vermin, pesticides, mycotoxins, heavy metals, and microorganisms.[1,12] Chemical contaminants are minimized by manufacturers through appropriate screening and selection of raw materials. Diet manufacturing methods often involve heat treatment that effectively minimizes the levels of microorganisms and vermin. Diets can be sterilized through autoclaving or irradiation, if required for immunodeficient models or specialized studies. Sterilization can affect the nutritional level of diets as some vitamins are heat labile. Facilities should ensure that they are using diets formulated for sterilization so deficiencies do not develop.[12,143–145]

Animal nutritional needs change with age and reproductive status,[143–145] and diet manufacturers typically produce *life stage* diets appropriate for different physiologic states including growth, reproduction, and aging. However, this approach is not commonly used in toxicology research. There appears to be a focus on eliminating diet formulation as a variable in studies rather than controlling body condition and energy balance. Facility managers typically select a diet that is a compromise between the higher energy and protein requirements required for growth and lactation and the lower levels needed as animals age. This likely is not an important variable for short-term studies using young animals, but it has been shown to be important as animals age, since excessive energy and protein levels affect the incidence of neoplasia, renal failure, and obesity, with its associated health problems.[146] Obesity also increases the amount of test article needed for an animal, and can introduce variability into drug metabolism and distribution because of the greater body fat compartment.[147]

Methods for managing the effects of excessive energy and protein levels have been developed that involve food restriction.[148,149] This is effective but can be costly because of the involved labor. Use of an appropriate diet for aging should be considered as an appropriate alternative, particularly where vendors can produce a line of diets modifying only selected components to adjust for age and reproductive state while leaving the remaining components fixed.

Most standard diets used in research are natural ingredient diets. That is, they use agricultural products, each of which contributes several dietary components to the final formulation.[144] Diets with purified ingredients providing only a single component are available and may be used for some studies. However, these diets are extremely expensive and relatively unpalatable, so they are not used for most purposes.

The presence of certain nonnutritive dietary constituents in natural ingredient diets can be variables in some studies. Nitrosamines are present in fish meal, which is an additive providing protein and omega fatty acids. Nitrosamines are known to be carcinogenic and should be avoided in diets used for oncogenicity testing.[150] Phytoestrogens are naturally occurring selective estrogen receptor modulators present in agricultural products typically used in diets, including soybean and alfalfa.[151] Scientists should determine whether the effects on reproduction and estrogen-responsive tumors are relevant in their studies and consider diets controlling the natural variability of phytoestrogens in natural ingredient diets, or use diets that restrict the use of these ingredients.

Dietary fiber is very important for proper gastrointestinal function in some species of animals. For rabbits, guinea pigs, and ruminants (sheep, goats, cattle), it is important for motility of the gastrointestinal tract and may prevent stasis and overgrowth of *Clostridium* sp. and other pathogens.[152–154] In nonhuman primates, fiber appears to be important for the management of episodic diarrhea common in these animals.[155] Dietary fiber can be managed to some extent through fiber content in commercial pelleted or biscuit diets. However, there appears to also be a requirement for physical *roughage* that can only be supplied through whole agricultural products such as hay, vegetables, and fruit. While animals can be maintained without this additional source of fiber, the incidence and severity of gastrointestinal problems seem to be higher than in animals that do not receive these items. A common concern with the use of agricultural products for dietary supplement is that they typically are not available commercially in palatable forms with defined nutritional content and certified to be free of contaminants.

Facilities can run their own analyses to define these parameters, but this is expensive and of limited value as there is also no control over the supply chain of these types of items and it is not practical to test *lots* that went through the harvest or preparation process as a unit. While control of nutrition and contaminants is important, if these types of additives are fed in small amounts it is unlikely they significantly impact the overall nutritional status of the animal or contribute to study variability. They are normally sourced from suppliers for human and agricultural animal use and typically are considered safe for consumption. Ultimately, the benefits of maintaining gastrointestinal functions in animals must be weighed against such risks.

Animals may be fed ad libitum or a defined amount of food each day (meal feeding). Ad libitum feeding has the advantage of less labor than meal feeding and allows the animal to eat the amount it desires. There is the risk of the animal becoming obese with ad libitum feeding, particularly if a high-energy diet is used. Another risk of ad libitum feeding is that the food can absorb moisture in a humid environment and mold or spoil in between cage changes. Ad libitum feeding is most commonly used with rodents, younger rabbits, and dogs.

The advantages of meal feeding are essentially the opposite of ad libitum feeding. It is possible to control the amount of food, and prevent food wastage and obesity. Food does not spoil as the feeders are emptied daily. Meal feeding is typically used for livestock, nonhuman primates, and is often required for older rabbits and dogs. With meal feeding, slightly more than the minimum requirement is provided to ensure adequate nourishment. Energy requirements for animals at different life stages have been published.[143–145] Vendors provide information on nutrient and energy levels of commercial diets so that the amount of feed needed can be calculated. This can be adjusted as animals are monitored for food consumption, body condition, and weight loss. While meal feeding is more labor intensive than ad libitum feeding, as discussed previously, the benefits of managing an animal's body condition are often overlooked and may be needed if it cannot be managed through diet selection.

Animals are often fasted prior to oral dose administration to prevent food from interfering with absorption of the test article. Fasting is also commonly performed for the collection of serum chemistry samples to ensure that blood glucose and triglyceride measurements are not affected by a recent meal. Fasting is also performed in many species prior to anesthesia to prevent regurgitation, and prior to necropsy to minimize the amount of ingesta in the gastrointestinal tract. The typical duration of a fast is over night, or approximately 12 h. This is adequate for most purposes and is safe in most species.[156] However, clinical hypoglycemia has occasionally been observed by the author in neonates, small rodents, or nonhuman primates following several hours of fasting. Therefore, the age and metabolic status of animals should be considered when fasting animals, and animals at risk of hypoglycemia should be carefully monitored both clinically and glucose monitoring may be indicated. For larger animals, fasts of up to 24 h are generally safe, and for ruminants, fasting for up to 48 h may be required to prevent regurgitation.[157]

Feed can also be restricted for some behavioral models to encourage them to perform tasks for a food reward. The amount of feed is typically restricted to a percentage of the amount the animal eats when fed ad libitum or food is restricted empirically to achieve a percentage of normal body weight when the animal is fed ad libitum. Protocols vary but approximately 75% is a common percentage used for either method.[1,156]

Water

As with diet, drinking water can potentially contain constituents serving as variables in toxicology studies,[1] but with modern water systems, this rarely occurs. For most purposes, use of municipal water with only minimal additional treatment and testing is appropriate.[12] Municipal water is tested periodically using the EPA drinking water testing standard for contaminants such as minerals, heavy metals, pesticides, total bacterial counts (heterotrophic plate counts or HPC), and coliform bacteria. Animal facility drinking water is typically tested for these contaminants at a certified EPA testing laboratory at quarterly to annual intervals to assure no contaminants have been introduced by the facility water system. This testing is also typically performed when a facility is first qualified for use, after the water system has been shut down for maintenance, or if a part of the facility has been decommissioned and put back into use.

Many facilities will add filtration systems with 1–2 μ efficiency to drinking water systems to remove particles. If the water source is extremely hard (high levels of minerals), water softening may be used to prevent mineral accumulation on caging, in watering systems, and in cage washers. Softening of water does add minimal levels of salt to the water, which typically is not enough to affect cardiovascular or renal physiology in normal animals, but may be important when considering different animal models/species and/or study types. Additional filtration or treatment is not normally necessary for immunocompetent animals. However, some facilities will choose to install additional water purification systems, such as higher efficiency filtration (down to 0.2 μ), reverse osmosis, deionization, or distillation if it is necessary to achieve a higher level of purity.[12] Maintenance of watering systems to prevent bacterial growth is described in the section on sanitation.

There are specialized animal models that require water additives. Hypertension can be induced in Dahl salt-sensitive rats by adding salt to drinking water.[158,159] Animals with pituitary, thyroid, or parathyroid glands removed may require addition of salt, glucose, or calcium to their water.[160] In addition, antibiotics or anticoccidial drugs are sometimes added to drinking water to control microbes for veterinary purposes in immunosuppressed models[161] and tetracycline is used with inducible gene expression systems.[162]

Water is typically provided to animals ad libitum. Some research models limit the amount of water provided to the animal to encourage them to perform tasks. It is important to

monitor animals carefully so they do not become excessively dehydrated when limiting water.[1,156] Water is not typically removed when animals are fasted prior to dosing, anesthesia, or necropsy.

Contact Bedding

Bedding is added to the cage of animals to provide a substrate for nesting, thermoregulation, and absorbance of waste products.[12] Bedding benefits many laboratory animal species,[164] but its use is most emphasized in rodents. The thermoregulatory benefit of bedding is important for smaller animals, and it allows rodents to practice species-specific behaviors of nest building, burrowing, and shelter seeking, reduces alopecia, and elicits positive vocalizations.[12,163,165] For larger animals, the logistics of managing the large volumes of bedding needed often precludes its use except where it is critical to the success of the study. However, it may be specifically indicated, for example, with breeding, or to provide padding and footing to animals recovering from orthopedic surgery.

Bedding is typically made of naturally occurring or processed fibrous materials such as wood shavings, wood pulp, paper, cotton, straw, or hay. As with diet and water, it is important to ensure that bedding products do not introduce contaminants or other factors that can affect animal health or introduce variability to studies.[21,166–168] Animals will consume bedding, and continuous contact with bedding products means that animals can be affected by dermal contact and inhalation of particles or aerosols.[1] Because of this, the dust content of bedding should be minimized. Resinous woods such as pine and cedar should not be used for animal bedding because these resins are known to be inducers of cytochrome P450 enzymes that are a key drug metabolism pathway.[169] The source and preparation of paper products need to be evaluated to ensure that there are no residual chemicals that can have physiologic effects on animals.[167,170,171] Bedding can be certified for contaminants in the same way diet is certified. Many commercial bedding products are heat-treated as part of the drying process, which also minimizes microorganisms. However, bedding can be autoclaved or irradiated to fully sterilize it.

Different products vary in their ability to meet the different requirements of bedding material. Some products have been shown to be more effective at controlling waste products such as ammonia.[172] Others may be better for nest construction and providing shelter.[173,174] It is not uncommon for facilities to use several types of bedding in the same cage to optimize bedding function.

Historically, toxicology studies have tried to minimize the use of bedding in rodent cages because it has been thought to obscure clinical signs of toxicity, such as abnormalities in feces, urine, general behavior, and the integument and to prevent consumption of bedding.[175] However, as the use of solid-bottom caging with bedding has increased within the industry, discovery research environments, and toxicology studies run in European facilities, this concern is not considered as significant as it once was. Bedding in solid-bottom caging does not appear to limit detection of clinical signs,[176] and data are now commonly submitted for drug registration that was developed using animals in solid-bottom caging with contact bedding.

Likewise, the historical concern about maintaining rodents on contact bedding due to the reingestion of feces (coprophagy) affecting exposure levels of test articles has not been supported by data. Coprophagy is a normal behavior of rodents and rabbits, and normally the animals consume the feces as it is being expelled, so the use of mesh floor caging does not completely eliminate the reingestion of feces.[177–179] For complete elimination of coprophagy, use of specialized devices is required.[180] Mesh flooring may reduce coprophagy, as animals are frequently observed eating feces from the cage bottom when housed in solid-bottom cages with bedding. However, if it is assumed that the rate of reingestion is similar among treatment groups, coprophagy is not a true variable. This assumption may not be accurate in all cases, and scientists should be aware of situations where coprophagic behavior can become an important variable. Mesh flooring by itself only controls at most 50% of this source of test article.[177–179] If dose levels between groups are within this range, or if there are other factors that can skew coprophagic behavior, it may be an important variable. For example, if the level of active test article or metabolite in the feces is high, the presence of the test article may actually stimulate fecal consumption behavior (e.g., substance abuse, drug-seeking behavior), and if the difference in dose levels between treatment groups is narrow, it is possible that coprophagic behavior could result in enough variability in test article exposure to result in the loss of the ability to discern important effects between dose levels. These multiple *if* scenarios are the exception rather than the rule in toxicological studies.

Cleaning

Animal cages need to be cleaned regularly to remove waste. Large animal slat floor cages are typically sprayed out with water at least daily to remove feces and urine.[11,12] It is important to coordinate daily cleaning between husbandry and research staff so abnormal feces, urine, or emesis are not removed before these observations can be recorded. Bedding or papers placed in waste pans under wire-floor caging is commonly replaced two to three times a week. If bedded cages are used, they must be checked daily and excessively soiled or wet bedding removed. At least weekly all of the bedding needs to be removed, the floor sanitized, and bedding replaced.[12] Bedding may need to be replaced more frequently to keep cages dry and clean. Larger, older animals and diabetic animals, as well as guinea pigs, often require more frequent bedding changes because of the larger amount of waste these animals produce. There is some evidence that frequent cage changing can be a stressor by itself, and scientists need to consider this as a potential variable.[181] Exceptions to animal welfare standards for cage changing frequency need to be approved by the IACUC based on scientific justification. There would need to be clear data demonstrating that the effects of cage changing directly interferes with study goals such that it is not possible to interpret data by correcting for these effects for such an exception to be considered scientifically justified.

Social Environment

Animal welfare regulations and guidelines require social species be housed socially unless there is scientific justification not to do so.[11,12] Justification for an exception may be based on the need to monitor individual food or water consumption; cage observations such as feces, urine, emesis, or hemorrhage; the effects of social housing on behavioral assays; or cross contamination of test articles. It is important to make these justifications based on data rather than supposition, as some expected challenges with social housing have not been supported by the data.[182,183] For example, the ability to accurately identify clinical observations has not been shown to be adversely affected by social housing in solid-bottom caging.[176] Individual food or water consumption may be of limited value in interpreting toxicologic effects of a test article, because other data such as body weight and body condition of clinical signs of hydration are also collected.

Most species of animals used in toxicology research are social. However, the conditions under which they can be housed socially are not necessarily practical in a research environment. Territoriality and dominance can be a particular problem with male members of species such as rabbits, hamsters, some strains of mice, nonhuman primates, and livestock.[12,34] It is possible to socially house these animals if they were introduced prior to sexual maturity,[12,184] if adequate space is provided, and if animals have physical structure in the cage.[12,185,186] When exceptions to social housing are made on a facility-wide basis based on the inability to provide an appropriate environment to facilitate such housing, this should be approved by the IACUC as part of a social housing policy. Exemptions in social housing policies should be reviewed at least annually. Exceptions to social housing may be approved by a veterinarian on an individual animal basis to provide medical care, or because the animal is not compatible with others. For nonhuman primates, these exceptions need to be reviewed and reapproved at least monthly.[11] There are provisions for documentation of permanent exemptions for individual animals based on health or behavioral needs, however, these cases should still be reviewed periodically, as it may be preferable to remove an animal from a colony and replace it with an animal that can be housed in such a way that housing or social stress is minimized.

Social environment can also include housing of behaviorally compatible species in the same enclosure or area.[12] It is not uncommon to house agricultural animals in the same enclosure in farm settings. Mixing of species in the same enclosure or room is less commonly performed in research settings for a variety of reasons, including disease control and separation of study activities. Mice and rats have historically been considered incompatible, because there is thought to be a predatory effect of rats on mice.[12] However, it appears that animals may be able to acclimate to this effect as cohousing of mice and rats has not been shown to adversely affect reproduction in these species[187] and, in fact, is common practice with commercial rodent breeding facilities.

While there are challenges with social housing of animals, for most situations it has been shown to be compatible with toxicology study designs and provides significant benefits to animals.[188] Rats demonstrate a stress-like response when they are removed from a social environment.[189] Animals may have an improved ability to adapt to stressful situations when housed socially,[190] and social groups are important for environmental control such as thermoregulation.[191] Ad libitum–fed rats are less prone to weight gain than singly housed counterparts.[192] There tends to be reduced levels of abnormal behaviors in socially housed animals.[193,194] Dominance behavior can result in occasional fighting with subsequent skin lesions and excessive allogrooming that affects the hair coat and can result in food exclusion and weight loss of submissive animals. Dominance can also temporarily affect sex hormone production, which could be a variable in some studies.[195] With the exception of extreme aggression that can result in injury or death as with socially housed hamsters or male mice, typically, it is possible to interpret study data in light of these signs and there are numerous examples of regulatory approvals based on data collected in socially housed animals.

Environmental Enrichment

Environmental enrichment is often defined as creating a housing environment that stimulates species-typical behaviors in animals that are thought to reduce stress and enhance the welfare of research animals.[12] Enriched environments have been shown to improve animal learning and reduce cognitive loss with aging.[192,196] Social housing is one strategy for environmental enrichment and appears to be a more significant form of enrichment for social animals than environmental modifications.[189] The use of solid-bottom caging with bedding is another method of environmental enrichment that has been previously discussed. Besides social housing, and providing a substrate for burrowing and nesting, other types of environmental enrichment are listed in Table 20.9. It is not within the scope of this chapter to describe all of the specific examples of enrichment that are used and their relative benefits to the animal. This is a specialty area by itself within laboratory animal science, and the inherent nature of successful environmental enrichment is to provide the animal with variety,[188,197] so facility managers and veterinarians are typically trying different items out on a regular basis. It is common to supply animals with several types of enrichment rather than just a single form. Therefore, it is important that scientists consider the potential for data variability from enrichment items and provide direction through the study protocol on the items that may be used or should be restricted.

Manipulanda or toys are a commonly used form of enrichment.[12] There are relatively few risks of common manipulanda items on study data. Many toys are made of inert materials such as stainless steel or plastic. Plastics can be chewed and consumed by animals so it is important to use

TABLE 20.9
Types of Environmental Enrichment for Laboratory Animals

Category	Behavioral Benefits	Examples
Manipulanda	• Provides variety. • Promotes chewing behavior. • Promotes play behavior. • Promotes noise-making behavior. • Interaction time with items reduces time spent on cage exploration that can result in injury or escape. • Reduces excessive self-grooming.	• Plastic chew toys • Hardwood chew sticks • Balls • Metal cage toys • Puzzles and activity boards • Paint rollers and grooming boards
Visual and auditory stimuli	• Provides variety. • Promotes watching and listening behaviors. • Promotes social interactions when not socially housed. • Provides white noise to reduce reactivity to external stimuli. • Acclimation to human noises. • Provides cues for routine facility activities. • Reduces baseline stress levels. • Reduces reactivity to sudden changes in light levels.	• Room, cage, and toy colors • Music, audio books, and nature sounds • Television and movies • Ability to see and hear other animals • Full-spectrum light • Moonlight • Dawn/dusk lighting control • Fresh produce
Food variety and foraging	• Provides variety of flavor and texture. • Provides fiber. • Stimulates appetite. • Stimulates foraging behavior. • Increases time spent on eating.	• Grass hay and alfalfa • Grains and cereals • Prepared snacks • Certified treats • Frozen items • Puzzle feeders • Foraging mats
Environmental control and shelter	• Control over visual and physical social interactions with other animals. • Control over thermoregulations. • Control over light exposure. • Reduces baseline stress levels.	• Nesting material • Shelters • Opaque cage panels • Separate cage compartments • Perches
Exercise	• Promotes play behavior. • Maintains muscular, cardiovascular, and respiratory health. • Maintains appropriate glucose and fat metabolism and energy balance. • Reduces baselines stress levels.	• Social exercise pens with increased cage space • Climbing swings and perches • Running wheels

materials that have been shown to be safe either through contaminant certification or through historical use, which has shown no association with physiologic effects or toxicity. Rodents and rabbits typically gnaw on these items, and while ingestion of small particles of plastic has not been shown to cause gastrointestinal lesions,[198] this material is visible in excreta or ingesta and must be distinguished from abnormal findings. Dogs and nonhuman primates may not be discriminatory when chewing on plastic items, which can result in accidental ingestion and gastrointestinal trauma.[199] It is important to only use items that are too large for them to fit completely in their mouth and are not excessively worn or cracked so that large pieces cannot be ingested. Some manipulanda can result in entrapment and subsequent injury of animals, particularly if they are suspended from the cage structure.[193,200] In an effort to find manipulanda that animals maintain interest in, there have been some natural product manipulanda that have been used, such as certain types of wood[201] and whole coconuts. These items are not easily sanitized and usually are discarded when they become soiled.

Many animals are stimulated by visual and auditory stimuli. Visual stimuli in the form of television, movies, and colored toys, cage, and room surfaces are often used for nonhuman primates.[193] This type of enrichment is unlikely to cause variability in study data. However, it is important to monitor animal reactions as there are anecdotal reports of particular videos that animals react negatively to, such as those showing predatory behavior or violence. Auditory stimuli in the form of music, human voices, nature sounds, or even white noise are often used to reduce animal responses to common noises in the facility. It is not clear whether this serves only to desensitize animals to other noises, or if there is a positive benefit from auditory enrichment. Music has not been shown to be preferred by nonhuman primates over silence[202] or food enrichment (D. Gauvin, 2012, unpublished data). The type of auditory materials should be reviewed and

approved by facility management, as some types of music, particularly very loud or rhythmical music, seem to increase rather than decrease animal stress responses.[202–215]

Another method for providing environmental enrichment is to provide food variety and foraging opportunities. The animals typically used in research are adapted to seek and consume a variety of food sources to meet their full nutritional needs. Providing a balanced diet in the laboratory environment does not alter the drive to express this behavior. Foraging is a specific activity that does not involve just food variety but instead is the work that animals need to perform to obtain the food.[12,193] Animals with continuously growing incisions (rodents and rabbits) require hard food to chew on, or a hard chew toy to prevent teeth from overgrowing.[216] Food may be mixed in with nonfood substrates to increase the time the animals spend obtaining food. Nonhuman primates and other intelligent animals will utilize puzzles with food hidden in them.[193] The challenge for scientists, facility managers, and veterinarians is to find food enrichment items that meet these behavioral needs without introducing dietary variables to studies. Odors are also important to animals and animals respond to olfactory enrichment in ways both positively and negatively.[217,218] Transfer of soiled objects to clean cages may reduce stress and aggression associated with animals reestablishing territories.[219]

Animals also benefit from being able to exercise control over their environment. This can include providing the animal with the ability to control exposure to temperature, light, elevation, and visibility to other animals.[12] Enrichment items that give the animal control over its environment include bedding and nesting material, shelters, elevated perches or vertical space in cages, opaque panels in cages, and sections of caging that are made of thermoneutral materials or are warmed. These items can be extremely important to animals but their use may be restricted because they can make it more difficult to observe animals, remove them from caging to perform procedures, or make the cages more difficult to clean.

It is important to provide animals with the ability to perform physical activity, particularly activities that are normal behaviors for those animals. Social animals will play with, groom, and nest with others.[12,193] Even some amount of agonistic behavior to establish dominance is normal and should not be interfered with unless it adversely affects the health of the animals.[12] Rodents benefit from bedding because they can burrow in it and build nests.[163,220] The use of running wheels provided in the cage results in certain physiologic changes that are generally considered beneficial to the animal but do represent potential research variables.[221–223] It is required that dogs be provided with sufficient space to allow them to exercise, preferably in groups; enforced activity such as treadmill performance does not meet this standard.[11] Cats and nonhuman primates need to have the ability to perch or climb.[11,12]

Acclimation

Animals experience physiologic stress when they are transported and are placed in a new environment. Therefore, acclimation to the new environment is required after animals are received from a supplier before they are put into a study.[12] The amount of time needed for acclimation varies by study needs and the research procedure as different systems take different amounts of time to stabilize.

Animals typically take several days to adapt to a new diet and source of water.[224] Some institutions will attempt to match the diet they use with that used by the animal vendor to minimize the risk of dietary change. Water, however, cannot normally be matched. It may have different constituents that change the odor or flavor, which can affect palatability. The method of delivery can also differ. Animals that are not adapted to the use of automatic watering systems may have difficulty in adapting and facility managers have found that water bottles, bowls, or gelatin can aid to facilitate this transition. Even if they are adapted to automated systems, differences in water pressure or the pressure needed to actuate the water valve may affect animals' ability or willingness to drink.

Socially housed animals need to establish new dominance hierarchies. This can take as little as 1–2 days.[225–228] However, behavioral adaptation to the environment and all of the activities that are performed on the animals can take longer. Training animals for study procedures can take from 1 to 2 weeks.[229,230] The effects of stress on clinical pathology and physiological parameters can range from several days to nearly 2 weeks after transportation.[231–235] This also makes animals more susceptible to infection during this time and they are at risk of anesthetic complications, particularly within the first few days of transportation (S. Adrian, DVM, personal communication), perhaps due to decreased immune function, or perhaps due to direct effects of transportation.[236]

Research Procedures

Many procedures for creating an animal model, administering test article, and collecting data have the potential to affect the welfare of animals and the quality of the data derived from the model. Methods to control these variables are refinement alternatives. Animal welfare regulations and standards specify some aspects of the conduct of research procedures, but in not nearly the detail that is specified for housing and husbandry requirements. Best practices within the industry have evolved. Some of these have been published, but not all, so each institution has IACUC-approved policies and standard operating procedures (SOPs) for the conduct of these procedures at that institution. If the decisions made to create these procedures are not based on data from controlled experiments, but rather from experience and collective wisdom, this information is unlikely to make it into the peer-reviewed literature. In addition, many journals do not include details of procedural refinements in the Methods sections of articles, except in the few publications dedicated to laboratory animal science and alternatives (Table 20.10). Textbooks and abstracts from professional meetings can be useful references for this type of information. In addition, policies from public institutions tend to be available via the internet and it is common practice for institutions to review these when developing their own policies.

TABLE 20.10
Literature Resources for Standards and Refinement Alternatives in Research Procedures

Publication	Publisher	Internet Address
Alternatives to Laboratory Animals	Fund for the Replacement of Animals in Medical Experimentation (FRAME)	http://www.frame.org.uk/page.php?pg_id=18
Animal Welfare	Universities Federation for Animal Welfare (UFAW)	http://www.ufaw.org.uk/animal.php
Comparative Medicine	American Association for Laboratory Animal Science (AALAS)	http://aalas.publisher.ingentaconnect.com/content/aalas/cm
CAAT: Organization links, resources, and searchable database on published animal testing alternatives	Johns Hopkins Center for Alternatives to Animal Testing	http://caat.jhsph.edu/
ILAR Journal	Institute for Laboratory Animal Research, National Research Council	http://nas-sites.org/ilarjournal/
Journal of the American Association for Laboratory Animal Science	American Association for Laboratory Animal Science (AALAS)	http://aalas.publisher.ingentaconnect.com/content/aalas/jaalas
Journal of Investigative Surgery	Academy of Surgical Research (ASR)	http://www.surgicalresearch.org/
Laboratory Animals	Elsevier	http://www.lal.org.uk/
Lab Animal	Nature Publishing Group	http://www.labanimal.com/laban/index.html
Numerous Titles	National Research Council, National Academies Press	Lab Animal Research: http://www.nap.edu/topics.php?topic=316 Nutritional Requirements for Most Species: http://www.nap.edu/topics.php?topic=296

These internal policies essentially represent the refinement alternatives that the institution has identified that are relevant to the majority of work it conducts. Additional refinements may still be needed on a study-by-study basis. Scientists are required to indicate within their Animal Care and Use Protocol the refinement alternatives they have considered and the justification for not incorporating available alternatives into their study design.[11,12]

Physical Restraint

Many research procedures require the animal to be physically immobilized to perform them properly and to prevent injury, pain, or distress to the animals in the process. Chemical restraint can and should be used where appropriate (see section on "Anesthesia and analgesia") but is not scientifically appropriate, practical, or necessary for many situations.

The most basic and commonly used form of physical restraint is manual restraint. Animals are normally removed from their cages, manipulated, and restrained by one or more persons to complete a variety of procedures such as cage changing, examination, body weight collection, dose administration, blood collection, or transfer to other data collection devices. It is essential that personnel are trained in proper methods of handling and manual restraint.[11,12] The reader is referred to published resources for these methods.[1,237–239] For some animals and some methods of restraint, PPE is needed for the handler to avoid injury. Protective gloves are appropriate to protect against bites and scratches. However, they can reduce dexterity, which in some cases has the potential to prevent proper restraint from being provided. In the author's experience, if personnel are initially trained in animal handling with the use of protective gloves, they do not experience the same problems as those who are experienced in performing manual restraint without protective gloves who then try to begin using them.

Most animals require some amount of acclimation to manual restraint. Acclimation to manual restraint may begin with the handler talking quietly to the animal, offering positive reinforcement such as a food treat or stroking the animal when the animal approaches the handler, and then handling the animal for short periods of time while also providing positive reinforcement before proceeding to longer periods of restraint.[12] All animals benefit from this type of acclimation but it is particularly critical for larger animals that are more capable of escaping or injuring the handler.

In addition to manual restraint, there are a variety of restraint devices that can be used to restrain animals for longer procedures such as intravenous (IV) infusion or repeat blood collection. These can include restraint tubes for rodents and rabbits, slings for dogs and swine, and chairs for nonhuman primates.[240–244] Animals require acclimation to restraint devices to prevent excessive stress or risk of injury. Animals need to be monitored while they are in restraint devices to make sure that they are breathing normally and extremities are not entrapped in the device. Waste should be cleaned regularly to prevent animals from becoming soiled. If animals are to be maintained in restraint devices for an extended period of time, they may need to be provided with food or water and it is recommended that they be released for a period of time to allow normal movement. This is a specific requirement for nonhuman primates.[11]

There are methods for facilitating dose administration and data collection that provide relatively free movement of animals such as the use of jackets, wraps, and tethers.[245,246] Animals may need acclimation to these methods as with other restraint devices, and the animals need to be monitored

regularly to ensure that the system is not confining the animal or creating a risk of entrapment or injury.

Dose Administration

Administration of test articles has the potential for complications that can result in pain or distress to animals. Refinements in dosing procedures include controlling the volume administered, the pH, and the osmolarity to prevent tissue damage, hemolysis, pain, or inflammation.[244,247,248,251] Recommendations for dose volume and needle size parameters are listed in Tables 20.11 through 20.13. These factors are interdependent. For example, it is not advisable to administer a test article near either extreme of the pH range at a high volume. IV administration can be used for administration of test articles that are closer to the extreme end of the range for pH (between pH 2 and 11) or osmolarity (between 100 and 900 mOsm/L) compared to other parenteral doing methods because the test article is rapidly diluted into the blood and because the blood has significant buffering capacity. However, blood vessels are relatively fragile and repeated or prolonged administration of irritating test articles intravenously can cause significant inflammation or phlebitis. Intramuscular (IM) and intraperitoneal (IP) dosing routes are more tolerant of irritating test articles than subcutaneous (SQ) routes because blood flow to these tissues is higher, which absorbs the material more quickly to prevent development of inflammation. However, the volume that can be dosed IM or IP is lower and the pain associated with the injections is greater than with the SQ route. Enteral (oral or PO) dosing is relatively tolerant of pH extremes and can accommodate a relatively larger volume compared to parenteral routes. For parenteral routes of administration, the needle needs to be sized appropriately. A smaller needle is less painful to inject than a larger one, but may require longer time to administer the dose, particularly if the dosing material is viscous. Care must be taken to ensure that the parenteral doses are administered aseptically to prevent infection.

Oral dosing can be accomplished by administration of a dry form in a tablet or capsule, or by liquid forms through an orogastric or nasogastric gavage tube. Tablets and capsules are relatively easy to administer to dogs or cats, but are relatively difficult to administer to other species. Gavage is the most common enteral dosing method for rodents, rabbits, and nonhuman primates. A risk of gavage dosing is accidental intratracheal (IT) administration of the test article. This can result in foreign body pneumonia and may change the absorption kinetics of the test article. Perforation of the

TABLE 20.11
Dose Administration Guidelines: Dose Volume and pH

	Oral (PO)	Subcutaneous (SC or SQ)	Intradermal (ID)	Intraperitoneal (IP)	Intramuscular (IM)	Intravenous (IV)	Intratracheal (IT)
pH	**2–9**	**5–9**	**7.4**	**7.4**	**3–8**	**2–11**	
Dose Volume	**mL/kg[a]**	**mL/kg[b]**	**mL/site**	**mL/kg**	**mL/kg[b]**	**mL/kg[c,d]**	**(Total mL)**
Mouse	10[e]	10	0.05	20	0.05 (mL/site)	5–25	0.05
Rat	10[e]	5	0.05	10	0.1 (mL/site)	5–20	0.1
Ferret	5	5	0.1	1	0.25	2.5–5	
Nonhuman primate	5	5	0.1	1	0.25	2.5–5	
Cat	5	5	0.1	1	0.25	2.5–5	
Rabbit	10	2	0.1	5	0.25	2–10	0.5
Dog	5	5	0.1	1	0.25	2.5–5	
Swine	10	1	0.1	1	0.25	2.5–5	
Sheep and goat	10	5	0.1		0.25	2.5–5	

Sources: MPI Research IACUC and as adapted from Turner, P.V. et al., *J. Am. Assoc. Lab. Anim. Sci*., 50, 614, 2011; Diehl, K.H. et al., *J. Appl. Toxicol*., 21, 15, 2001.

Note: Blank cells indicate no data or no specific recommendation—refer to similar size species.

[a] Gavage dosed 4 times at any one dosing interval, 12 times total per day.

[b] Volumes over 5 mL should be divided into two injection sites, maximum of two IM sites/day and three SQ sites/day.

[c] Low end of range for bolus, high end for slow infusion (up to 30 min).

[d] 10 mL/kg/h over 6 h, 4 mL/kg/h over 24 h.

[e] 4 mL/kg limit for 100% oil vehicles.

Other dose routes

Intranasal dosing: pH > 3, 0.05 mL total volume in mice, 0.5 mL total volume in other species.[251,337]

Intracerebroventricular dosing: Mice pH 7.4; volume 2% of brain volume.[336]

Intrathecal dosing: 25 µL total volume in mice,[338] 1 mL total volume in nonhuman primates.[339]

Epidural: 0.15–0.2 mL/kg, maximum of 6 mL total in animals up to 35 kg.[244]

Ophthalmic: 0.03–0.05 mL in rabbits.[336]

TABLE 20.12
Dose Administration Guidelines: Capsule Dose Guidelines

	Single Dose	7-Day Dose	28-Day Dose		
Species	Maximum Number of Capsules	Maximum Number of Capsules/Day	Maximum Number of Capsules/Day	Minimum Body Weight (kg)	Maximum Capsule Size
Canine	10	8	4	6.0	12
	10	8	4	20.5	11
	10	8	4	27.0	12el
	10	8	4	32.0	10
Macaque	3	2	1	3.5	00
Mini Pig	1	1	1	9.0	13
	1	1	1	15.9	12
Rat	1	2	2	0.2	9 or 9el

Source: MPI Research IACUC and as adapted from Torpac, Inc., Capsule Size and Weight Chart—Large Animal [Chart on the Internet]. Cited March 6, 2014. Available at: http://www.torpac.com/Reference/sizecharts/Capsule%20Size%20By%20Species%20Weight%20Large%20Animal.pdf; Capsule Size and Weight Chart—Small Animal [Chart on the Internet]. Cited March 6, 2014. Available at: http://www.torpac.com/Reference/sizecharts/Capsule%20Size%20By%20Species%20Weight%20Small%20Animal.pdf.

TABLE 20.13
Dose Administration Guidelines: Recommended Needle Sizes for Blood Collection and Parenteral Dose Administration

Location	Rat	Mouse	Rabbit	Canine	Primate	Ferret	Swine	Primate
SQ	26G	26G	26G	21G	21G	25G	21G	23G
IV	26G	27G	25G	21G	21G[b]		21G	23G
Indwelling catheter	24G	24G	24G	22G	24G	24G	22G	24G
IM	26G	26G	25G	23G	25G	25G	21G[b]	25G
IP	26G	26G						
Jugular	23G		22G	21G			20G	
Cephalic				23G		23G		
Saphenous			23G	23G				
Tail vein	21G[a]							
Central ear vein			25G					
Femoral vein/artery					3 mL syringe—23G 5 mL syringe—21G			23G
Cranial vena cava						23G		
Sublingual	25G							
Cardiac	23G	23G						

Source: MPI Research IACUC.
Note: Blank cells indicate not applicable or no specific recommendation.
[a] Butterfly needle.
[b] Butterfly or straight needle.

esophagus is another risk of gavage dosing that results in localized infection and also alters the absorption of the test article. With enteral routes, selection of the appropriate gavage tube is important. A larger gavage tube will allow the dose to be administered more rapidly, but there is more risk of injury to the animal. Smaller tubes can be safer to use, but they are more flexible and may be harder to insert and guide into the esophagus. This can increase the risk of accidental IT administration. Rigid metal gavage tubes have traditionally been used in rodents, but there may be a greater risk of esophageal perforation when used by inexperienced personnel than newer flexible plastic designs. However, some individuals find it harder to appropriately direct a flexible gavage tube and feel the risk of IT instillation is higher with a flexible tube than a rigid one. It is also possible for flexible tubes to be bitten off by the animal and the cut end needs to be retrieved from the esophagus, possibly with the use of anesthesia.

Test articles may need to be administered into tissues or spaces that are difficult to access, such as the bone marrow, a joint space, the CNS, a region of the heart, or other specific organs or tissues. Often animals must be anesthetized for these procedures, both because of the potential for pain with the method of administration and because of the need for complete immobility of the animal to allow precision placement. They typically also require a greater emphasis on aseptic methods because of the invasiveness of the procedure. Some of these methods benefit from the use of imaging technologies such as fluoroscopy, ultrasonography, and even magnetic resonance imaging to ensure precise delivery of the test article.

For some studies, the test article must be administered very frequently or continuously to achieve and sustain the desired level of systemic exposure. Because frequent dose administration increases the risk of dosing error or injury with each dose, indwelling catheters are indicated for this purpose. Catheters are typically placed intravenously, but they may also be placed into other tissues such as the SQ, intracerebroventricular, intrathecal, osteomedullary, or IP spaces; the gastrointestinal lumen; or intravascular with the tip localized to specific sites. Placement and maintenance of these catheters must be performed with appropriate anesthetic and analgesic protocols using aseptic technique. If indwelling catheters are exteriorized, they have a relatively limited duration of use because infections commonly develop in and around the catheter site. Animals also may not be able to be socially housed when an exteriorized catheter is in place, as there is risk of cage mates damaging the catheter or surgical site. However, the author has observed successful social housing of animals with exteriorized catheters when the animals were provided additional environmental enrichment to divert their attention from the catheter. The use of subcutaneously implanted vascular ports significantly improves the longevity of indwelling catheters. They require routine maintenance and there is still risk of infection, but it is lower than with exteriorized catheters.[249–251]

Vehicle selection is also an important factor in dose administration. Some vehicle components that are used to improve solubility of the test article or improve absorption may have adverse effects when administered.[244] For example, surfactants can result in histamine release.[244] This type of reaction can occur at low dose levels and can be serious when they occur. However, it is relatively easy to treat or prevent through the use of antihistamines. Other risks include tissue inflammation and osmotic damage at the site of administration or hemolysis with IV dosing.[251] A full discussion of vehicles is beyond the scope of this chapter and the reader is referred to publications on this subject.[244,247]

Because of the numerous factors when making decisions around dose administration, institutions should have centralized training resources or institutional policies to collect and share experience with dosing methods to ensure the best outcomes for studies and for animals.

Collection of Biologic Samples

Refinement of procedures for the collection of biological samples is also an important consideration for animal welfare and data quality. Many of the considerations for sample collection are similar to those for dose administration. Typically, blood and other body fluids are accessed through a hypodermic needle using manual restraint. But depending on the fluid space, volume required, and frequency of collection, indwelling catheters may also be used. Both exteriorized catheters and those with vascular access ports are appropriate for body fluid collection.[244] These catheters may require chemical restraint or anesthesia to implant, but then require minimal restraint for subsequent use. Technology for automated blood sample collection now exists that significantly reduces the labor associated with repeat sample collection.[244] Aseptic procedures are important for preventing infections when collecting biological samples by any parenteral route.[244]

There are numerous routes for blood collection, and the preferred method varies by the species, size of the animal, and the parameters being measured.[252] There can be some variability in clinical pathology parameters and test article distribution depending on the site of collection.[253–257] In most cases, these differences do not affect the interpretation of data as long as all samples are collected by the same method. However, it can be problematic when comparing results among samples collected using different methods. It can be done, but there is more intersample variability that reduces sensitivity for detecting significant differences.

Handling, physical restraint, and anesthesia can all affect clinical pathology parameters and other data.[1,258–265] This means that a scientist cannot avoid these effects completely, but must choose the method that has the least significant or variable effect on the parameters being measured. Modifications to standard procedures such as the use of a rapidly acting anesthetic like carbon dioxide inhalation, or a physical method of euthanasia such as decapitation, cervical dislocation, or microwave irradiation, may be needed for some endpoints. Indwelling catheters can be useful for the collection of this type of data as the animal may require minimal to no restraint. However, the effects of anesthesia and surgery on the parameters being measured can persist for up to a week after implantation of these catheters, and more study planning is required when using these methods (A. Aulbauch, Dipl. ACVP, ASVCP, personal communication).

Another source of variability in biological data resulting from the sample collection method is associated with tissue damage or contamination that can occur during collection. Needle collection of samples typically results in the least amount of tissue damage to the sample, but if a collection needle is inappropriately sized or the sample is aspirated too quickly, hemolysis can result, for example, with blood collection.[266,267] Hemolysis can interfere with the instrument reading of some assay results, but can also result in artificial elevation of intracellular constituents such as aspartate amino transferase, glucose, or alkaline phosphatase.[268] Some serum chemistry parameters can also be elevated through muscular injury from physical restraint (e.g., in fractious nonhuman primates) or sampling methods that cause leakage of intracellular fluid such as tail or retroorbital sinus collection.[269,270]

One method of blood collection that has created significant controversy within the laboratory animal field is retro-orbital sinus blood collection in rodents.[1,270] This is a method that was used historically without anesthesia, but in recent years animal welfare oversight bodies view this as a painful procedure that requires anesthesia. However, even with anesthesia, injury can occur to the eye or adjacent structures, which results in pain and distress to the animal, and may require euthanasia. These injuries can be treated with antibiotics and anti-inflammatory drugs, but this may be contraindicated depending on the study requirements. The incidence of these injuries can be reduced with some simple preventative measures, such as the use of appropriate size collection tubes, application of vasoconstrictors and direct pressure placed on the orbit to prevent hemorrhage, and early detection and treatment of any evidence of orbital damage.[271] Nevertheless, many institutions have policies restricting the use of retro-orbital blood collection unless there are no acceptable alternatives. Methods such as submandibular, saphenous, sublingual, or tail vein collection can provide useful samples in most situations.

It is important to manage the volume of blood and other body fluids collected to prevent health problems in animals and adverse impacts on study data. There are limited data to support volume standards for most fluids except blood; however, the considerations are similar. If blood is removed faster than the animal can replace it, anemia, hypoproteinemia, or hypovolemia can result.[252,270,272,273] Typically, these will have adverse effects on data quality before they affect animal health. While up to 40% has been removed in rats without adverse clinical effects,[252] clinical pathology data can be affected by removal of as little as 10% of the blood volume.[270] Variability in serum albumin can have significant effects on drug distribution and effects.[274] If larger volumes are required for assays, isotonic fluids or colloidal fluids may be administered to prevent the acute hypovolemic effects of blood loss.[272] However, this may require waiting for several weeks to allow blood constituents to be replaced before additional samples are taken. If large volumes of blood are required for an assay and it must be obtained from small animals, separate cohorts of animals may be required and the blood collected as a terminal procedure. As a general rule, it is safe to collect a volume of blood equivalent to 1% of an animal's body weight (i.e., 10 mL/kg) at a single time, or as a cumulative volume over a 2–4-week period.[252,275]

There is less information on guidelines for collection of other body fluids than for blood. Approximately 200 µL of joint fluid has been collected from rabbits[276] and 300 µL serially from larger species such as sheep and dogs (S. Adrian, DVM, personal communication). Aqueous and vitreous humor has been collected from swine at volumes of 200 µL.[277] Between 50 and 500 mL of fluid can be instilled and collected in dogs for bronchoalveolar lavage,[278] and a volume of 0.1 mL has been used in rats.[279]

Cerebrospinal fluid (CSF) is commonly collected for the measurement of test article distribution into the CNS and the biological effects of the test article. This procedure is typically performed on an anesthetized animal, particularly when collected from the cisterna magna. Collection from the lumbar region of livestock is sometimes performed on conscious, restrained animals using local anesthesia. At least one dose of postprocedural analgesia is recommended. Repeat collections of CSF may be performed using a percutaneous method, but there is risk of inflammation, infection, injury to the spinal cord, and hemorrhage into the CSF, which is detrimental to sample quality. In the author's experience, between two and four collections in an initial 24 h period are possible, and subsequent collections spaced several days to a week apart can be successful as there is sufficient healing of the collection site to prevent chronic traumatic injury. The risk of repeated anesthetic episodes is at least equal to the risk of direct complications from the procedure. Implantation of indwelling epidural or intrathecal catheters should be considered where frequent collection and uncontaminated samples are needed.

The volume of CSF that can be collected tends to be limited more by the small amount of intrathecal or epidural space and the practical aspects of safely collecting an uncontaminated sample, rather than by the risk of CSF depletion. Single collection volumes of 5–15 µL have been reported in mice[280]; 50–100 µL in guinea pigs and rats[281–283]; 1.5–2 mL in rabbits[1]; and 1 mL in rhesus macaques,[284] and 1 mL/5 kg body weight is recommended as a maximum volume in dogs, cats, and horses.[285] These volumes can generally be repeated within the recommended intervals as CSF production rates have been reported to range between approximately 2 and 13 µL/kg body weight/minute[286,287] in different species, and therefore, it is rapidly replaced. It is possible to exceed this rate of collection through a catheter, but there is little risk with percutaneous collection. One author describes replacement of collected CSF volume with saline.[284] This may be indicated to maintain intracerebral pressure when large volumes are removed, but this may affect the quality of future samples until the infusate is replaced by CSF.

In some cases, solid tissue samples may be required to assess the effects of a test article. Biopsy specimens can be collected as a survival procedure with the appropriate use of anesthesia, analgesia, and aseptic collection methods. Historically, repeated collection of intra-abdominal biopsy specimens, such as liver, was constrained by the potential for pain or distress due to repeat major survival surgery. However, the use of less invasive methods, such as endoscopic surgery or ultrasound-guided percutaneous biopsy, has allowed these samples to be collected in a manner that is consistent with animal welfare standards.[12]

Collection of some biological samples, such as feces and urine, does not directly impact the welfare of animals. However, in order to obtain uncontaminated samples, specialized caging may be required. This caging may not meet all of the regulatory standards for cage size and construction, or animals may require individual housing, therefore, specific approval by the IACUC will be required. Caging or housing that does not meet standards should be used for the minimum time necessary to obtain the needed samples, and animals should be returned to normal caging once the collection is complete.

Physiologic and Behavioral Monitoring

Monitoring animals for physiological or behavioral changes resulting from test article administration can present a challenge if the method of monitoring affects the parameters being measured or results in pain or distress to the animal. For example, cardiovascular and respiratory parameters, body temperature, and electronic activity of the heart, brain, or muscle all require instrumentation that is attached to, or implanted into the body to measure them. With acclimation, animals can be maintained in restraint devices while these parameters are measured. Sedation or anesthesia can be used for some measurements as long as the effects of the anesthetic used are known. However, the development of portable external and implantable telemetry has greatly improved the sensitivity of these measurements.[288] While surgical implantation of telemetry devices is invasive, appropriate postoperative care minimizes animal welfare concerns, and animal welfare is improved for subsequent research as there is no restraint required for data collection. In addition, the enhanced sensitivity of these systems allows safety assessments to be performed using lower dosages of drugs that would be required with a less sensitive system, which also represents an animal welfare refinement and provides data on subclinical effects of the test article.

Monitoring of respiratory parameters may require a device that encloses the head or the entire animal.[289] As these chambers may be restrictive, it is important to acclimatize animals to them and to monitor the animals during use. They must also be cleaned and disinfected appropriately so they do not serve as reservoirs for microorganisms that can cause disease.

Behavioral monitoring does not usually require direct instrumentation of the animal but may require housing that does not meet animal welfare standards.[290,291] This monitoring may need to be performed outside of the housing room due to the monitoring equipment being used or the need to isolate the animal from environmental variables that may be present in a general housing area. These monitoring facilities still need to meet the general requirements for animal housing in which they need to have adequate ventilation and environmental control and be kept clean and be sanitized periodically. However, because the number of animals and the duration of housing in these areas are typically lower than in a general housing space, the infrastructure needed to meet standards may be somewhat less involved. The cage space available in operant conditioning chambers may be less than that required by standards and manual cleaning may be required to prevent damage to the electronics. If a swimming test is used, the water needs to be maintained at a temperature that will prevent hypothermia.[291] Animals may need to be dried or provided heat at the completion of the test. Scientists need to have their veterinary staff and IACUC review these housing areas and monitoring equipment and approve them for use. Periodic inspections are performed in these areas as part of the semiannual facility inspection and program review.

Pain Research and Aversive Stimuli

Some research procedures are performed to assess physiologic mechanisms of pain, pain response, or use aversive stimuli to elicit certain behaviors in animals. Refinement of these procedures to minimize pain or distress is challenging as pain is an expected and necessary outcome. The level of pain created should be limited to that which can be reliably measured and that will respond to clinically relevant exposure levels of the test article. The painful stimulus should also not cause tissue injury unless chronic pain is the goal of the study. A model of chronic pain or inflammation need not cause more than localized clinical signs of pain to be biologically relevant. In fact, if the model is severe enough to result in signs of distress, such as weight loss or decreased activity, it is likely that the model will have more variability than desired because there are systemic effects of the model that are more difficult to control than local effects.[291]

Anesthesia, Analgesia, and Surgery

One of the key requirements of animal welfare regulations and standards is that surgical procedures are performed aseptically using appropriate anesthesia, and that analgesics be used following surgery and any time the animal experiences pain, unless doing so will interfere with the goals of the study.[11,12]

Surgery

Aseptic technique for surgery includes a variety of procedures, all of which work together to minimize the exposure of tissues to microorganisms and prevent postoperative infection.[12] Details of aseptic surgery requirements are provided in other resources but the general approach to asepsis is to prevent organism from the animals' skin, the surgeon, or the environment from entering tissues that are exposed during surgery. This begins with proper surgical facilities, as previously described, to isolate the operating room, sterile instruments, and the surgeon from the animal while it is being prepared. Animal hair needs to be removed and the skin prepared antiseptically to reduce the microorganisms on the skin. The surgeon dons clean clothing, a hair cover, mask, and sterile gloves to minimize exposure of the surgical site to skin bacteria, hair, saliva, and other contaminants.[12,292]

Surgical instruments, supplies, and any implanted devices need to be sterile. Sterilization is normally achieved by autoclaving, or with chemicals such as ethylene oxide, vaporized hydrogen peroxide, glutaraldehyde, or hydrogen peroxide/peracetic acid mixtures. Sufficient exposure time for these sterilization methods is critical to their success and sterility needs to be monitored for each batch and each item or package that is sterilized. Less effective antiseptics or disinfectants, such as alcohols, iodophors, or chlorhexidine, are not sufficient for sterilization of surgical materials because they do not eliminate bacterial spores.[12,292]

Surgical technique is also critical for maintaining asepsis. The surgeon needs to be extremely careful not to touch anything outside of the prepared surgical field. This can be

challenging when using specialized equipment for surgical conduct, monitoring, or data collection. This is not a one-person task. Having assistants to manage anesthesia and surgical equipment is critical. In addition to maintenance of asepsis, surgeons need to be skilled in general surgery techniques as well as the specific procedure being performed. Excessive tissue damage and extended surgical time result in devitalization of tissues, increasing the risk of postoperative infection.[292]

Historically, the application of these methods of surgical asepsis has been applied to nonrodent species without question, but rodent surgical technique has often been abbreviated, perhaps in part to accommodate the relatively high volume of nonrodents that are surgically modified for a typical study. However, rodents are equally susceptible to infection and suffer from the same physiologic consequences as any other species. In fact, they are the standard model for studying surgical site infections. Therefore, aseptic technique is just as important to the success of the model and maintenance of animal welfare with rodents as with nonrodents. The methods for achieving asepsis can be modified slightly and still be effective due to the smaller surgical field and shorter surgical times.[292] However, doing so puts particular emphasis on attention to detail and the skill level of the surgical team. To some extent, application of traditional aseptic technique is more forgiving of error than methods that are developed for production purposes.

Because of all of these requirements, surgical programs benefit greatly from being centralized within an institution rather than having each investigator attempt to establish the required facilities and maintain the equipment and skills needed to meet surgical standards.

Anesthesia and Analgesia

The need for anesthesia during surgery or other painful procedures is well established. Animal welfare standards are specific about the requirement for the use of appropriate anesthetic and additional monitoring when neuromuscular blocking agents are used during anesthesia, due to the risk of providing an insufficient depth of anesthesia and the loss of ability to monitor depth through reflex activity.[11,12]

Historically, there has been more controversy over the requirement for analgesics, particularly when performing surgery in rodents. This is likely because the signs of pain in rodents are not as well recognized as those in nonrodents and perhaps also because the relative moral value ascribed to rodents tends to be lower. However, rodents feel and respond to pain and are a standard model for pain research.[293]

A common argument against the use of analgesics following surgery and when animals are experiencing pain for other reasons is concern that the drugs used will interfere with study data interpretation. However, the consequences of *not* relieving pain are often overlooked. Pain has immediate and long-term effects on neurobiology, behavior, and cardiovascular function, some of which are well defined and some of which are not.[293] By contrast, analgesic drugs have relatively well-defined effects and typically only require administration for a short period of time to prevent or relieve pain in animals.[1,293,294] When used at therapeutic levels, their effects on the research model can be predicted and interpreted along with study data.

It may seem self-evident that analgesic drugs cannot be used with models of pain. However, this is not necessarily true for all models. For example, surgically induced neuropathic pain models may be provided with analgesia in the immediate postoperative period to relieve the acute pain of surgery.[295] The drug will be eliminated before data measurement begins as the condition takes a week to develop following surgery. Also if pain is resulting from an injury or condition that is not a planned part of the model, then the pain represents a variable that can adversely affect the quality of data from the model. Short-term treatment to remove this variable may be necessary for the long-term success of the model.

Veterinary Care and Euthanasia

While considerable effort is put into refinement of study procedures to minimize the potential for pain or distress in research animals, the ultimate goal of toxicology research is to study mechanisms and manifestations of serious adverse events associated with the test article. This means that at some point, many animals on these studies will reach the point where they require veterinary care or even euthanasia to relieve the pain or distress. As discussed previously, there must be scientific justification approved by the IACUC to allow animals to experience pain or distress without providing appropriate relief.[11,12] The approval is for pain or distress specifically related to the study that is inherent with the study goals. If pain or distress results from another reason, such as spontaneous disease, injury, or unexpected adverse events, the original IACUC approval may not include these outcomes as being scientifically justified reasons for withholding veterinary care or euthanasia.

Humane Endpoints

In order to accurately assess the toxicological effects of a test article and compare these effects across studies and different classes of test article, it is necessary to have an endpoint that is measurable and consistent. In the early days of toxicology research, one of the clearest endpoints was the death of the animal, and traditionally, the toxicity of the drug was expressed as a lethal dose. However, the advancement of science and the advancement of animal welfare have gone hand in hand. When it was recognized that microscopic examination of tissues provided important information on the mechanisms of toxicity, it became desirable to euthanize and necropsy animals rather than allow them to die spontaneously and allow tissues to degrade through autolysis. Currently, toxicity is often defined through clinical signs, changes in clinical pathology parameters, gene expression, and other biomarkers of changes at the systemic and cellular levels. These data can often be collected prior to the presentation of significant clinical signs that represent pain or distress in animals. When these types of parameters are used

to define toxicity, it is termed a *humane endpoint* and it represents a significant refinement alternative. Technically, the endpoint is the point at which the study data can be collected. In practical terms for a toxicity study, it usually means the point at which dosing is completed and animals are euthanized. Euthanasia is the ultimate way to prevent pain or distress in animals experiencing toxicity but veterinary care can be provided to relieve pain or distress to get animals past a point of acute toxicity to another study endpoint.

Humane endpoints should be applied whenever possible in toxicity studies and should be discussed in the application to the IACUC, particularly when describing justification for doses selected and the plan for veterinary care or euthanasia. Humane endpoints have been embraced by most scientific and regulatory agencies, and various standards have been published.[296,297] The key to applying humane endpoints is to understand when it is and is not scientifically valid to do so. Definition of an MTD for an investigational new drug (IND) application must take into account a number of considerations that make the clear definition of a safety study endpoint challenging. The initial presentation of toxicity may subside with repeated dose administration.[298,299] Therefore, it may not be appropriate to stop dosing in the face of adverse effects until it is known whether the effects will be sustained. For some test articles, such as antineoplastic drugs, the accepted safety margin is narrow and a certain level of toxicity is expected in animal studies. Animals may be dosed in the face of this toxicity so that underlying, additional toxicological effects can be detected. Another challenge with safety study endpoints is seen when trying to define the mechanism of toxicity but the clinical signs or clinical pathology are not definitive. This is often a problem when toxicity presents peracutely after dosing. Continued dosing may be needed to allow development of signs, physiological changes, or tissue lesions that will provide this information. An additional case that complicates humane endpoint determination is when the high dose on a study is showing significant signs of toxicity, but the mid dose is showing no signs. In this case, it may be necessary to reduce the high dose slightly to identify the dose–response level rather than discontinue dosing or euthanize the animals. The scientist may also choose to stop dosing an animal experiencing significant toxicity, but must determine if the toxicity is reversible and cannot euthanize the animals immediately. A final consideration is that it may be better to allow more severe toxicity to develop during studies that are early in the development of a particular test article so they can be thoroughly characterized, which then allows for better definition of a humane endpoint in later studies. Early stage studies typically use smaller numbers of animals, and are not generally conducted according to GLP standards. There is also some flexibility to allow collection of data that was not originally planned, which improves the mechanistic understanding of the cause of the toxicity.

Clinical Care

In all cases where pain or distress is seen in animals, there should be an attempt to provide veterinary care to relieve pain or distress in animals, but the specific care may be limited so that therapeutic drugs do not directly interact with the test article or obscure assessment of the mechanism of toxicity. Historically, veterinary care for animals on toxicology studies, particularly those run according to GLP standards, was severely limited by concerns about interference with the interpretation of study results. However, this is changing along with the mechanistic approach to characterization of the safety profile of a test article. The effects of standard methods of veterinary care are well understood because they were developed using the same process by which new test articles are evaluated and these data are published and available to help with interpretation of study results in light of the care provided.[300] Veterinary care normally only needs to be provided for a limited period of time to relieve pain or distress. For a longer-term study, the effects of several days of treatment are not likely to interfere with data interpretation. If they are, and if the animal cannot maintain a physiological balance without veterinary care, this is often sufficient to define the MTD for the test article. Finally, veterinary therapeutic drugs are administered at clinical dosages that are magnitudes of order lower than the dosages of the test articles that are causing toxicity. These drugs are not likely to cure the animal of the toxicity, but instead are intended to relieve the most serious effects of the test article and allow the animal to maintain a more normal physiological balance until it reaches the planned study endpoint. Veterinary care is not normally provided for minor clinical signs of toxicity, so the likelihood that it will obscure subtle evidence of toxicity is low. However, therapeutic levels of drugs could be a concern where the data collected are physiological in nature rather than toxicological, as with safety pharmacology, fertility, or combined efficacy/safety studies. However, even in these situations, the physiological effects of untreated disease, pain, or distress on study data must be weighed against the effects of the veterinary care. As previously stated, veterinary drugs are well defined, whereas the effects of unrelieved pain or distress can be extremely variable.[301]

Diagnostic tests may need to be performed to diagnose the nature of a problem an animal is experiencing, determine the prognosis, and develop a veterinary care plan. Diagnostic test results contribute to the study dataset, and therefore, scientists and veterinarians need to discuss this testing to ensure that it is collected in such a way that it can be appropriately interpreted. Some testing equipment may not be validated or results may be needed immediately to aid in the veterinary care plan without time for proper quality control checks. Interpretation of some tests, such as radiography or ultrasonography, is subjective, requiring professional veterinary interpretation that can be difficult to consistently repeat. These data are appropriate for the evaluation of animal health, but if there are findings that might add additional information to the study dataset, these tests may need to be repeated on all animals in the study using appropriately controlled and validated assays or equipment. Veterinary records and standard operating procedures should also be written so that it is clear that veterinary observations and diagnoses are

provisional, based on limited datasets, and that final interpretation is dependent on final collection of all study data and evaluation by the scientist.

The most common type of veterinary care that is provided to animals on toxicity studies is supportive care. This is normally nonpharmacologic nursing care that is provided to assist the animal in maintaining its own physiological functions. A common example of this supportive care is providing food items with greater palatability or enhanced nutritional levels to compensate for decreased food consumption. Food supplementation can, however, interfere with the interpretation of body weight and body condition data, and of course quantitative food consumption.

Animals may become dehydrated when they are not eating, or when there is vomiting, diarrhea, renal toxicity, or blood loss as a consequence of test article toxicity. Supplemental fluids provided subcutaneously, intravenously, orally, or through high water content gels or supplemental food items provide immediate and significant improvement in the animals' condition. Aside from fluid consumption measurement, the primary ways that fluid supplementation will interfere with data interpretation are with the detection of clinical signs of dehydration or changes in clinical pathology associated with hemoconcentration or fluid loss. Subcutaneously administered fluids can be mistaken for tissue edema at necropsy and the necropsy team needs to be aware of any veterinary care provided for this reason. Supplemental heat or nesting material to improve the animals' ability to thermoregulate can also be important for supportive care. This is unlikely to interfere with study data interpretation unless body temperature is the primary data on the study. Thermal burns are a risk with heat supplementation and if this occurs, it needs to be communicated to the pathology team.

Pharmacological veterinary care may be indicated for some conditions and study types. It is not possible to address all of the potential therapeutic protocols that may be used, but some common types of treatment are discussed here. While there are scant data in the literature on the potential effects of veterinary therapeutics on the interpretation of toxicological study data,[1] the mechanism of actions and safety levels of these drugs are well characterized[300] and educated decisions can be made when faced with a decision between providing veterinary care and having to remove the animal from study.

The use of analeptic drugs for animals experiencing seizures is indicated to gain immediate control of the situation and prevent injury or death. A choice needs to be made whether to continue dosing the animal at the same level with the hope that the animal will accommodate to this effect, to reduce the dose, or to stop dosing completely. Typically, a single IV dose of a benzodiazepine drug will control a seizure seen on a safety study. As exposure drops below the peak level, these signs normally resolve. If the effect is prolonged, a second dose of the analeptic drug may be indicated, or a longer acting drug like a barbiturate may be required. In the author's experience, if a seizure persists beyond the duration of a barbiturate, the animal is not likely to recover within the same day and euthanasia is indicated. It is generally not practical or scientifically necessary to provide prolonged intensive care for an animal with significant neurological signs.

Gastrointestinal toxicity and emesis may benefit from veterinary care with supplemental fluids, antiemetics, gastrointestinal protectant drugs, or antidiarrheal drugs. These treatments prevent the animal from becoming dehydrated, malnourished, or developing permanent secondary problems such as gastrointestinal hemorrhage or bacterial translocation and sepsis. These drugs are relatively short acting, and once the animal is stabilized, they may be discontinued and the drugs will be eliminated without persistent effects. Dosing may be continued while these drugs are in use but orally administered test articles need to be given several hours before or after administration of gastrointestinal protectant drugs or absorption will be reduced.

Antihistamines are indicated in the event of an anaphylactoid reaction to a test article or vehicle. Once anaphylactoid reactions have been seen, they will likely continue with each subsequent dose. It is not uncommon for antihistamines to be used prophylactically prior to dosing to prevent these reactions. Antihistamines that are rapid acting and have short half-lives such as diphenhydramine are available. They are used relatively commonly with general safety or immunotoxicology assessments of test articles and their use has not been identified as a significant study variable.

Antibiotics and other anti-infective drugs are often indicated in animals for perioperative surgical care, to treat spontaneous infections, or to prevent sepsis when animals are injured, are experiencing gastrointestinal toxicity, or immunosuppressive effects of a test article. As with other therapeutic drugs, considerable information is available on the pharmacology and pharmacokinetics of antibiotics. In addition, the available drug classes for antibiotics are perhaps wider than with any other type of drug, which allows scientists and laboratory animal veterinarians to work around specific restrictions for the test article that is being evaluated.

Anesthetics, sedatives, and analgesics are another group of drugs that are frequently used to provide veterinary care. There are multiple classes of anesthetics and sedatives available, which also allow them to be used with relatively little risk of interference with study data aside from CNS evaluations. There are fewer options with analgesics, particularly with drugs that have a long duration of action. This is desirable when the analgesics need to be administered to a large number of animals. The two primary classes of analgesic drugs used are opioids and NSAIDs. Opioids will impact the assessment of any test article that acts at the opiate receptor and can affect behavior, pulmonary function, gastrointestinal motility, and the immune system.[1,302] NSAIDs act on both the cyclooxygenase and lipoxygenase branches of the arachidonic acid metabolism pathway. In addition to impacts on test articles with activity on these pathways, NSAIDs affect platelet activity, can cause gastrointestinal mucosal bleeding, and in a dehydrated animal can increase the risk of renal toxicity.[302] Despite these challenges, analgesics are important for the relief of pain and distress in laboratory animals

and there must be clear evidence that the use of analgesics will interfere with data interpretation to scientifically justify withholding their use. It cannot be based on a possibility of interference.

Under some circumstances, a variety of other drugs may be used in laboratory animals. Corticosteroids may be required for dermal conditions, acute inflammation, immunologic reactions, or intracerebral edema. Drugs to treat abnormal cardiac function, blood pressure, pulmonary edema, or respiratory arrest may be indicated in emergency situations. Clearly, these drugs can have a myriad of effects on systems that may be evaluated as part of a safety study. Their use needs to be balanced against the risks of not treating the condition or losing the animal from the study.

Euthanasia

The final decision that typically must be made as part of veterinary care or refinement of a study is euthanasia of an animal that is experiencing pain or distress. Criteria for euthanasia of animals should be detailed in the IACUC-approved protocol or, in the case of unexpected signs of toxicity, generic facility guidelines for endpoints that balance animal welfare and scientific needs.

In some cases, the condition of the animal is such that euthanasia must be performed immediately to prevent the animal from suffering unnecessarily, and to collect viable samples for data analysis. It is important that any final data that need to be collected be clearly detailed in the study protocol or that there is communication between the scientist, veterinary staff, research staff, and necropsy team to determine if specific samples need to be collected before the animal is euthanized. In order to prevent delays in euthanizing animals that are in acute distress, it is important that trained staff are available at all hours to euthanize all species of animals that are being used, and that supplies and processing equipment for the samples that need to be collected are also readily available. If necropsy cannot be performed immediately, the animal should be placed into a refrigerator to preserve tissues until the necropsy team can complete the procedure. Freezing of the animal will damage tissues for microscopic examination. However, if microbiological or DNA samples are important, freezing may be indicated to preserve sample quality.

Methods for euthanasia need to be consistent with guidelines from the AVMA.[38] Euthanasia methods need to cause rapid unconsciousness before death and should not result in distress to other animals that may be present at the time of the procedure. Most euthanasia methods use an overdose of anesthesia to cause rapid depression of CNS function and cardiopulmonary arrest. For some studies, these drugs will interfere with endpoint data that must be collected and a physical method of euthanasia that rapidly disrupts CNS function may be needed (e.g., cervical dislocation, decapitation, or captive bolt). Because physical methods of euthanasia are more difficult to perform properly than an anesthetic overdose, and the inherent aesthetic concerns with them, scientific justification is required for their use, and procedures for training of staff in their use and maintenance of equipment are required.

The effects of exposure of animals to the euthanasia of other animals are controversial. If animals experience distress, pheromones can transmit stress responses to other animals.[1] However, the relative stress response associated with observation of euthanasia may be no more significant than that experienced during normal husbandry activities.[303]

Animal Reuse

Not all studies require euthanasia of animals to collect final data. Animals may be used for more than one study if doing so does not result in additional risk of pain or distress to the animal and if the prior use of the animal does not adversely affect the ability to collect quality data on a subsequent study. Appropriate reuse of animals represents a reduction alternative and is commonly employed with species that are costly or in short supply, as with nonrodents, or models that have specialized training or instrumentation. While reduction of overall animal use is in of itself an important goal, there is a direct trade-off between reduction and refinement, as multiple uses of animals expose them to repeated risks of pain or distress from study procedures and endpoints.[12] Animals are commonly reused for acute toxicity, pharmacokinetics, safety pharmacology, and behavioral studies and for staff training or blood donation for in vivo assay development. In order to minimize the risk of pain or distress that is compounded by reuse, it is important to periodically perform an animal welfare assessment on the animals and assure their suitability for continued use. This assessment should consider clinical signs, examination findings, alterations in organ function or clinical pathology that could result from toxicity in a study, the level of pain or distress experienced by the animal in a prior study, continued function of catheters or instrumentation, or the presence of medical or behavioral problems resulting from, or coincidental to the prior study use.

In order to reuse animals effectively from the standpoint of cost control, it is important to carefully manage these stock colonies. Maintenance of animal colonies is expensive and if the animals are not being used regularly, it may be more cost-effective to use them once and euthanize them rather than maintaining them until the next study. Each facility needs to analyze its own costs to make the determination of the maximum interval between studies that justifies reuse from a cost standpoint, but in the author's experience, animals should not be maintained if they are not being used at least several times in a year. Regular use of animals that are in stock colonies is also important for nonfinancial reasons. Animals that are not handled regularly can develop abnormal behaviors and become unsuitable for study use. It is also possible to overlook health problems in animals that develop in between their scheduled health assessments when they are not handled regularly. Ultimately, if there are animals in stock colonies that are not being used regularly, and they are consistently not meeting study selection criteria, it is preferable to humanely euthanize them rather than maintain them indefinitely. With a carefully managed colony, it is usually possible to find a terminal use for these animals, such

as donation of blood or tissues for in vitro assay use, or to provide staff training.

Some institutions have programs to retire research animals from their colonies without euthanizing them.[304] Options for retirement can include transfer to another institution for which they meet study requirements, and adoption to animal retirement centers or to private owners. Retirement of research animals fulfills an important emotional need in some staff, but retirement options must be carefully evaluated by scientists and veterinarians before being approved. It is not appropriate to allow animals to leave an institution if they have health or behavioral problems, or contain hazardous or proprietary agents that will affect their future use. Time may be equally well spent counseling staff members who have concerns about euthanasia of research animals on the reasons that it is indicated for a specific animal and providing ideas for appropriate redirection of their efforts to provide for the welfare of animals.

CONCLUSIONS

Animal welfare is an important issue for our society that has been expressed in the form of regulations and standards for the humane care and use of animals in research. It is important for research institutions to be able to communicate the benefits of the research being performed and the justifications for the way it is being done and to be able to address any potential concerns by both internal staff and the public. Public perception has direct effects on the ability to perform research using animals through the implementation of regulatory requirements and animal welfare standards.

When managed appropriately, meeting animal welfare standards also assures the quality of the data collected from the animals. The focus of animal welfare standards and regulations is to require that the use of animals be justified scientifically through the consideration of *replacement alternatives*, that the number of animals used be minimized through application of *reduction alternatives*, and that the pain or distress be minimized through the use of *refinement alternatives*. Because pain and distress are themselves a source of variability in study data, refinement of husbandry and study procedures improves the reliability of data derived from research animals. Refinement methods may be withheld only when there is scientific justification that their use will directly interfere with the interpretation of study data. This justification must be based on actual evidence or a clear mechanistic argument to support the justification, rather than just a supposition based on the possibility of unknown effects that could interfere with data interpretation.

Animal models used in the safety assessment of pharmaceuticals should be able to closely predict the potential for off-target effects. Through historical use, specific stocks and strains of rats and mice have been established as the most reliable rodent species for this purpose, and the dogs and nonhuman primates as the most reliable nonrodent species. Swine have been growing in use and offer certain advantages for safety assessment. As the understanding of various disease states and human and animal physiology increases, better animal models including genetically modified rats and mice, and nontraditional animal species such as chinchillas, ferrets, woodchucks, armadillos, and zebrafish are being employed to more accurately assess toxicity and predict safety margins of certain pharmaceuticals in humans. The use of environmental toxicity test species provides regulators with data for appropriate risk management decisions to aid in the protection of environmental species from harmful levels of pharmaceuticals, chemical products, and wastewater.

Because of the complexity of regulatory requirements for humane animal care and use and the considerable knowledge that has been accumulated regarding the needs of animals and the effects of study activities on animal health and welfare, institutions need specialists in these areas. Typically, an institution will require one or more veterinarians trained in laboratory animal medicine and personnel with knowledge of laboratory animal science to manage the compliance process, the facilities, and animal husbandry. While scientists may perform their own research procedures, there are significant advantages to having a technical resource staff to conduct studies and research procedures, and allow scientists to focus on study design and data interpretation.

Standards have been developed for all aspects of the humane care and use of research animals. These are codified in animal welfare regulations and accreditation standards. Standards for animal care and use encompass many areas: animal facility design, construction, operation, environmental control, cleaning, and sanitation; animal model selection, acquisition, and genetic, health, and microbial status; feeding, watering, daily care, and monitoring; and behavioral management, research procedures, veterinary care, and euthanasia. The IACUC at each institution is responsible for reviewing each proposal for the use of animals, standard operating procedures at the facilities, and any exceptions to the standards. These standards are designed to promote animal welfare by maintaining animals in a physiological and behaviorally normal state, which in turn serves to minimize the potential for variability on research results resulting from animal pain or distress. Because husbandry and veterinary care procedures themselves can be study variables, it is important that the relative effects of these procedures on study data be carefully evaluated, and simultaneously considered along with the variability that will occur if animals are exempted from animal welfare standards.

Ultimately, what is important is that all aspects of the animal model, its environment, and scientific needs be balanced to determine what is most appropriate from both humane and scientific perspectives. That is the purpose of a committee process for management of animal care programs and for outside regulatory oversight. There is no one person who can understand the interaction of all of the variables that are present within a typical animal study and all parties have biases that can limit their appreciation for some of these variables. It must be remembered that in our society, the use of animals in research is a privilege and not a right. No matter how important and scientifically appropriate the work is, if it cannot

be adequately communicated to the public and their concerns about animal welfare addressed, this privilege can be revoked, which would have a significant impact on the development of new chemicals, drugs, and medical treatments that improve the quality of life for both humans and animals.

QUESTIONS

20.1 Why is it important for scientists to be aware of public opinions regarding animal use and be able to communicate how and why animals are used for toxicological research?

20.2 Give three examples each of *reduction*, *refinement*, and *replacement* alternatives for animal use.

20.3 What are the two primary agencies that regulate animal research in the United States? What are the three primary documents that contain animal welfare regulations and standards used by these agencies?

20.4 What are the examples of animal welfare requirements for the prevention of disease in research animal facilities?

20.5 What effects do pain and distress have on animals and the data collected from them?

20.6 Describe the roles of the IACUC, the veterinarian, and the scientist in determining whether an animal requires veterinary care or euthanasia if it is experiencing toxicity while on a study.

20.7 List three considerations for selection of a species or strain of animal for use as a model for safety evaluation of both small- and a large-molecule test articles.

20.8 Name five specific animal models used in toxicologic research and reasons why each model would be selected for a particular study.

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21 Validation and Regulatory Acceptance of Toxicological Testing Methods and Strategies

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CONTENTS

INTRODUCTION

Toxicological test methods are necessary to assess the hazard and safety of various substances such as medicines, consumer products, and industrial chemicals. Many of these methods have traditionally used animals as the test system. However, there has been increasing interest in the development of alternative methods that incorporate advances in new science and technology and that utilize knowledge of adverse outcome pathways at the molecular and cellular level [1–3]. New testing methods and strategies are sought that can provide improved accuracy and efficiency compared to existing methods. There are also continuing national and international interests and legislative mandates to develop alternative test methods that can replace animal use, reduce the number of animals required for a test procedure, and refine testing procedures to lessen or eliminate unrelieved pain and distress [4]. For any new or revised test method to be used to meet regulatory testing requirements, including alternative methods, the method must first undergo adequate validation and then be determined to be acceptable by regulatory authorities. This chapter discusses the criteria and processes for validation and regulatory acceptance of new, revised, and alternative methods and testing strategies. In addition,

examples of new alternative test methods and testing strategies that have been accepted by national and international authorities are reviewed.

CONCEPT OF ANIMAL USE ALTERNATIVES IN TOXICOLOGY

The concept of animal use alternatives was first described in 1959 by Rex Burch and William Russell in their book *The Principles of Humane Experimental Technique* [5]. Commonly referred to as the *3Rs of alternatives*, this concept involves *reducing* the number of animals needed for a specific study, *replacing* animals with nonanimal systems and approaches, and *refining* animal use to lessen or avoid unrelieved pain and distress. In the 1980s, animal protection groups began to emphasize the need to identify and use alternative methods for animal testing. Industry responded with various initiatives that included support to establish a Center for Alternatives to Animal Testing (CAAT) at the Johns Hopkins University in 198l. Public concern and increased awareness about animal use contributed to the passage of new laws requiring consideration of alternative methods prior to the use of animals in the United States in 1985 [6,7], and in Europe in 1986 and 2010 [8,9]. Additional laws in 1993 and 2000 directed the National Institutes of Health (NIH) to conduct research on alternative methods, to develop and validate alternative methods for testing, and to establish a formal process for acceptance of proposed alternative testing methods [10,11].

Regulatory Requirements for Consideration of Alternative Methods

In the United States, Animal Welfare Act regulations implemented in 1989 require investigators to consider alternative methods prior to the use of animals for research or testing whenever proposed procedures involve more than slight or momentary pain or distress [12]. Before animals can be used, the investigator must provide evidence of the sources used to determine if alternative methods to procedures that cause more than slight or momentary pain or distress are available. The investigator must document this search, and both the search for alternatives and the proposed animal use must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC). Institutions using animals for research and testing must register with the U.S. Department of Agriculture (USDA) and are subject to periodic compliance inspections by the Animal Care Unit of the USDA Animal and Plant Health Inspection Service (APHIS).

Investigators subject to the provisions of the Public Health Service (PHS) Policy on the Humane Care and Use of Laboratory Animals must also consider refinement, reduction, and replacement alternatives prior to the use of animals [13]. These include organizations that receive funding from PHS agencies (e.g., NIH, FDA, CDC, ATSDR), as well as organizations that participate in the voluntary animal facility accreditation program of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The PHS Policy implements relevant provisions of the Health Research Extension Act of 1985 and requires that studies using animals comply with the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (Table 21.1) [13]. These principles effectively require incorporation of refinement, reduction, and replacement alternatives into animal studies to the extent that they are consistent with obtaining testing and research objectives.

Refinement Alternatives

Toxicity testing often involves pain and distress as a result of direct or indirect local or systemic tissue damage from the test article. Additional pain and distress may occur as systemic toxicity progresses toward a lethal outcome as a result of significant disruption of normal homeostatic mechanisms. The goals of refinement alternatives are to minimize or eliminate unrelieved pain and distress and to enhance the well-being of animals used in testing and research [4]. Refinements not only provide for improved animal welfare but also enhance the quality of experiments by reducing or eliminating pain and distress as an experimental variable [4,14–16].

Death has been used historically as an experimental endpoint in toxicity testing; however, considerable pain and distress may precede death, and therefore, the occurrence of spontaneous deaths should be avoided during toxicity studies [16–18]. With recent changes to national and international testing guidelines, death is no longer a required endpoint for toxicity studies conducted for regulatory safety testing purposes. Toxicity testing regulations and guidelines now allow for humane euthanasia of moribund animals, as well as animals that show evidence of severe pain and distress [4,16–18]. These include national and international test guidelines for acute oral toxicity conducted to provide an estimate of the oral LD_{50} [19–22]. International guidance has also been developed for selecting appropriate endpoints for toxicity studies [23].

Refinement can be achieved in toxicity studies by identifying earlier, more humane endpoints that are predictive of traditional study endpoints involving pain and distress [4,16–18]. Clinical signs, physiologic parameters, biochemical measurements, and other parameters can serve as potential earlier biomarkers of humane endpoints. Detailed data should be collected to confirm the validity of the earlier biomarker. When it has been determined that the earlier biomarker provides the same or better accuracy as the traditional biomarker, it can be proposed for acceptance by regulatory authorities.

The local lymph node assay (LLNA) is an example of an alternative test method where the use of an earlier mechanistic endpoint completely eliminates the pain and distress previously involved in the determination of allergic contact dermatitis (ACD) potential of chemicals [4,16,24,25]. In the traditional test method using the Buehler test or guinea pig maximization test (GPMT), the test requires observation for actual elicitation of allergic dermatitis manifested by redness, swelling, and pruritus. In contrast, the LLNA uses an earlier, more sensitive biomarker that avoids the need to evoke the potentially painful elicitation phase. This method is discussed in greater detail later in this chapter.

TABLE 21.1
U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training

The development of knowledge necessary for the improvement of the health and well-being of humans as well as other animals requires in vivo experimentation with a wide variety of animal species. Whenever U.S. government agencies develop requirements for testing, research, or training procedures involving the use of vertebrate animals, the following principles shall be considered, and whenever these agencies actually perform or sponsor such procedures, the responsible institutional official shall ensure that these principles are adhered to:

I. The transportation, care, and use of animals should be in accordance with the Animal Welfare Act (7 U.S.C. 2131 et seq.) and other applicable federal laws, guidelines, and policies.
II. Procedures involving animals should be designed and performed with due consideration of their relevance to human or animal health, the advancement of knowledge, or the good of society.
III. The animals selected for a procedure should be of an appropriate species and quality and the minimum number required to obtain valid results. Methods such as mathematical models, computer simulation, and in vitro biological systems should be considered.
IV. Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings might cause pain or distress in other animals.
V. Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia, or anesthesia. Surgical or other painful procedures should not be performed on anesthetized animals paralyzed by chemical agents. (For guidance throughout these principles, the reader is referred to *The Guide for the Care and Use of Laboratory Animals* prepared by the Institute for Laboratory Animal Research, National Academy of Sciences.)
VI. Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed at the end of the procedure or, if appropriate, during the procedure.
VII. The living conditions of animals should be appropriate for their species and contribute to their health and comfort. Normally, the housing, feeding, and care of all animals used for biomedical purposes must be directed by a veterinarian or other scientist trained and experienced in the proper care, handling, and use of the species being maintained or studied. In any case, veterinary care shall be provided as indicated.
VIII. Investigators and other personnel shall be appropriately qualified and experienced for conducting procedures on living animals. Adequate arrangements shall be made for their in-service training, including the proper and humane care and use of laboratory animals.
IX. Where exceptions are required in relation to the provisions of these principles, the decisions should not rest with the investigators directly concerned but should be made, with due regard to principle II, by an appropriate review group such as an institutional animal care and use committee. Such exceptions should not be made solely for the purposes of teaching or demonstration.

Reduction Alternatives

Reduction alternatives are approaches and methods that result in attainment of study objectives with fewer animals. For example, minimizing one or more experimental variables can often improve statistical power, allowing for fewer animals per group [4,14,15,26]. Using inbred rodent strains is one way to reduce experimental variation associated with genetic differences found in outbred stocks. Optimal statistical designs of studies will also contribute to ensuring the use of the most appropriate number of animals.

One approach to reduction is to periodically conduct a retrospective review of testing results to determine if the number of animals can be reduced without significantly affecting the outcome of the study. For example, six rabbits were routinely used to conduct an ocular irritation assay; however, a retrospective statistical evaluation determined that the number could be reduced to a maximum of three in most situations [27–29]. Further reductions can be accomplished by testing one animal at a time sequentially and stopping if evidence indicates severe irritation or ocular corrosion in one animal [29].

The up-and-down procedure is an example of how animal use for assessing acute oral toxicity has been drastically reduced by up to 80% by use of an innovative statistical approach and sequential animal testing [4,19,22,30]. The acute toxic class method and the fixed dose procedure also provide for reduced animal use for acute oral toxicity studies [21,22,30].

Replacement Alternatives

Replacement alternatives are those that use nonanimal methods, such as cell, tissue, and organ cultures, or nonsentient phylogenetically lower species such as insects [4,5,15]. Nonanimal alternatives can sometimes be used to reach a regulatory hazard decision without animals for a given endpoint, for certain test results, or for certain defined types of substances commonly referred to as the applicability domain. For example, nonanimal test methods for assessing dermal corrosivity potential have been approved [31–35], and no animal or other further testing is required when the substance is identified as a corrosive.

In vitro test methods are also increasingly being incorporated as components in integrated approaches to assess the safety or potential toxicity of various chemicals, medicines, and products [1,3,36]. Their development has been stimulated by advances in new technologies and an enhanced understanding of the molecular and cellular mechanisms of toxicity. Advances in cell and tissue culture methods and the development of genetically modified stable cell lines have contributed to improved in vitro model systems.

New scientific tools such as toxicogenomics, proteomics, and metabonomics are facilitating the identification of more sensitive and earlier biomarkers of toxicity that will likely be incorporated into future in vitro and animal safety testing methods [1,3]. Efforts are underway to investigate the validation strategies that would be required to adopt such new technologies for regulatory decision-making purposes [1,3,36–38]. The number and diversity of in vitro test systems incorporating sensitive biomarkers will undoubtedly expand greatly in the coming years.

Integrated Testing and Decision Strategies

Integrated testing and decision strategies utilize a systematic assessment process that considers all available information at each stage and determines whether the available information is sufficient for a safety decision or whether additional information is needed in order to make a decision [1,3,39,40]. In cases where there is insufficient certainty to make a decision, the ideal ITDS will provide information on what would be the most value-added next additional test that may provide sufficient information to make a decision regarding a specific toxicity. If, based on a weight-of-evidence evaluation, the information is not sufficient, then testing progresses to provide additional information. Testing progresses until sufficient information is available to make a decision. Utilization of integrated testing and decision strategies can sometimes allow for toxicity hazard classifications to be made with fewer or no animals. Proposals for integrated testing strategies for ocular and dermal irritation and corrosion testing have been incorporated into the Globally Harmonized System (GHS) for chemical hazard classification and labeling and as supplemental guidance for international test guidelines [41,42].

VALIDATION OF NEW AND ALTERNATIVE SAFETY ASSESSMENT METHODS AND TESTING STRATEGIES

Prior to using data from new and alternative methods and testing strategies for regulatory safety assessment decisions, the test methods strategies used to generate such data must be determined to be scientifically valid and acceptable for their proposed use [43–46]. Adequate validation is therefore a prerequisite for test methods to be considered for regulatory acceptance. Demonstration of scientific validity requires evidence of the relevance and reliability of a test method and is necessary to determine the usefulness and limitations of a test method for a specific intended purpose. This requires validation studies to determine their accuracy and reliability compared to currently approved reference tests or strategies, and evaluation of whether their proposed use will provide equivalent or improved hazard identification as the currently used test or strategy. Regulatory acceptance involves reviewing the results of validation studies to determine the extent to which a test method can be used to fulfill specific regulatory needs and requirements. This section reviews established criteria for validation.

Test Method Validation Criteria

Validation is defined as the scientific process by which the relevance and reliability of a test method are determined for a specific purpose [43–47]. *Relevance* is defined as the extent to which a test method correctly measures or predicts a biological or toxic effect of interest. Relevance incorporates consideration of the accuracy of a test method for a specific purpose and consideration of mechanistic and cross species or other test system relationships. *Reliability* is an objective measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. A test method is considered adequately validated when its accuracy and reliability characteristics have been adequately determined for a specific purpose.

Criteria that should be met for a new or revised test method to be considered adequately validated for regulatory risk assessment purposes have been developed by national and international authorities (Table 21.2) [44–46]. These criteria serve as principles that should be followed in the validation of new test methods and provide clarity as to the critical information that should be collected and provided to substantiate the validity of test methods to regulatory authorities. The extent to which these criteria are addressed in validation studies will vary with the test method and its proposed use. Accordingly, there must be flexibility in designing validation studies to ensure that the information generated is appropriate given the intended purpose of the test method and consideration of all existing supporting data. Test methods can be designed and used for different purposes by different organizations and for different categories of substances. Accordingly, the determination by regulatory authorities as to whether a specific test method is adequately validated and useful for a specific purpose will be on a case-by-case basis. Regulatory acceptance of new test methods and testing strategies generally requires a determination that decisions made using results from the test method or testing strategy will provide equivalent or improved hazard identification, dose–response assessment, or risk assessment compared to currently used test methods and strategies. Further guidance on adequately addressing established validation criteria is provided in this section.

Test Method Purpose and Regulatory Rationale

New proposed test methods and testing strategies should have a clearly stated regulatory rationale and a clearly defined specific proposed use. The proposed use should describe how a test method is to be used for decision making in the context of current or anticipated regulatory requirements, regulations, and guidelines. National regulatory authorities and international organizations have developed guidance and numerous standardized test guidelines that can be used to meet regulatory safety and hazard assessment requirements for various toxicity endpoints [44,47,48]. Reference data generated for an appropriate range and number of substances using these standardized test method protocols can serve as the basis for

TABLE 21.2
Test Method Validation Criteria

For a new or revised test method to be considered validated for regulatory risk assessment purposes, it should generally meet the following criteria (the extent to which these criteria are met will vary with the method and its proposed use); however, there must be flexibility in assessing a method given its purpose and the supporting database:

1. The scientific and regulatory rationale for the test method, including a clear statement of its proposed use, should be available.
2. The relationship of the test method's endpoints to the biologic effect of interest must be described. Although the relationship may be mechanistic or correlative, tests with biologic relevance to the toxic process being evaluated are preferred.
3. A detailed protocol for the test method must be available and should include a description of the materials required, a description of what is measured and how it is measured, acceptable test performance criteria (e.g., positive and negative control responses), a description of how data will be analyzed, a list of the species for which the test results are applicable, and a description of the known limitations of the test, including a description of the classes of materials that the test can and cannot accurately assess.
4. The extent of within-test variability and the reproducibility of the test within and among laboratories must have been demonstrated. Data must be provided describing the level of intra- and interlaboratory reproducibility and how it varies over time. The degree to which biological variability affects this test reproducibility should be addressed.
5. The performance of the test method must have been demonstrated using reference chemicals or test agents representative of the types of substances to which the test method will be applied and should include both known positive and known negative agents. Unless it is hazardous to do so, chemicals or test agents should be tested under code to exclude bias.
6. Sufficient data should be provided to permit a comparison of the performance of a proposed substitute test with that of the test it is designed to replace. Performance should be evaluated in relation to existing relevant toxicity testing data and relevant toxicity information from the species of concern. Reference data from the comparable traditional test method should be available and of acceptable quality.
7. The limitations of the method must be described; for example, in vitro or other nonanimal test methods may not replicate all of the metabolic processes relevant to chemical toxicity that occur in vivo.
8. Ideally, all data supporting the validity of a test method should be obtained and reported in accordance with GLPs. Aspects of data collection not performed according to GLPs must be fully described, along with their potential impact.
9. All data supporting the assessment of the validity of the test method must be available for review.
10. Detailed protocols should be readily available and in the public domain.
11. The methods and results should be published or submitted for publication in an independent, peer-reviewed publication.
12. The methodology and results should have been subjected to independent scientific review.
13. Because tests can be designed and used for different purposes by different organizations and for different categories of substances, the determination of whether a specific test method is considered by an agency to be useful for a specific purpose must be made on a case-by-case basis. Validation of a test method is a prerequisite for it to be considered for regulatory acceptance.

comparing the performance of a new test method proposed to evaluate the same toxicity endpoint.

The specific purpose of test methods and testing strategies currently included or proposed for inclusion in regulations and guidelines can vary widely [37,44–46]. For example, many methods serve as definitive test methods that provide sufficient information for regulatory hazard classification and labeling decisions, while others may serve as screening tests, mechanistic adjunct tests, or components of a testing battery or testing strategy. A new test method or testing strategy may be proposed as a complete replacement for all testing situations for an existing test method, or it may be proposed to substitute for an existing test method in certain testing situations for a defined applicability domain, such as the evaluation of test articles for specific well-defined product or chemical classes or those with specific physical or chemical properties.

Definitive test methods are those that provide sufficient data to characterize the specific hazard potential of a substance for hazard classification and labeling purposes without further testing. Examples include specific animal tests for skin irritation, eye irritation, ACD, acute oral toxicity, multigenerational reproductive toxicity, and the rodent carcinogenicity bioassay.

Screening test methods are those that may in some situations allow for hazard decisions in a tiered testing strategy or that may provide information helpful in making decisions on prioritizing chemicals for more definitive testing. As an example, a test method could be proposed as a screening test in a tiered testing strategy where positive results can be used to classify and label the hazard of a substance without further testing, while negative results would undergo further defined testing, or testing using the currently accepted definitive testing procedure. Whenever a test method is proposed to be used as a screening test, the specific decisions that will be made with each possible test result must be clearly defined.

Several in vitro screening tests have been accepted for determining if a substance has the potential to cause dermal corrosion [31–35]. Positive results can be used to classify and label substances as corrosives, while negative results may need to undergo additional testing to identify any false-negative corrosive substances and to determine the dermal irritation potential. The use of information from screening tests to meet regulatory requirements must take into consideration the precautionary principle and the public health need to avoid potential underclassification and inadequate or lack of labeling for hazardous substances.

Mechanistic adjunct test methods are those that provide data that add to or help interpret the results of other assays or that otherwise provide information useful for the hazard assessment process. An example is the estrogen-receptor-binding assay [49]. A positive result in this assay indicates that a substance has the potential to bind to the estrogen receptor in an in vitro system; however, it does not definitively indicate that the substance will be active in vivo because it does not take into account absorption, distribution, metabolism, and excretion (ADME) factors. When considered in conjunction with other testing information, such as a positive rodent uterotrophic bioassay, a positive result in this in vitro assay contributes mechanistic information for a weight-of-evidence decision supporting the likelihood that the in vivo bioassay response resulted from an estrogen-active substance. When an adjunct test method is proposed to generate data for use in a weight-of-evidence decision, it is important to provide data that substantiate and quantitatively characterize the weight, or likelihood, that a toxic effect will be associated with the outcome from the mechanistic test.

A *testing battery* is a series of test methods that are generally performed at the same time or in close proximity to reach a decision on hazard potential. This contrasts with a testing strategy, which typically involves several potential test methods, but incorporates the potential for decisions to be made in some situations with only one or more of the methods. In evaluating the scientific validity of test batteries and testing strategies, it is critical that the component test methods undergo validation as individual test methods. For the individual test methods proposed for inclusion, it is essential that each individual test method validation study use the same reference substances or at least a sufficient number of the same substances to adequately evaluate the usefulness and limitations of the proposed test battery or strategy. This is necessary to allow calculation of the accuracy of each possible combination of component test methods and to identify the most accurate combination for given classes or other categories of substances.

Test methods or testing strategies proposed to replace an existing definitive test method will require evidence from validation studies that the use of the proposed method will provide for a comparable or better level of protection than the currently used test method or strategy. In some cases, there may be limitations of a new test method or strategy with regard to certain types of physical or chemical properties (e.g., solubility in an in vitro system) that do not allow for it to completely replace an existing test. In this case, it may be determined to be an adequate *substitute* for the existing test method for many but not all test substances or testing circumstances.

Test Method Scientific Rationale and Relationship of the Test Method Endpoint to the Biological Effect of Interest

The scientific rationale for a new test method should always be provided [44–46]. This should include the mechanistic basis and relationship of the biological model used in the test system compared to that for the species of interest for which the testing is being performed (e.g., humans for health-related testing). The extent to which the mechanisms and modes of action for the toxicity endpoint of interest are similar or different in the proposed test system compared to that in the species of interest must be considered. Other uncertainties regarding mechanisms and modes of action and their potential impact on the relevance of the test method must be discussed. The potential role and impact of in vivo ADME on the toxicity of interest must also be considered, as well as the extent to which each of these parameters is or is not addressed by the proposed test method. For an in vitro test system, the impact of any ADME limitations of the in vitro test system must be discussed. It is also important to consider what is known or not known about similarities and differences in responses between the target tissues in the species of interest, the surrogate species used in the currently accepted test method, and the cells or tissues of the proposed in vitro test system. The extent to which a critical event in any defined adverse outcome pathway for the toxicity endpoint of interest has been incorporated into the test method should be discussed and explained.

Detailed Test Method Protocol

Protocol

The outcome of a validation study should be a detailed standardized, optimized, and transferable test method protocol that has been adequately evaluated to characterize its accuracy and reliability for a specific defined purpose. The test method protocol should be sufficiently detailed that it can be reproduced in other appropriately equipped laboratories with trained personnel. Because most testing conducted for regulatory purposes must be conducted in accordance with national or international good laboratory practice (GLP) regulations [50–53], the test method protocol should be prepared so it can be used as the basis for a GLP-compliant study protocol in specific laboratories.

The test method protocol should provide a detailed description for all aspects of the proposed test method (Table 21.3) [46], including a description of all materials, equipment, and supplies. Detailed procedures for dose selection for animal studies or concentration selection for in vitro studies should be provided. Where appropriate, this should include procedures for dose-range-finding studies and solubility testing to select appropriate solvents, as appropriate. Criteria should be provided for selection of the highest concentration or dose that should be used. For in vivo studies, this may be a maximum tolerated dose, with carefully defined criteria or a defined upper-limit dose. For in vitro methods, this may be a defined limit concentration (e.g., 1 mM), the highest noncytotoxic concentration, or the highest soluble concentration. The duration and basis for test substance exposure and postexposure incubation for in vitro systems should be provided. The nature of data to be collected and the methods and procedures for data collection must be specified.

TABLE 21.3
Selected In Vitro Test Method Protocol Components

1. Biological systems, materials, equipment, reagents, and supplies
2. Concentration selection procedures: for example, defined limit concentration, range-finding studies, procedures for determining limit of solubility, highest noncytotoxic concentration
3. Test system endpoints measured
4. Duration of test article exposure, postexposure incubation
5. Positive, vehicle, negative, and benchmark control substances; basis for their selection
6. Acceptable response ranges for positive, vehicle, and negative control substances, including historical control data and basis for acceptable ranges
7. Decision criteria for interpreting the outcome of a test result, basis for the decision criteria for classifying a chemical, accuracy characteristics of the selected decision criteria
8. Information and data to be included in the study report
9. Standard data collection and submission forms

Positive, Vehicle, and Negative Controls

Nearly every toxicological test will have untreated controls that serve as the basis for detecting whether cells or animals treated with the test article results in an increased response above the control response. When a vehicle or solvent is used with the test article, a vehicle or solvent control should also be used. In addition, it may also be desirable or necessary to have concurrent positive and negative controls. For in vitro test systems, vehicle and positive controls should be designated and used for every test. For some in vivo tests, it may also be necessary to use positive controls. These are necessary to ensure that the test system is operating properly and capable of providing appropriate positive and negative responses. A positive control substance should normally be selected that is intermediate in the potential dynamic response range of the test system. For in vitro tests, an acceptable positive control response range should be developed for each laboratory. Test results are not normally considered acceptable if the positive control is outside of the established (historical) acceptable positive control range.

Benchmark Controls

In some cases, it may be desirable to include substances for which potential toxicity has previously been established in human, animal, and/or in vitro test systems. These substances, commonly referred to as benchmark controls, could include substances that are in the expected response range of the test articles or that have similar chemical structure or physical–chemical properties as the test articles [37]. Benchmark controls can be helpful in providing information about the relative toxicity of a test article compared to other well-characterized similar substances, and can also be used to ensure that the test system is functioning properly in specific areas of the response range.

Decision Criteria

For test methods that determine the hazard classification category of a test substance, the test method protocol must describe the decision criteria used to determine the classification category based on results from the test system. For methods that provide qualitative assessments of toxicity, these may be the criteria used to determine if the results indicate that a substance is positive, negative, or if the result is inconclusive. For example, in Corrositex®, a test method for determining the corrosivity category of substances, the corrosivity hazard category is based on the time that it takes for the substance to penetrate a biobarrier membrane [34,35]. A formula or algorithm that incorporates the decision criteria for a test outcome is often used to convert test method results into a prediction of the toxic effect [44,46]. Accordingly, decision criteria are sometimes referred to as a *prediction model*. Test method decision criteria should address four elements: (1) a definition of the specific purpose of the test method, (2) specifications of all possible results or outcomes that may be obtained when using the test method, (3) an algorithm that converts each study result or outcome into a prediction of the effect of interest, and (4) specification of the accuracy associated with the selected decision criteria (i.e., sensitivity, specificity, false-positive and false-negative rates) [44–46]. Decision criteria should always be carefully described in the test method protocol. It is important to note that decision criteria and prediction models for the final proposed test method protocol may have to be revised following a validation study in order to obtain a sensitivity and specificity appropriate for the intended regulatory use. Such modifications should seek to minimize false-negative and false-positive rates appropriate for the toxicity endpoint being assessed. The regulatory tolerance for false negatives in a test system will depend on the nature, duration, and severity of the injury or disease that could result from any potential false negatives. False negatives can potentially result in exposure to a hazardous substance because it will either be not labeled or underlabeled with regard to the potential health hazards that it could cause.

Test System Selection and Description

The basis for selection of the test system should be described in the test method protocol and should include a detailed description and specifications for animals, cells, tissues, or other critical components used. Procedures for ensuring the correct identity and critical parameters of animal stocks and strains, cells, and tissues should be provided in the test method protocol, including the basis for determining that the components are of acceptable quality and responsiveness [37,51]. Good cell culture practices have been developed to provide guidance on practices necessary to ensure high-quality in vitro testing using cell cultures [54,55].

Evaluation of Test Method Reliability

Test method reliability involves determining the intralaboratory repeatability and intra- and interlaboratory

reproducibility of a test method [37,44,45]. *Intralaboratory repeatability* of a test method is the closeness of agreement between test results obtained in a single laboratory when the test method is used to evaluate the same substance under identical conditions at the same time. These data provide an estimate of the variation that is inherent in the biological responses of a test system and the study conditions in a single laboratory. *Intralaboratory reproducibility* is the determination of the extent that qualified personnel within the same laboratory can successfully replicate results using a specific test method protocol at different times. Acceptable intralaboratory reproducibility should be achieved before evaluating interlaboratory reproducibility.

Interlaboratory reproducibility is a measure of the extent to which different qualified laboratories using the same test method protocol and the same substances can produce qualitatively and quantitatively similar results. This assessment is necessary to determine if the test method protocol contains sufficient procedural detail that will result in qualified laboratories obtaining similar and consistent results, and indicates the extent to which a test method can be transferred successfully among laboratories. Interlaboratory reproducibility should be assessed using the same or a subset of the reference substances used to assess test method accuracy. Most importantly, reference substances representing the full range of possible test outcomes, chemical and physical properties, and mechanisms of toxicity should be evaluated. This can sometimes be accomplished with a smaller number of reference chemicals than used to characterize accuracy; however, there should be a compelling scientific and statistical rationale for using a reduced number of substances for this determination. Interlaboratory reproducibility has typically been assessed using three qualified laboratories. The impact of the results of test method reliability assessments on laboratory transferability and erroneous results should always be considered. Situations where evidence indicates poor reproducibility, such as for certain chemical classes, physicochemical properties, or specific areas of response, should be identified as potential limitations of the proposed test method.

Reference Substances

Reference substances are those for which the response of the substance is known in the existing reference test method or target species; they are used to characterize the accuracy and reliability of the proposed test method [37,44–46]. Test method reliability and accuracy must be evaluated using reference substances representative of the types of substances to which the test method will be applied and should include both known positive and negative substances. The selection of appropriate reference substances is a critical aspect of validation studies. The ideal reference chemicals are those for which high-quality testing data are available from both the reference test method and from the species of interest (e.g., humans); however, adequate human testing data are rarely available for ethical reasons. Exceptions are for substances and endpoints that do not result in severe or irreversible effects, such as ACD and mild to moderate dermal irritation. However, human studies are usually limited to the premarketing assessment of products that are intended for human contact, such as cosmetics and some mild consumer products. For test methods proposed for predicting human health effects, reference substances for which there are accidental human exposures and toxic effects should also be considered.

The number and types of reference substances selected must adequately characterize the accuracy and reproducibility of a test method for its specific proposed use [37,44–46]. Reference chemicals should represent the range of chemical classes, product classes, and physical and chemical properties (e.g., pH, solubility, color, solids, liquids) for which the test method is expected or proposed to be applicable. Reference chemicals should also represent the range of expected responses proposed for the test method, including negatives and weak to strong positives. Reference chemicals and formulations should ideally be of known purity and composition and should be readily available from commercial sources. Formulations should provide detailed information on the type, purity, and percentage of each ingredient. Unless justified, chemicals should not normally pose an extreme environmental or human health hazard, should not be prohibitively expensive, and should not involve exorbitant disposal costs.

Evaluation of Test Method Accuracy

Accuracy reflects the closeness of agreement between results from a new proposed test method and reference values from a currently accepted test method. A two-by-two table can be used to calculate accuracy and the associated parameters (Table 21.4) [46]. *Sensitivity* is the proportion of all positive substances that are correctly classified as positive by the new test method. *Specificity* is the proportion of all negative substances that are correctly identified as negative substances in the new test method. The *false-positive rate* is the proportion of all negative (inactive) substances that

TABLE 21.4
Two-by-Two (2 × 2) Table: Accuracy Statistics

		New Test Outcome		
		Positive	**Negative**	**Total**
Reference Test Classification	**Positive**	a	c	$a + c$
	Negative	b	d	$b + d$
	Total	$a + b$	$c + d$	$a + b + c + d$

Notes: The 2 × 2 table can be used for calculating accuracy (concordance) $((a + d)/(a + b + c + d))$, negative predictivity $(d/(c + d))$, positive predictivity $(a/(a + b))$, prevalence $((a + c)/(a + b + c + d))$, sensitivity $(a/(a + c))$, specificity $(d/(b + d))$, false-positive rate $(b/(b + d))$, and false-negative rate $(c/(a + c))$.

are falsely identified as positive, and the *false-negative rate* is the proportion of all positive (active) substances that are falsely identified as negative.

Ideally, test methods should be highly accurate, have a high level of sensitivity and specificity, and have negligible false-positive and false-negative rates; however, in toxicity testing, this is rarely achievable. Accordingly, decision criteria for interpreting the outcome of a test method must be adjusted depending on the desired performance characteristics and the impact of an erroneous result; for example, from the perspective of protecting public health, it is most desirable to use decision criteria for a test method that provide for a high level of sensitivity and have no or minimal false negatives. This is because a false-negative result incorrectly indicates a lack of hazard or lower hazard than actually exists for a substance. The real hazard of the substance will subsequently not be indicated on packaging or in worker safety information. Products without a proper warning of their real hazard, such as skin and eye corrosives, could then result in human injury or disease to exposed persons. Conversely, a low level of specificity and high false-positive rate can result in overlabeling the true hazard of a substance. This has economic implications in that some hazards require more expensive packaging and shipping precautions. It is also desirable not to overlabel the hazard of a substance because this could lead to complacency in consumer and worker compliance with recommended exposure precautions if accidental exposures frequently do not result in adverse effects.

Test Method Limitations

The limitations of a test method and test system must be described [44,45]; for example, the extent to which an in vitro test system does not replicate all of the metabolic processes relevant to the in vivo toxicity for a specific toxicity endpoint should be discussed. Similarly, there may be uncertainties or known limitations with regard to cross species differences in ADME for certain substances that must also be considered. Furthermore, limitations may be identified with regard to the ability of the test method to reliably and accurately detect the toxicity or biological activity of specific chemical classes and specific physical or chemical properties.

Quality of Validation Data

Ideally, all data supporting the validity of a test method should be obtained and reported in accordance with national or international GLP regulations and guidelines [44,45,50–53]. Because nearly all safety testing for regulatory purposes must be accomplished in accordance with GLP requirements, it is logical that validation studies for a test method proposed for safety testing should be carried out in accordance with those same GLP requirements. This will provide increased confidence in data quality and documentation as to the extent of laboratory adherence to the test method protocols under evaluation. GLPs provide a formal quality assurance system for data collected in the study. If validation studies are not conducted in accordance with GLPs, then aspects of data collection or auditing not performed according to GLPs should be documented. International guidance for the application of GLPs to in vitro testing is available [51]. In any case, all laboratory notebooks, raw and transformed data, and all other relevant test-related information should be retained and available for audit if requested by the reviewing authorities.

Availability of Validation Data

All data supporting the assessment of the validity of a proposed test method should be made available for review [44,45]. This includes raw data collected from the test system, as well as transformed data that are derived from the raw data. All test method protocols used to generate data must also be available. Ideally, the test method protocols and results from validation studies should be subjected to independent scientific peer review and published or submitted for publication in an independent peer-reviewed publication. Additional information on scientific peer review is provided in the section on the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [44,46].

EVOLUTION AND VALIDATION OF A TEST METHOD

Validation of a test method is one of many stages involved in the evolution of a test method from concept to regulatory acceptance (Figure 21.1) [44]. These stages may begin with determination of the need for a new test method. New test methods are often sought that provide for improved prediction of adverse effects, that are more humane or do not use animals, or that involve less expense and time to conduct. Additional research may be needed to understand critical mechanisms and critical modes of action for a toxicity endpoint of interest and to identify potential biomarkers that can be included in a test method. The test method development stage involves incorporation and evaluation of one or more promising predictive biomarkers in a test system. This usually involves testing a limited number of substances with well-known toxicity to determine if the critical biomarker is capable of detecting the toxic effect. If so, then a decision may be made to initiate validation of the test system. Validation of a proposed test method is an iterative process that typically evolves through several phases (Table 21.5) [44].

Levels of Validation

When considering validation strategies for new test systems and methods, three progressive levels of validation must be

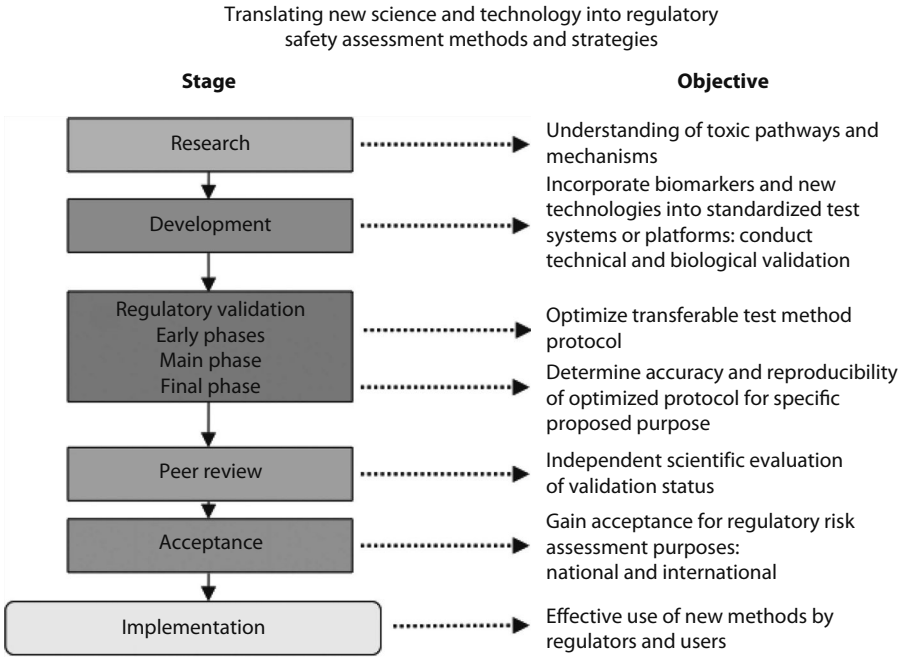

FIGURE 21.1 Test method evolution process. (Adapted from Interagency Coordinating Committee on the Validation of Alternative Methods, Guidelines for the nomination and submission of new, revised, and alternative test methods, NIH Publ. No. 03-4508, National Institute of Environmental Health Sciences, Research Triangle Park, NC, 2003.)

considered [3,56]. First, *technical validation* focuses on the extent that a new technology platform or test system can provide reproducible and reliable results. For example, testing of a limited number of chemicals across a range of responses is repeated to determine if the technology platform provides consistent and reproducible answers. Technical validation occurs early in the test method development process. Test platforms with excessive variation will require modifications to achieve an acceptable range of reproducibility before advancing to biological validation.

Biologic validation evaluates whether the underlying biology of interest is accurately reflected in the outcomes obtained from the new technology platform or test system [3,56]. This determines the extent that the measured qualitative and quantitative responses in the test system are indicative of the true biologic response and whether there are other factors causing unrelated positive, negative, or quantitatively altered responses.

Regulatory validation is often considered following successful technical and biologic validation and when there is potential applicability of test methods using the new technology platform or system to regulatory decision making [3,56]. Regulatory validation determines the extent that the test system generates information useful for regulatory decisions on safety or hazard and the extent that the use of a proposed standardized test method protocol produces similar results in different qualified laboratories. The following sections describe the processes applicable to regulatory validation.

Regulatory Validation: Test Method Standardization, Optimization, and Transferability

Regulatory validation is most efficiently conducted in several phases. The early phases are aimed at test method standardization and optimization of a transferable test method protocol. The first objective is to initially evaluate the standardized test method protocol and then to optimize the test method protocol to maximize accuracy and reliability first within and then across different laboratories. Careful planning is essential prior to the initiation of any validation study. The validation study plan must adequately address established validation criteria [44–46]. The objectives of each phase of the validation study should be clearly defined and a validation study design selected that will adequately address the defined objectives. Test method modifications are often necessary during the early validation phases to reduce sources of intra- and inter-laboratory variation and to optimize the accuracy of the test method to measure or predict the toxicity or biological activity of interest. The goal of the early validation phases is to optimize a test method protocol that is sufficiently accurate and reproducible and that can be used without further modification in the final phase of validation. Because the objective of the formal validation phase is to determine the reproducibility and accuracy of this optimized and standardized test method protocol, no changes should be made to the protocol during the final phase of validation.

TABLE 21.5
Test Method Validation Process

I. Test Development
- A. *Technical validation*
  1. Define the purpose of test.
  2. Assess test system reproducibility and reliability.
- B. *Biological validation*
  1. Determine the extent that test system results accurately reflect the underlying biology of interest.

II. Regulatory Validation
- A. *Preliminary validation study planning*
  1. Establish validation management team; evaluation and oversight procedures.
  2. Define basis and specific purpose of test.
  3. Develop detailed test method protocol for use in all laboratories:
     a. Identify positive and negative control substances.
     b. Establish initial decision criteria.
  4. Design validation study:
     a. Determine number and identity of reference substances for each phase.
     b. Determine number of replicates for each laboratory for each phase.
     c. Determine record-keeping and data submission procedures.
  5. Select participating laboratories; identify experienced lead lab.
  6. Code and distribute chemicals for initial phase.
- B. *Lab training and qualification phase*
  1. Lead laboratory provides training on test system and test method protocol:
     a. Establish initial historical positive control acceptance range for each laboratory.
  2. Labs test replicates of small number of coded chemicals (e.g., 3).
  3. Data reviewed; decision on whether each lab is sufficiently accurate and reproducible, or if not, procedures optimized and retested; repeated until satisfactory results.
- C. *Final protocol optimization phase*
  1. Test selected number of coded chemicals representing range of potency (e.g., 9).
  2. Assess intra- and interlaboratory reproducibility and accuracy.
  3. Further optimize test method protocol to maximize reproducibility and accuracy.
  4. Finalize optimized test method protocol for main validation study.
- D. *Main validation study phase*
  1. Test larger number of coded chemicals representing range of chemistry and potency in all labs, three replicates per lab.
  2. Assess intra- and interlaboratory reproducibility and initial accuracy.
  3. If reproducibility and initial accuracy are satisfactory, progress to final phase.
- E. *Final validation study phase*
  1. Test expanded number of coded chemicals necessary to adequately characterize accuracy of test method protocol in one or more qualified labs.
  2. Assess final accuracy.
  3. Prepare validation study report.
  4. Submit study report to national or international organizations for review.
- F. *Conduct independent scientific peer review*
  1. All data and study results made publicly available.
  2. Peer-review report made publicly available.

III. Recommendations on Test Method and Usefulness and Limitations
1. Test method recommendations supported by validation study provided to regulatory authorities for acceptance decisions.
2. Regulatory authorities announce acceptance decisions.
3. Regulatory testing guidelines updated to incorporate new test methods.

Coding and Distribution of Test Substance

Test substances should normally be coded during all phases of the validation study to exclude bias [37,44,45]. This can be accomplished by the use of a chemical distribution facility not directly associated with the participating laboratories. Each substance should be uniquely coded for each different laboratory so the identity is not readily available to laboratory personnel; however, provisions must be made to ensure that the designated safety officer in each laboratory has the safety data sheets available for each coded substance in case the need arises to access the information. One approach is to provide participating laboratory testing staff with sealed packages containing all relevant health

and safety data, including instructions for accidental exposures or other laboratory accidents. The envelopes can then be returned to the study sponsor at the end of study, with an explanation for any opened envelopes. Laboratories will need to ensure that all environmental, safety, handling, and disposal procedures are in compliance with regulatory requirements.

Selection of Laboratories for Validation Studies

Laboratories selected for validation studies should be adequately equipped and have personnel with appropriate training; for example, validation of an in vitro test method that involves aseptic tissue culture should utilize laboratories that have demonstrated proficiency in successfully conducting tissue culture experiments or testing. The use of three laboratories has generally been found to be adequate for assessing the interlaboratory reproducibility of test methods during validation studies. While there may be a desire to use more laboratories, this can often complicate the management of the study. If more than three laboratories are used, it is vital that all laboratories agree to follow the established protocols and validation study design and to adhere to the quality provisions of the study in order to ensure that high-quality data are generated and to avoid excessive delays. It is helpful to designate the laboratory most experienced with the test method as the *lead laboratory* during early validation studies to serve as a resource for technical issues that develop during the studies.

Phased Validation Studies

In a recent in vitro validation study managed by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), dividing the early validation study into three phases was found to aid in efficiently optimizing the test method protocol [57]. The first phase involved a series of multiple testing in each laboratory with the positive control, with cycles of modifications and additions to the protocol until all laboratories were able to obtain reproducible results. This phase also was used to establish acceptance criteria for the test system, including positive control acceptance values for each laboratory. The second phase tested three coded substances representing three different areas of the response range (low, moderate, and high toxicity) and was again followed by minor protocol revisions to minimize variation within and among the participating laboratories. The third phase tested nine coded substances, again representing the range of responses as well as range of solubility. Additional minor protocol revisions were made after this phase, and an optimized test method protocol was finalized for testing the 60 remaining reference chemicals in the formal validation phase. The test methods and validation study results underwent scientific peer review and were determined to have sufficient reproducibility and accuracy to use in setting initial starting doses for in vivo acute oral toxicity studies [58–60]. Standardized test method protocols and an OECD guidance document incorporating the two test methods were published [61,62].

REGULATORY ACCEPTANCE CRITERIA FOR NEW SAFETY EVALUATION METHODS

After a new test method has been evaluated in a validation study, regulatory authorities do not automatically accept it; rather, the regulatory authorities must determine if there is sufficient evidence that the use of test method will provide for equivalent or improved protection of human health, animal health, or the environment, as defined by the intended purpose of the test method [11,44,60]. Furthermore, although a validated test method can be found to be technically acceptable, the extent that the test method may be useful for specific regulatory bodies is often determined by the nature and type of chemicals and products they each regulate. Regulatory acceptance criteria that should be adequately addressed by a test method proposed for regulatory applications have been developed for the United States (Table 21.6) [44,46], as well as internationally [45]. This section discusses the regulatory acceptance criteria for new test methods.

Independent Scientific Peer Review

The test method should have undergone independent scientific peer review by a group of persons that includes experts in the respective field and includes experts who are knowledgeable about the test model used in the validation study [44–46]. These individuals should not have financial or other conflicts of interest such that they, any family members, or their organizations stand to gain financially or professionally from either a positive or negative outcome of the peer-review process. Transparency of the validation and review process is essential. All data substantiating the scientific validity of the test method should be made available to the public and the public should have the opportunity to comment on the test method, data, and proposed recommendations on the validity of the test method [44,46,60]. The peer-review panel should conduct its deliberations in public session, and the report of the peer-review panel should be made available to the public [44,60]. ICCVAM has implemented a transparent peer-review process that incorporates these features [43,44,60].

Detailed Test Method Protocol

A detailed test method protocol must be provided that includes all relevant standard operating procedures (SOPs), operating characteristics, and decision criteria (e.g., assay acceptance criteria, response criteria) [44–46]. The performance of the test method protocol should be substantiated by appropriate validation studies, and any changes in the protocol from the version used in the validation study should be scientifically

TABLE 21.6
Test Method Regulatory Acceptance Criteria

Validated methods are not automatically accepted by regulatory agencies; they need to fit into the regulatory structure. Flexibility is essential in determining the acceptability of methods to ensure that appropriate scientific information is considered in regulatory risk assessment. A test method proposed for regulatory acceptance generally should be supported by the following attributes:

1. The method should have undergone independent scientific peer review by disinterested persons who are experts in the field, knowledgeable in the method, and financially unencumbered by the outcome of the evaluation.
2. There should be a detailed protocol with SOPs, a list of operating characteristics, and criteria for judging test performance and results.
3. Data generated by the method should adequately measure or predict the endpoint of interest and demonstrate a linkage between either the new test and an existing test or the new test and effects in the target species.
4. There should be adequate test data for chemicals and products representative of those administered by the regulatory program or agency and for which the test is proposed.
5. The method should generate data useful for risk assessment purposes (i.e., for hazard identification, dose–response assessment, and/or exposure assessment). Such methods may be useful alone or as part of a battery or tiered approach.
6. The specific strengths and limitations of the test must be clearly identified and described.
7. The test method must be robust (relatively insensitive to minor changes in protocol) and transferable among properly equipped and staffed laboratories.
8. The method should be time and cost effective.
9. The method should be one that can be harmonized with similar testing requirements of other agencies and international groups.
10. The method should be suitable for international acceptance.
11. The method must provide adequate consideration for the reduction, refinement, and replacement of animal use.

justified. The test method protocol should provide sufficient information to allow for development of laboratory-specific GLP-compliant test protocols.

Adequate Measurement or Prediction of the Endpoint of Interest

Data generated by the test method should adequately measure or predict the endpoint of interest and demonstrate a linkage between either the new test and an existing test, or the new test and effects in the target species of interest [44–46]. Such a determination should ideally include an objective assessment of the accuracy of the existing test for measuring or predicting the toxic effect of interest; however, much of the data generated for an existing test method may be proprietary and therefore not readily available for such an assessment. In such instances, regulatory authorities may not be able to provide the actual data to the public that they use for making regulatory acceptance decisions.

Adequate Test Data

Adequate testing data should be available for the chemicals and products for which the test method is proposed for use [44–46]. In some cases, these data may not adequately represent the complete spectrum of chemicals and products regulated by a specific regulatory agency or program. This lack of data might serve as the basis for nonacceptance by one or more regulatory authorities. However, it is possible for test methods to be found valid and acceptable for some defined chemical classes or physical and chemical properties that may not encompass the entire range of substances regulated by a specific agency. In such cases, acceptance may include specific restrictions on the substances for which the test method may be used.

Usefulness for Risk Assessment

A test method should generate data useful for risk assessment purposes in order to be accepted [44–46]. This will most commonly be for hazard identification purposes but could also include dose–response or exposure assessment purposes. The specific use for which the test method is proposed should be provided, such as whether the test method is proposed as a substitute or complete replacement for an existing test method, whether the test method is proposed as a screening test in a tiered testing strategy, or if the test is proposed as part of a battery of tests using a weight-of evidence approach.

Identification of Strengths and Limitations

The specific strengths and limitations of the test method must be clearly identified and described [44–46]. This description may be in terms of demonstrated usefulness and limitations based on high-quality data, or it may be based on the fact that certain types of substances have not yet been evaluated in the test system. The limitations associated with a test method will necessarily influence its regulatory utility and could restrict its applicability in certain regulatory domains.

Robustness and Transferability

The validation study must have demonstrated that the test method is sufficiently robust and that it is transferable among properly equipped and staffed laboratories [44–46].

There must be sufficient evidence to assure regulatory authorities that similar results will be obtained with the same substance regardless of the geographic area and laboratory where the test method is conducted or the laboratory personnel performing the test.

Time and Cost Effectiveness

The test method should be time and cost effective compared to the test method that it is proposed to substitute or replace [44–46]. Obviously, a test method that takes considerably more time and expense to conduct than an existing test would have to have sufficient other advantages to warrant acceptance by regulatory authorities and use by the regulated industry.

Harmonized for Use by Other Agencies and International Groups

The test method should be capable of being harmonized with similar testing requirements of other agencies and international groups [44–46]. This criterion is especially important in light of the worldwide implementation of the GHS of Classification and Labeling of Chemicals [41]. Accordingly, new test methods should include assessment of the test method accuracy for the GHS hazard classification scheme where appropriate.

Suitability for International Acceptance

New test methods should be suitable for acceptance by international authorities, such as the United Nations (UN), the Organization for Economic Cooperation and Development (OECD), and the International Organization for Standardization (ISO) [44–46]. This is to ensure that the new method can be accepted for use internationally and thereby avoid the need for redundant testing for countries that have not adopted the new test method.

Adequate Consideration of the 3Rs

New test methods must provide for adequate consideration of the reduction, replacement, and refinement (3Rs) of animal use if they involve animals or test system components derived from animals [44–46]. Such consideration is necessary to comply with the United States and European animal welfare regulations, policies, and guidelines [8,9,12,13].

TEST METHOD PERFORMANCE STANDARDS

Many new and alternative test methods are proprietary in nature and are protected by intellectual property laws such as patents, trademarks, and copyrights. Such intellectual protections stimulate innovation by providing financial incentives for companies to develop and market new products, such as in vitro testing methods that may reduce, refine, or replace animal use. U.S. laws, however, require that government regulatory authorities cannot simply endorse or approve proprietary methods until they first convey the basis by which the proprietary methods have been determined to be acceptable for use [46,63,64]. ICCVAM subsequently incorporated procedures to routinely recommend performance standards for all new test methods that it evaluates, regardless of whether they include proprietary components or not. ICCVAM has now developed and recommended performance standards for both in vitro and in vivo test methods, including in vitro corrosivity test methods, the murine LLNA, and in vitro estrogen receptor agonist and antagonist transactivation assays [63–67].

Defining Test Method Performance Standards

Performance standards are defined as the basis by which a proprietary or nonproprietary test method has been determined to have sufficient accuracy and reliability for a specific testing purpose [46,63,64]. Performance standards are based on an adequately validated test method and provide a basis for evaluating the comparability of mechanistically and functionally similar test methods. This process involves first determining that a test method has sufficient accuracy and reliability for a defined specific testing purpose. This information is then used to develop performance standards that can be used as the basis for evaluating the acceptability of proposed test methods based on similar scientific principles and that measure or predict the same biological or toxic effect. If a similar test method adequately addresses and meets these standards, then it would be considered to be comparable, in terms of performance, to the test method used to establish the performance standards. Performance standards can then be used by regulatory authorities to communicate the basis by which they find the original reference test method to be acceptable for specific regulatory testing purposes, as well as to judge the acceptability of subsequent similar methods. ICCVAM now routinely develops and proposes performance standards during test method evaluations for both proprietary and nonproprietary methods that have undergone adequate validation [46,63,64]. The availability and use of test method performance standards can significantly streamline the validation and regulatory acceptance of structurally and functionally similar methods, thus providing more efficient and faster validation [63,64].

Components of Performance Standards

Performance standards consist of three elements: (1) essential test method components, (2) a minimum list of reference chemicals, and (3) accuracy and reliability values [46,63,64]. *Essential test method* components are the requisite structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed mechanistically and functionally similar test method. These components include unique characteristics of the test method, critical procedural details, and quality control measures.

If there are deviations from the recommended essential test method components, then a scientific rationale must be provided and any potential impact of the deviations discussed. Incorporation of and adherence to essential test method components will help ensure that a proposed test method is based on the same concepts as the corresponding validated test method.

The *minimum list of reference chemicals* is used to assess the accuracy and reliability of a mechanistically and functionally similar test method that incorporates all of the essential test method components [46,63,64]. These chemicals are a representative subset of those used to demonstrate the reliability and accuracy of the validated reference test method on which the performance standards are based. To the extent possible, these reference chemicals should:

- Represent the range of responses that the validated test method is capable of measuring or predicting (e.g., negative and weak to moderate to strong positives)
- Produce consistent results in the validated test method and in the in vivo reference test method or target species of interest
- Reflect the accuracy of the validated test method
- Have well-defined chemical structures
- Be readily available (i.e., can be purchased from commercial sources)
- Not be associated with excessive hazard or prohibitive disposal costs
- Represent the range of known or suspected mechanisms or modes of action for the toxicity measured or predicted by the test method
- Represent the range of physical and chemical properties for which the test method is proposed to be capable of testing (e.g., solubility, pH, volatility)

These reference chemicals are the minimum number that should be used to evaluate the performance of a proposed mechanistically and functionally similar test method. Reference chemicals should not be used to develop the decision criteria or prediction model for the proposed test method. If any of the recommended reference chemicals are unavailable, other chemicals for which adequate reference data are available could be substituted with adequate scientific justification. To the extent possible, any substituted chemicals should be of the same chemical class and potency activity as the original chemicals. If desired, additional chemicals representing other chemical or product classes and for which adequate reference data are available can be used to more comprehensively evaluate the accuracy of the proposed test method; however, these additional chemicals should not include those used to develop the proposed test method. Table 21.7 provides a list of internationally harmonized performance standards reference chemicals for the LLNA [24,66]. These reference chemicals have now been used to evaluate two new similar versions of the LLNA that were subsequently adopted as OECD test guidelines [68–71].

Accuracy and *reliability* values are the comparable performance that should be achieved by the proposed test method when evaluated using the minimum list of reference chemicals [46,63,64]. Reference chemicals should be designated for performance standards that will result in accuracy and reliability values similar to the overall values determined from the entire validation database for the reference test method.

Process for Developing Performance Standards

The ICCVAM has developed a process for establishing performance standards during the evaluation of proposed new test methods [46,63,64,72]. The process is designed to ensure rigorous scientific review and to provide the opportunity for broad stakeholder and public comment. The ICCVAM process for developing performance standards for new test methods is as follows:

- The NICEATM and the appropriate ICCVAM interagency working group develop proposed performance standards for consideration during the ICCVAM evaluation process. If a sponsor proposes performance standards, these are considered by ICCVAM at this stage. Generally, the proposed performance standards will be based on the information and data provided in the test method submission or on other available applicable data.
- The ICCVAM/NICEATM peer-review panel evaluates the proposed performance standards for completeness and appropriateness during its evaluation of the validation status of the proposed test method. The proposed performance standards are made available with the test method submission to the public for comment prior to and during the peer-review panel meeting.
- The appropriate ICCVAM working group, with the assistance of NICEATM, prepares the final performance standards for ICCVAM approval, taking into consideration the recommendations of the peer-review panel and public comments.
- Performance standards recommended by ICCVAM are incorporated into ICCVAM test method evaluation reports, which are published, provided to federal agencies, and made available to the public. The availability of ICCVAM test method evaluation reports is announced routinely in the *Federal Register*, NTP newsletters, and ICCVAM/NICEATM e-mail LISTSERV groups.
- Regulatory authorities can then reference the performance standards in the ICCVAM report when they communicate their basis for their acceptance of a new test method. In addition, performance standards adopted by regulatory authorities can be provided in guidelines issued for new test methods, which is now done routinely for new OECD test guidelines [34,73].

TABLE 21.7
LLNA Performance Standards: Reference Substances for Validation of Modified LLNA Methods

Substance Name	LLNA	Vehicle	EC3[a]	*N*[b]	Guinea Pig[c]	Human
CMI/MI	+	DMF	0.009	1	+	+
2,4-Dinitrochlorobenzene	+	AOO	0.049	15	+	+
4-Phenylenediamine	+	AOO	0.11	6	+	+
Cobalt chloride	+	DMSO	0.6	2	+	+
Isoeugenol	+	AOO	1.5	47	+	+
2-Mercaptobenzothiazole	+	DMF	1.7	1	+	+
Citral	+	AOO	9.2	6	+	+
Hexyl cinnamic aldehyde	+	AOO	9.7	21	+	+
Eugenol	+	AOO	10.1	11	+	+
Phenyl benzoate	+	AOO	13.6	3	+	+
Cinnamic alcohol	+	AOO	21	1	+	+
Imidazolidinyl urea	+	DMF	24	1	+	+
Methyl methacrylate	+	AOO	90	1	+	+
Chlorobenzene	–	AOO	NC	1	–	–[d]
Isopropanol	–	AOO	NC	1	–	–
Lactic acid	–	DMSO	NC	1	–	–[d]
Methyl salicylate	–	AOO	NC	9	–	–
Salicylic acid	–	AOO	NC	1	–	–
Optional substances to demonstrate improved performance relative to the LLNA						
Sodium lauryl sulfate	+	DMF	8.1	5	–	–
Ethylene glycol dimethacrylate	+	MEK	28	1	–	+
Xylene	+	AOO	95.8	1	NA	–
Nickel chloride	–	DMSO	NC	2	+	+

Abbreviations: AOO, Acetone:olive oil (4:1); CMI/MI, 3:1 5-chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one (*KathonCG*); DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EC3, estimated concentration needed to produce a SI of 3; LLNA, murine local lymph node assay; MEK, methyl ethyl ketone; NA, not available; NC, not calculated because SI < 3.

[a] Arithmetic means where the number of LLNA studies >1.

[b] Number of LLNA studies from which data were obtained.

[c] Results obtained from GPMT and/or Buehler test.

[d] Presumed to be a nonsensitizer in humans based on the fact that no clinical patch test results were located; it is not included as a patch test kit allergen, and no case reports of human sensitization were located.

Performance Standards for Dermal Corrosivity Test Methods

Performance standards are available for three proprietary—Corrositex, EPISKIN™, and EpiDerm™—and one nonproprietary—rat skin transcutaneous electrical resistance (TER)—in vitro dermal corrosivity test methods [65]. Due to the structural and functional differences of the four methods, three different sets of performance standards were developed [65]. EPISKIN and EpiDerm are structurally and functionally similar; therefore, one set of performance standards was developed for these two methods. The standards were based on EPISKIN, as this method had a larger validation database than EpiDerm (60 vs. 24). In addition to the essential test method components, a minimum list of 24 reference chemicals was selected from the 60 chemicals used for the validation of EPISKIN. This list included 12 corrosives and 12 noncorrosives. All of the selected reference chemicals are commercially available. Accuracy and reliability values for the 24 minimum reference chemicals closely matched the overall performance for the 60 chemicals in the validation database. For the rat skin TER, a minimum list of 24 reference chemicals was selected, which also provided accuracy and reliability values similar to those for the total validation database of 60 chemicals.

Performance standards based on Corrositex were developed for a generic in vitro membrane barrier test system for skin corrosion [34]. This in vitro membrane barrier test system is capable of identifying the three subcategories of corrosivity described by the UN Packing Group (PG) classification system [41]. Accordingly, the validation database contained a larger number of substances (129). The selected minimum list of reference chemicals contained a total of 40 chemicals, including 12 noncorrosive methods and 28 corrosive chemicals [65]. As with the other in vitro methods, the

accuracy and reliability values for the minimum list of reference chemicals were similar to those for the total validation database. These performance standards were subsequently included in the Test Guideline 435 for an in vitro membrane barrier test system for skin corrosion [34].

Using Performance Standards for Validation Studies

The availability of performance standards can significantly expedite the validation and acceptance of new test methods that are structurally and functionally similar to previously accepted methods for which there has been adequate validation [46,63,64]. For example, using performance standards, validation studies on a generic version of Corrositex could potentially be accomplished with 40 substances compared to the over 129 chemicals used for the original validation [29]. Performance standards should also facilitate more efficient and faster validation of improved versions of existing tests. The concept and definition of test method performance standards are now included in international guidance on validation [45].

ICCVAM ROLE IN VALIDATION AND REGULATORY ACCEPTANCE

History

The ICCVAM was first established as an ad hoc interagency committee in 1994 [15,44,60]. It consisted of representatives from 15 federal agencies and programs that require, generate, use, or disseminate toxicological testing information (Table 21.8) [15,44,60]. This committee was created by the National Institute of Environmental Health Sciences (NIEHS) in response to directives in the NIH Revitalization Act of 1993 that charged NIEHS with developing validation and regulatory acceptance criteria and recommending a process for achieving the regulatory acceptance of scientifically valid alternative test methods [10,44,60]. The principles embodied in the validation and regulatory acceptance criteria are based on good science and the need to ensure that the use of new test methods will provide for equivalent or better protection of human health and the environment than previous testing methods or strategies. ICCVAM issued its report in 1997 [44,60].

To implement a process for achieving regulatory acceptance of proposed new, revised, and alternative test methods with regulatory applicability, a standing ICCVAM was established to evaluate the scientific validity of such test methods in 1997 [60]. The NIEHS also established the NICEATM to administer ICCVAM and to provide scientific and operational support for the committee and its activities [60]. The ICCVAM Authorization Act of 2000 established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM [11]. NICEATM collaborates with ICCVAM to carry out scientific peer review and interagency consideration of new test methods of multiagency interest. The center also performs other functions necessary to ensure compliance with provisions of the ICCVAM Authorization Act of 2000 and conducts independent validation studies on promising new test methods [72].

Purposes and Duties

The specific purposes and duties of the ICCVAM prescribed by the ICCVAM Authorization Act of 2000 are provided in Tables 21.9 and 21.10 [11]. In accordance with the ICCVAM Act and its original mandate, ICCVAM also continues to coordinate interagency issues on test method development, validation, regulatory acceptance, and national and international harmonization. The public health goal of NICEATM and ICCVAM is to promote the scientific validation and regulatory acceptance of new toxicity testing methods that are more predictive of human health, animal health, and ecological effects than currently available methods. Methods are emphasized that provide for improved toxicity

TABLE 21.8
Member Agencies of the ICCVAM

The ICCVAM consist of the following 15 federal regulatory and research agencies that require, generate, use, or distribute toxicological testing results:

Agency for Toxic Substances and Disease Registry (ATSDR)
Consumer Product Safety Commission (CPSC)
Department of Agriculture (USDA)
Department of Defense (DOD)
Department of Energy (DOE)
Department of the Interior (DOI)
Department of Transportation (DOT)
Environmental Protection Agency (EPA)
Food and Drug Administration (FDA)
National Institute for Occupational Safety and Health (NIOSH)
National Institutes of Health (NIH), Office of the Director
National Cancer Institute (NCI)
National Institute of Environmental Health Sciences (NIEHS)
National Library of Medicine (NLM)
Occupational Safety and Health Administration (OSHA)

TABLE 21.9
Specific Purposes of the ICCVAM (P.L. 106-545, Section 3(b))

1. Increase the efficiency and effectiveness of federal agency test method review
2. Eliminate unnecessary duplicative efforts and share experiences between federal regulatory agencies
3. Optimize utilization of scientific expertise outside the federal government
4. Ensure that new and revised test methods are validated to meet the needs of federal agencies
5. Reduce, refine, and replace the use of animals in testing, where feasible

Source: 42 U.S.C. §285l-2, ICCVAM Authorization Act of 2000 (P.L. 106-545), 2000.

TABLE 21.10
Duties of the ICCVAM (P.L. 106-545, Section 3(e))

1. Review and evaluate new or revised or alternative test methods, including batteries of tests and test screens, that may be acceptable for specific regulatory uses, including the coordination of technical reviews of proposed new or revised or alternative test methods of interagency interest.
2. Facilitate appropriate interagency and international harmonization of acute or chronic toxicological test protocols that encourage the reduction, refinement, or replacement of animal test methods.
3. Facilitate and provide guidance on the development of validation criteria, validation studies, and processes for new or revised or alternative test methods and help facilitate the acceptance of such scientifically valid test methods and awareness of accepted test methods by federal agencies and other stakeholders.
4. Submit ICCVAM test recommendations for the test method reviewed by the ICCVAM, through expeditious transmittal by the Secretary of Health and Human Services (or the designee of the secretary), to each appropriate federal agency, along with the identification of specific agency guidelines, recommendations, or regulations for a test method, including batteries of tests and test screens, for chemicals or class of chemicals within a regulatory framework that may be appropriate for scientific improvement, while seeking to reduce, refine, or replace animal test methods.
5. Consider for review and evaluation petitions received from the public that
   (A) Identify a specific regulation, recommendation, or guideline regarding a regulatory mandate
   (B) Recommend new or revised or alternative test methods and provide valid scientific evidence of the potential of the test method
6. Make available to the public final ICCVAM test recommendations to appropriate federal agencies and the responses from the agencies regarding such recommendations.
7. Prepare reports to be made available to the public on its progress under this act. The first report shall be completed not later than 12 months after the date of the enactment of this act, and subsequent reports shall be completed biennially thereafter.

Source: 42 U.S.C. §285l-2, ICCVAM Authorization Act of 2000 (P.L. 106-545), 2000.

TABLE 21.11
ICCVAM Prioritization Criteria

ICCVAM prioritizes proposed test method submissions and nominations based on consideration of the extent to which the proposed test method is:

1. Applicable to regulatory testing needs
2. Applicable to multiple agencies/programs
3. Warranted, based on the extent of expected use or application and impact on human, animal, or ecological health
4. The potential for the proposed test method, compared to current test methods accepted by regulatory agencies, to
   a. *Refine* animal use by decreasing or eliminating pain and distress or enhancing animal well-being
   b. *Reduce* animal use
   c. *Replace* animal use
5. The potential for the proposed test method to provide improved prediction of adverse health or environmental effects, compared to current test methods accepted by regulatory agencies
6. The extent to which the test method provides other advantages (e.g., reduced cost and time to perform) compared to current methods
7. The completeness of the nomination or submission with regard to ICCVAM test method submission guidelines

characterization and savings in time and costs, and that provide for the animal welfare goal of refinement, reduction, and replacement of animal use whenever feasible.

Test Method Nomination and Submission Process

Any organization or individual can submit a test method for which adequate validation studies have been completed to ICCVAM for evaluation [11,46,60]. ICCVAM has published guidelines for the information that should be submitted and has developed an outline to organize the information and data supporting the scientific validity of a proposed test method [46]. Any organization or individual can also nominate test methods for which adequate validation studies have not been completed to the ICCVAM for further study [46]. Nominations are prioritized based on established ICCVAM prioritization criteria (Table 21.11) [46,72]. Specific activities, such as workshops and validation studies, are then conducted for those test methods with the highest priority and for which resources are available [46,72].

ICCVAM Contribution to Regulatory Acceptance of Alternative Test Methods

ICCVAM and NICEATM and their member agencies and international partners have now contributed to the adoption of over 63 in vitro and in vivo alternative test methods by national and international authorities [72,74]. These methods have resulted in significant refinement, reduction, and partial replacement of animal use. Examples of in vitro test methods reviewed and recommended by ICCVAM and adopted by U.S. agencies and the OECD where applicable include

four in vitro methods for identifying dermal corrosives [31–35], two in vitro methods for eye safety testing [75–77], five in vitro pyrogen tests [76], and two in vitro methods for assessing estrogen receptor agonist and antagonist activity of chemicals [49,67,77,78]. Important examples of reduction and refinement alternative methods recommended by ICCVAM and adopted by the OECD include four versions of the LLNA assay for assessing ACD [68–71,73,79,80], the revised up-and-down procedure for determining acute oral toxicity [22,81], and recommendations for the routine use of pain-relieving medications and humane endpoints whenever it is still necessary to use animals for eye safety testing [29,82]. Many other reduction, refinement, and replacement alternative test methods have been evaluated or are currently undergoing evaluation, while still others are being developed for a wide range of human health and ecological testing purposes [72,83].

OTHER ORGANIZATIONS INVOLVED IN VALIDATION

In addition to the United States, several other countries have also established government centers to conduct independent validation studies of new alternative methods. These include, among others, the European Centre for the Validation of Alternative Methods (ECVAM) in the European Union, the Japanese Center for the Validation of Alternative Methods (JaCVAM) in Japan, the National Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) in Germany, and the Korean Center for the Validation of Alternative Methods (KoCVAM). In 2009, an international memorandum of cooperation was signed by the United States, the European Union, Canada, and Japan to establish the International Cooperation on Alternative Test Methods (ICATM) [84,85]. In 2011, KoCVAM also became a signatory to the ICATM agreement.

International Cooperation on Alternative Test Methods

The ICATM is an agreement to establish and promote international cooperation by national validation centers in the critical areas of validation studies, independent peer review, and development of harmonized test method recommendations for alternative test methods [84,85]. The goal of these cooperation activities is to promote more efficient and more rapid international adoption of scientifically valid alternative methods. The five current national organizations participating in the ICATM are NICEATM/ICCVAM, Health Canada, EURL-ECVAM, JaCVAM, and KoCVAM [85].

EURL-ECVAM

The ECVAM was established by the European Union in 1992 as a component within the European Commission [86]. In 2011, ECVAM was redesignated as the European Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) as a result of EU Directive 2010/63/EU [9]. EURL-ECVAM is administratively located within the Institute for Health and Consumer Protection within the European Commission's Joint Research Centre in Ispra, Italy. The duties of the EURL-ECVAM are to promote the development and use of alternative methods in both basic research and applied research and in the regulatory field and to coordinate the process of validation of alternative methods at the European level. EURL-ECVAM has received significant support to assist with the development and validation of in vitro methods to meet EU Parliament deadlines that will prohibit the use of animals for the testing of cosmetic ingredients in 2013 [87]. EU legislation adopted in 2004 already bans the use of animals for testing cosmetic products [88]. ICCVAM and EURL-ECVAM work together on projects and validation studies of common interest to facilitate harmonized methods and to leverage resources [29,89].

JaCVAM

The JaCVAM was officially established in November 2005 [90,91]. The center is a component of the National Institute of Health Sciences, which is part of the Ministry of Health and Welfare, and is located in Tokyo, Japan. The center was established to develop, validate, and review alternative test methods in Japan and interacts closely with the Japanese Society for Alternatives to Animal Experimentation (JSAAE) [90–92]. The JSAAE has provided national leadership in the development and validation of alternative methods in Japan and provides an important scientific network for related in vitro studies in Japan.

ZEBET

The German Centre for the Documentation and Validation of Alternative Methods was established in Germany in 1989 [93]. Since 2002, it has been a component of the Federal Institute for Risk Assessment (BfR) located in Berlin, Germany. ZEBET has been instrumental in the development and validation of several important in vitro alternative methods, including methods for dermal irritation and corrosivity, embryotoxicity, ocular irritation, and photosensitization.

ALLERGIC CONTACT DERMATITIS

The murine LLNA is an example of a reduction and refinement alternative method for assessing the ACD potential of chemicals [4,16,24]. The LLNA was the first alternative test method evaluated and recommended by ICCVAM [25,94–96]. The LLNA uses fewer animals and completely avoids pain and distress compared to the traditional animal tests previously used for ACD testing such as the Buehler test and the GPMT [4,16,24,25,94–98]. The LLNA also provides dose–response information and can be completed in 6 days compared to over 4 weeks for the traditional tests that use guinea pigs [24,25,94]. Based on the results of a comprehensive scientific peer review and technical evaluation, ICCVAM determined that the LLNA was a valid substitute to currently accepted test methods that use guinea pigs and

concluded that the LLNA provides for the refinement and reduction of animal use [25,94]. ICCVAM forwarded test recommendations to agencies for their consideration, and the LLNA was subsequently accepted in 1999 by the U.S. Environmental Protection Agency (EPA), U.S. Food and Drug Administration, Consumer Product Safety Commission, and Occupational Safety and Health Administration. A new internationally harmonized test guideline (Test Guideline 429) on skin sensitization using the LLNA was then adopted in 2002 by the Test Guidelines Programme at the OECD [73].

In 2008 and 2009, ICCVAM evaluated and subsequently recommended several new versions and expanded applications of the LLNA [66,68,69,79,99]. These included an updated version of the LLNA that uses 20% fewer animals and a reduced LLNA (rLLNA) protocol that reduces the number of animals required by an additional 40% [66,79]. The improvements were adopted by U.S. agencies and incorporated in an updated OECD TG429 that also incorporates LLNA performance standards [73]. Finally, two versions of the LLNA that do not use radioactive substances, the LLNA-DA and the LLNA:BrdU-ELISA, were adopted by U.S. regulatory agencies and the OECD in 2011 [70,71]. The LLNA is now commonly used worldwide and is the preferred method to determine the ACD hazard for most types of substances [100,101]. The following sections describe the LLNA test method protocol and recent updates, the rLLNA, the LLNA-DA, and the LLNA:BrdU-ELISA.

Regulatory Rationale for the LLNA

ACD is associated with chemical exposures in the workplace and at home. ACD is a serious public health problem, with over 4000 chemicals and substances known to cause allergic dermatitis in humans [102]. Accordingly, regulatory agencies require testing to determine if substances may have the potential to cause ACD, and require that positive substances must be labeled to warn consumers and workers of the hazard potential for ACD along with appropriate precautions to take to avoid exposures [103–106]. An assessment of the potential for chemicals to cause ACD is therefore an important component of routine chemical and product safety testing. Traditionally, guinea pigs have been used to assess the ACD potential of chemicals, pharmaceuticals, and consumer products [94]. Although these test methods vary, the GPMT and the Buehler assay (BA) have been the most commonly used methods for ACD testing. Both of these tests rely on the induction and elicitation phases of ACD and require about a month to perform. The GPMT also may involve the use of complete Freund's adjuvant, which can be highly irritating to animals. The endpoint measured in the guinea pig methods is a visual assessment of erythema and edema at the challenge location and requires substantial technical expertise [107].

Mechanistic Basis of Allergic Contact Dermatitis

The LLNA is a mechanism-based assay for ACD testing that measures one of the early key biological pathway events that are necessary for the development of chemically induced ACD [108–110]. The sequence of key events that must occur in order to produce ACD are collectively referred to an adverse outcome pathway (Figure 21.2) [110]. The key event measured in the LLNA is the threefold or higher level of lymphocyte proliferation that occurs in the lymph

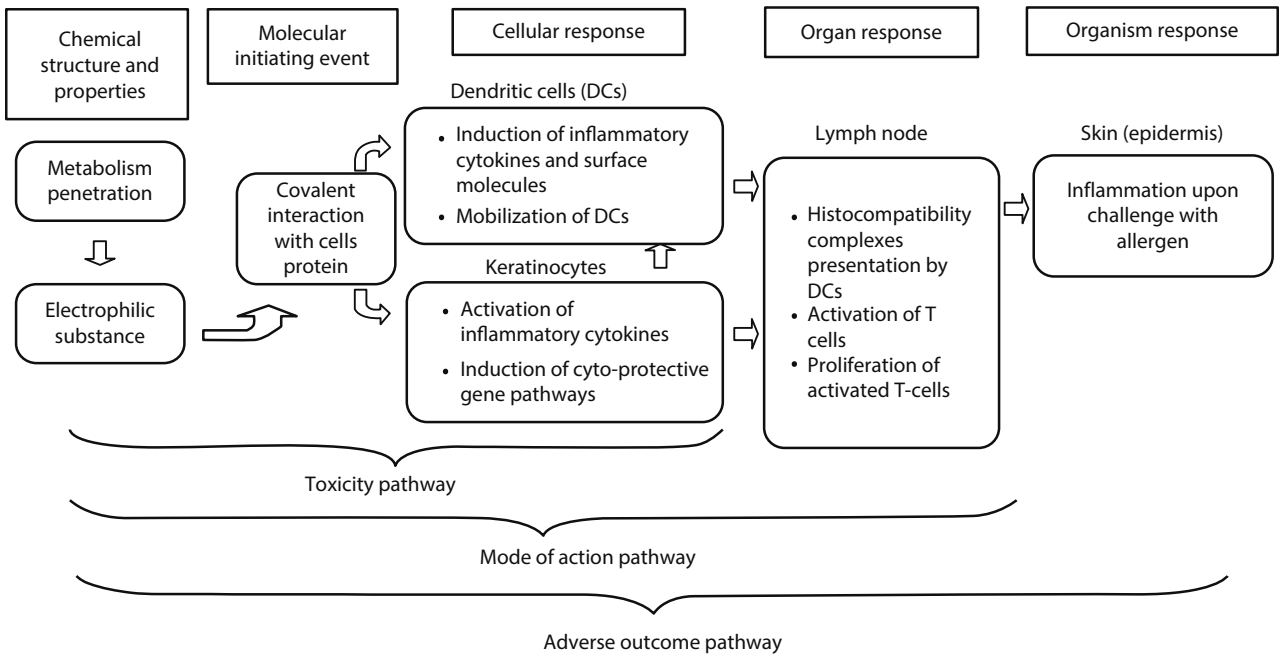

FIGURE 21.2 Adverse outcome pathway for skin sensitization. (From OECD, The adverse outcome pathway for skin sensitization initiated by covalent binding to proteins, Part 1: Scientific evidence, OECD Environment, Health, and Safety Publications: Series on Testing and Assessment No 168, OECD, Paris, France, 2012.)

nodes draining the skin area where a test article is repeatedly applied [94,108,110]. This compares with the assessment in the traditional GP assays of the actual adverse outcome of erythema and edema that occur 24–72 h after a challenge exposure to a sensitizing substance [95].

ACD develops in two phases: an initial induction phase followed by an elicitation phase. The induction phase begins with initial skin contact with a sensitizing agent. Following penetration of the epidermis, reactive electrophilic chemicals undergo covalent bonding to protein in a process called haptenization [110–112]. Allergenic substances that require metabolism in the skin to become reactive are called prohaptens, while substances that require oxidation or other chemical activation to become active are called prehaptens. Biological and chemical assays are now available to assess the peptide reactivity of chemicals, including the direct peptide reactivity assay (DPRA) and a recombinant human keratinocyte cell line with a reporter gene that is activated by electrophilic chemicals [111,113].

Following haptenization, an immediate release of signaling factors and activation of the skin dendritic cells occur. Dendritic cells process the chemical and subsequently mature and migrate to the draining lymph node where they serve as antigen-presenting cells. Lymphocytes within the nodes, upon antigen presentation, undergo cellular proliferation. Lymphocyte proliferation is the mechanistic endpoint assessed in the LLNA and indicates that the induction phase of ACD has taken place [97]. Following proliferation, T-lymphocytes are considered primed, as they have a specific recall for the sensitizing agent. Upon subsequent exposure to the agent, an antigen-specific response occurs, which is referred to as the *elicitation phase*. This second phase occurs only if there is elicitation of specific mediators that cause an inflammatory cell influx to the dermal site. Elicitation is a systemic response that can occur at locations other than the original site of sensitization. The elicitation phase is characterized by erythema and edema and occurs 24–72 h after the challenge exposure. This response is the endpoint assessed in traditional guinea pig tests [110]. Since the LLNA only assesses events that occur during the induction phase of ACD, it therefore avoids the need for later chemical challenge exposures necessary to elicit an allergic response and that can result in pruritus, erythema, and edema for sensitizing chemicals.

Traditional LLNA Procedure

ICCVAM has updated its detailed test method protocol for the LLNA [66] that has been adopted by U.S. regulatory agencies and that serves as the basis for the updated OECD test guideline [73] for the LLNA. The following sections briefly review the LLNA technical procedures in the updated test method protocol, but users should always ensure that they are compliant with the most recent version available and that it is consistent with any additional guidelines from the applicable regulatory authorities for which the test data are being generated. The basic principle underlying the traditional LLNA is that sensitizers induce proliferation of lymphocytes in the lymph node draining the site of chemical application [97,98,108,109]. Generally, this proliferation is proportional to the dose applied and potency of the sensitizer. The test measures cellular proliferations as a function of in vivo radioisotope incorporation into the DNA of dividing lymphocytes in the draining lymph nodes proximal to the application site (Figure 21.3) [72]. The lymphocyte proliferation in the test groups is compared to that in concurrent vehicle-treated controls. A positive control group of animals is included in each assay to indicate whether all biologic and technical aspects of the assay are sufficiently adequate such that the assay is capable of producing a sufficiently positive result for the positive control. If the positive control does not result in a positive test outcome, the test is not considered valid and must be repeated.

Animals

Young adult female CBA/Ca or CBA/J strain mice (nulliparous and not pregnant) 8–12 weeks of age are used for the assay [66,73,94]. Females are used because the existing database is predominantly based on this gender. Other strains and males can be used following evaluation that demonstrates that they can provide equivalent results to the strains and/or gender used in the validation studies. Mice are carefully observed for any clinical signs, including local irritation at the application site and signs indicative of systemic toxicity. Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded, with records being maintained for each individual mouse.

Test Articles

Solid test substances are dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be prepared daily unless stability data demonstrate the acceptability of storage. The solvent or vehicle should be selected on the basis of maximizing the test concentrations while producing a solution or suspension suitable for application of the test substance. In order of preference, recommended solvents and vehicles are acetone/olive oil (4:1 v/v), *N*,*N*-dimethylformamide (DMF), methyl ethyl ketone (MEK), propylene glycol (PG), and dimethylsulfoxide (DMSO), but others may be used [66,73,94]. Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off [99,114,115]. It may be necessary for regulatory purposes to test the chemical in the clinically relevant solvent or product formulation.

Four successfully treated animals are used per dose group, with a minimum of three consecutive concentrations of the test substance plus a solvent or vehicle control and a positive control group. Test substance treatment doses should be based on adopted recommendations and guidelines [66,73]. Doses are selected from the concentration series 100%, 50%, 25%,

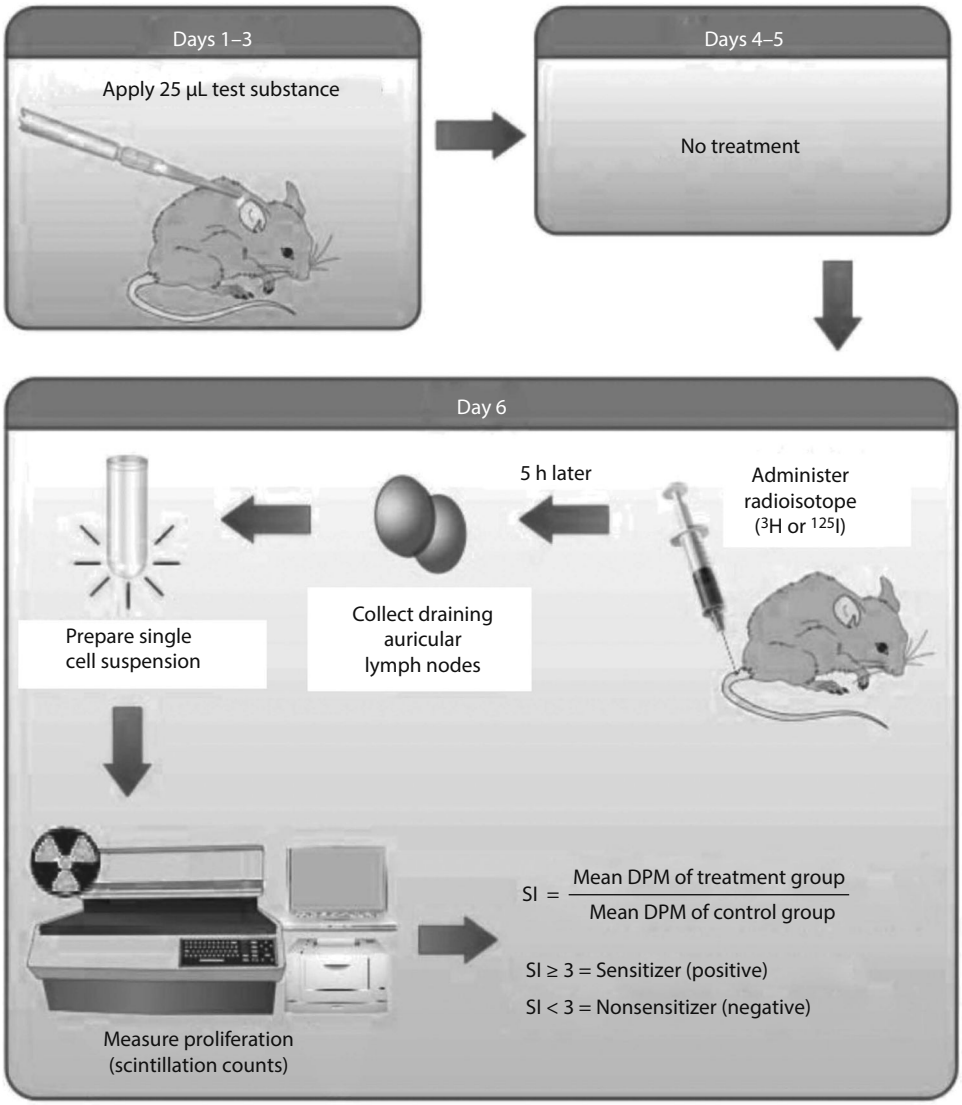

FIGURE 21.3 Testing procedure: traditional murine LLNA. (From Stokes, W.S. and Strickland, J., in Wilhelm, Z. and Maibach, H., eds., *Dermatotoxicology*, 8th edn. Informa Healthcare, London, U.K., 2012, pp. 473–485. With permission.)

10%, 5%, 2.5%, 1%, 0.5%, etc. The maximum concentration tested should be the highest achievable level that does not result in local irritation or overt systemic toxicity. To identify the appropriate maximum test substance dose, an initial toxicity test, conducted under identical experimental conditions except for an assessment of lymph node proliferative activity, may be necessary. To support an ability to identify a dose–response relationship, data must be collected on at least three test substance treatment doses, in addition to the concurrent solvent or vehicle control group. For negative LLNA studies, the concurrent positive control must induce a stimulation index (SI) greater than 3 relative to its vehicle-treated control.

Controls

Concurrent solvent/vehicle and positive controls should be included in each test [66]. In some circumstances, it may be useful to include a naïve control. Except for treatment with the test substance, animals in the control groups should be handled in a manner identical to that for animals of the treatment groups. Inclusion of a positive control group with each test is recommended to ensure that all test protocol procedures are being conducted properly and that all aspects of the test system are working sufficiently adequately that the test is capable of producing a positive response [66]. The positive control should produce a positive LLNA response at an exposure level expected to give an increase in the SI greater than 3 over the negative control group. The positive control dose should be chosen such that the induction is clear but not excessive. Preferred positive control substances are hexyl cinnamic aldehyde (HCA) or mercaptobenzothiazole.

Protocol Schedule

Day 1—Individually identify and record the weight of each mouse prior to dermal applications. Apply 25 µL/ear of the appropriate dilution of the test substance, the positive control, or the vehicle alone to the dorsum of both ears (Figure 21.3).

Days 2 and 3—Repeat the application procedure as carried out on day 1.

Days 4 and 5—No treatment.

Day 6—Record the weight of each mouse. Inject 250 μL of sterile phosphate-buffered saline (PBS) containing 20 μCi of ^{3}H-methyl thymidine (^{3}H-TdR) or 250 μL PBS containing 2μCi of ^{125}I-iododeoxyuridine (^{125}IU) and 10^{-5} M fluorodeoxyuride into each experimental mouse via the tail vein [74,75]. Five hours later, the draining (auricular) lymph node of each ear is excised and pooled in PBS for each animal [51]. Both bilateral draining lymph nodes must be collected.

Lymphocyte Measurements

A single-cell suspension of lymph node cells (LNCs) is prepared for each mouse. The single-cell suspension is prepared in PBS either by gentle mechanical separation through 200-mesh stainless steel gauze or by another acceptable technique for generating a single-cell suspension. LNCs are washed twice with an excess of PBS and the DNA precipitated with 5% trichloroacetic acid (TCA) at 4°C for approximately 18 h. For the ^{3}H-TdR method, pellets are resuspended in 1 mL TCA and transferred to 10 mL of scintillation fluid. Incorporation of tritiated thymidine is measured by β-scintillation counting as disintegrations per minute (dpm) for each mouse and expressed as dpm/mouse. For the ^{125}IU method, the 1 mL TCA pellet is transferred directly into gamma counting tubes. Incorporation of ^{125}IU is determined by gamma counting and also expressed as dpm/mouse.

Calculation of the Stimulation Index

The LLNA measures lymphocyte proliferation in the draining lymph nodes of mice topically exposed to the test article using the incorporation of radioactive thymidine or iododeoxyuridine into DNA. The results are expressed as a ratio, the SI, of the mean number of disintegrations per minute for treated mice as compared to controls. Chemicals with a SI of 3.0 or more are considered positive, and those with a SI less than 3.0 are considered negative. This scoring differs from the scoring in guinea pig assays, where a test substance is classified as positive based on the percentage of animals in a group that are responders (at least 15% in a nonadjuvant assay and at least 8% in an adjuvant test) [70].

Results for each treatment dose group are expressed as the mean SI. The SI is the ratio of the mean dpm/mouse within each test substance treatment group and the positive-control-treated group against the mean dpm/mouse for the solvent- or vehicle-treated control group. The investigator should be alert to possible outlier responses for individual animals within a group that may necessitate the use of an alternative measure of response (e.g., median rather than mean) or elimination of the outlier. Each SI should include an appropriate measure of variability that takes into account the interanimal variability in both the dosed and control groups [51].

In addition to an assessment of the magnitude of the SI, a statistical analysis for the presence and degree of dose–response may be conducted, which is only possible with the use of data from individual animals [66]. Any statistical analysis should include an assessment of the dose–response relationship, as well as suitably adjusted comparisons of test groups (e.g., pairwise dosed group vs. concurrent solvent or vehicle control comparisons) such as by using linear regression analysis to assess dose–response trends or Dunnett's test for pairwise comparisons. When choosing an appropriate method of statistical analysis, the investigator should be aware of possible inequality of variances and other related problems that may necessitate a data transformation or a nonparametric statistical analysis.

Individual mouse disintegrations per minute data should be presented in tabular form, along with the group mean dpm/mouse, its associated error term, and the SI (and associated error term) for each dose group compared against the concurrent solvent or vehicle control group.

Evaluation and Interpretation of Results

In general, when the SI for any single treatment dose group is 3 or greater, the test substance is regarded as a skin sensitizer [66,73,94]; however, the magnitude of the SI should not be the sole factor used to determine the biological significance of a skin sensitization response. A quantitative assessment performed by statistical analysis of individual animal data may provide a more complete evaluation of the test agents. Factors that should be considered in addition to the SI include statistical analyses, the strength of the dose–response relationship, chemical toxicity, solubility, and the consistency of the vehicle and positive control responses. Equivocal results, such as when the SI approaches but does not reach an SI of 3 and there is a positive dose–response relationship, should be clarified by performing statistical analysis and by also considering structural relationships, available toxicity information, and dose selection.

Training and Preparation for Node Identification

There are several methods that can be used to provide color identification of the draining nodes [66,94,95,98]. These techniques may be helpful to provide training for initial identification and should be performed to ensure proper isolation of the appropriate node. Examples of such treatments that can be used for training are listed as follows:

- *Evan's blue dye treatment*—Inject approximately 0.1 mL of 2% Evan's blue dye (prepared in sterile saline) intradermally into the pinnae of an ear. Humanely kill the mouse after several minutes and then proceed with the dissection to remove the auricular lymph nodes.
- *Colloidal carbon and other dye treatments*—Colloidal carbon and India ink are examples of other dye treatments that may be used [78]. For the purpose of node identification during training, a strong sensitizer is recommended. This agent should be applied in the standard acetone–olive oil vehicle (4:1). Suggested sensitizers used for this training exercise include 0.1% oxazolone, 0.1% (w/v)

2,4-dinitrochlorobenzene, and 0.1% (v/v) dinitrofluorobenzene. After treating the ear with a strong sensitizer, the draining node will dramatically increase in size, thus aiding in the identification and location of the node. Due to the exaggerated response produced by the suggested sensitizers for node identification, they are not recommended as positive controls for the assay performance and should only be used for training.

The node draining the ear (auricular) is located distal to the masseter muscle, away from the midline, and near the bifurcation of the jugular vein. Nodes can be distinguished from the glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. The application of sensitizing agents will cause an enlargement of the node.

Expanded Applicability Domain of the LLNA

Following the 1998 ICCVAM evaluation of the LLNA, the LLNA was not recommended for testing metals, due to some false-negative results, or aqueous substances, because they would not adhere to the skin and allow for sufficient skin contact. There was also no data provided to evaluate the usefulness of the LLNA for formulations. After a number of years of international experience with the LLNA and the accumulation of additional data, ICCVAM reevaluated the potential applicability domain of the LLNA in 2008 [99]. The predictivity of the LLNA was compared to available guinea pig and/or human ACD testing data for a wide range of substance and formulations. Based on this comparison, ICCVAM recommended that the LLNA could be used for testing metal compounds (with the exception of nickel), pesticide formulations, dyes, natural complex substances (i.e., fragrance oil and extracts), and substances tested in aqueous solutions [99]. ICCVAM also recommended that any substance could be tested in the LLNA, unless the substance has unique physicochemical properties that could interfere with identification in the LLNA as a sensitizing substance. U.S. federal agencies concurred with the ICCVAM recommendations on the expanded applicability domain (http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm), and the U.S. EPA updated their policy to accept LLNA data for testing pesticide products [116].

Reduced LLNA Test Method Protocol

The rLLNA is a modified version of the traditional LLNA that reduces animal use by 40% per test [24,73,79,116,117]. The test method protocols for the multidose LLNA and the rLLNA differ only in the number of dose levels tested. In the multidose LLNA, at least three dose levels are tested, while in the rLLNA, only the highest dose of a substance is tested. In either version, it is important that the highest dose is selected based on the maximum soluble concentration that avoids excessive local irritation and/or systemic toxicity [66,79]. The validity of the rLLNA was determined by comparing the outcome of multidose LLNA results to outcomes based only on the highest dose used in the LLNA tests [79]. The validation database included 457 unique substances tested in 471 multidose LLNA studies. Compared to the multidose LLNA, the rLLNA had an accuracy of 98.7% (465/471), a false-positive rate of 0% (0/153), and a false-negative rate of 1.9% (6/318). All six substances that were considered false negative in the rLLNA had an SI < 3 for the highest dose, while multidose LLNA had positive results based on a low- or mid-dose SI ≥ 3. However, the maximum SI in all cases was less than 4, which is considered a weak response. Based on these results, ICCVAM recommended that the rLLNA could be used to distinguish between skin sensitizers and nonsensitizers if dose–response information was not required [79].

ICCVAM also recommended that the rLLNA should be used routinely to determine ACD potential of chemicals and products *before* conducting the traditional LLNA in order to minimize the number of animals used for ACD testing [79]. This is because the majority of products and chemicals are expected to produce negative results. Using the rLLNA results, negative substances can therefore be classified as nonsensitizers and positive substances can be classified as sensitizers with 40% fewer animals. The exceptions to using the rLLNA for the initial test are testing situations where dose–response information is required *and* there is evidence suggesting that the substance is likely to be a sensitizer. In such situations, these substances should therefore be tested initially in the multidose LLNA.

Because of a small possibility of a false-negative result in the rLLNA, negative results should prompt a weight-of-evidence evaluation of all available information [79]. Items that could be considered in such an evaluation include factors that could reduce skin absorption at the high dose, structural relationship to known sensitizers, test results with similar substances, peptide-binding activity, molecular weight, and other in vitro/in silico/in chemico data. The U.S. EPA accepts rLLNA data in situations where dose–response data are not needed, such as when results are expected to be negative [116].

Updates to the LLNA Test Method Protocol

The LLNA test method protocol was updated in 2009 to include several improvements and approaches that could further reduce animal use [66]. The updated OECD test guideline for the LLNA adopted in 2010 incorporates these improvements, as well as the rLLNA, the expanded LLNA applicability domain, and LLNA performance standards [66,73,79,99].

The updated LLNA protocol now uses a minimum of four animals per dose group rather than five. This reduction was based on a retrospective evaluation of data that showed that there was no significant difference in classification outcome when the LLNA was conducted using four animals compared to using five or more animals [79,118].

The updated protocol emphasizes the importance of collecting data from individual animals rather than pooling lymph nodes from all animals in a dose group [66,79].

Pooled data are discouraged because a review of actual data revealed the possibility of low outlier values that could result in false-negative results [66,118]. Outlier analysis is now recommended whenever an unusually high or low value is observed in a dose group [66,73,79]. However, individual animal data must be collected in order to conduct an outlier analysis.

The updated protocol also included more detailed guidance on procedures to select the highest dose used in the LLNA [66,73,79]. In testing situations where there is inadequate basis for selection of the highest dose, then a dose-range finding study is recommended and criteria are provided to select the highest dose that does not cause excessive local irritation or systemic toxicity.

Nonradioisotopic LLNA Test Methods

Two versions of the LLNA are available that do not use radioactive substances: the LLNA:DA and the LLNA:BrdU-ELISA [68,69]. U.S. regulatory agencies and the OECD have adopted both versions [70,71]. These LLNA test methods now allow broader use of the LLNA, as they can be used instead of the traditional guinea pig test methods in laboratories that are not approved to use radioisotopes. These LLNA methods now allow for use of the LLNA for nearly all skin sensitization testing situations, resulting in significantly expanded animal welfare benefits of the LLNA in terms of reduced animal use and avoidance of pain and distress. The nonradioactive LLNA test methods also offer environmental advantages by avoiding the generation of radioactive wastes.

LLNA:DA Test Method

The LLNA:DA (*D* for Daicel Chemical Industries, Ltd., and *A* for ATP) is a nonradioactive LLNA method developed by Idehara and colleagues at Daicel Chemical Industries, Ltd. [24,70,119,120]. The LLNA:DA measures increases in ATP content in the draining auricular lymph nodes removed from treated and control mice. ATP content is quantified using a luciferin–luciferase assay to measure bioluminescence. Because ATP content correlates with living cell number, its measurement serves as an indicator of cell number at the time of sampling. The protocol for the LLNA:DA is similar to that for the LLNA (Figure 21.4). A minimum of four mice

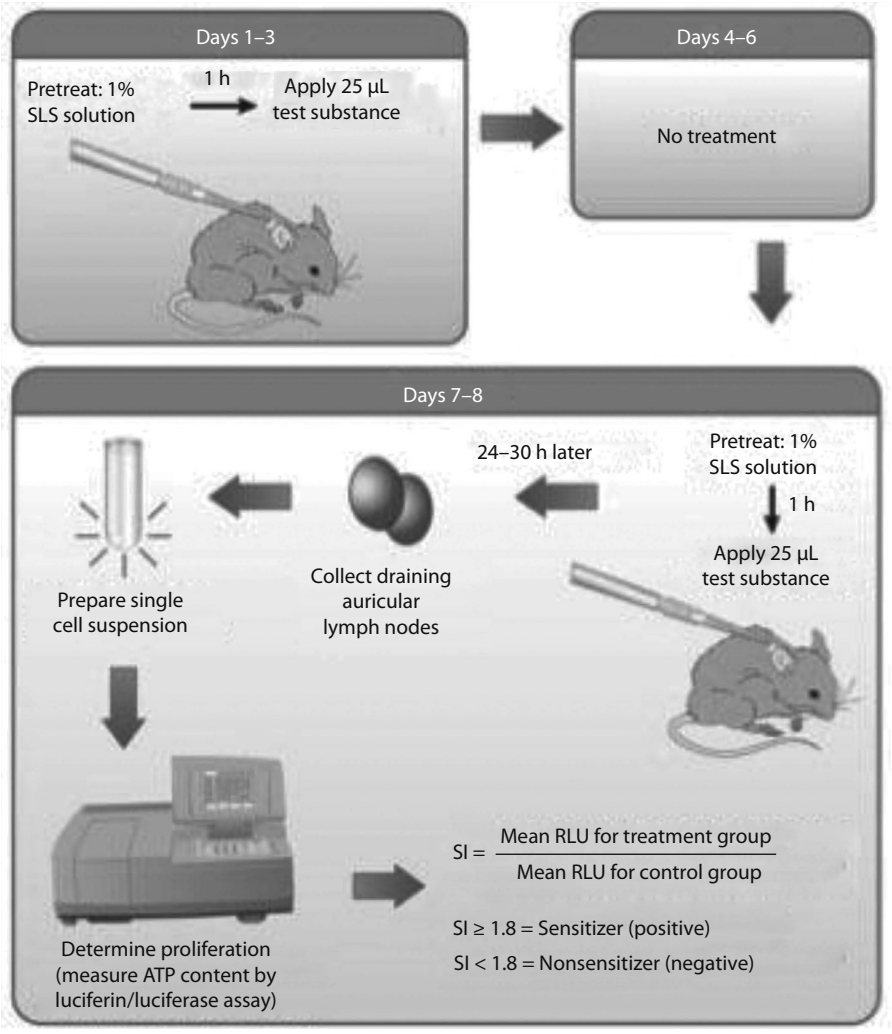

FIGURE 21.4 Testing procedure: murine LLNA:DA. (From Stokes, W.S. and Strickland, J., in Wilhelm, Z. and Maibach, H., eds., *Dermatotoxicology*, 8th edn. Informa Healthcare, London, U.K., 2012, pp. 473–485. With permission.)

are used per dose group, with at least three dose groups and concurrent vehicle and positive control groups [24,68,70].

Test and control substances (25 μL) are applied to the dorsum of each ear on days 1, 2, 3, and 7 one hour after pretreatment with 1% sodium lauryl sulfate (SLS). The SLS increases the absorption of the test substance across the skin. On day 8, the mice are humanely killed, the lymph nodes are excised, and an LNC suspension is prepared for each mouse. ATP content, which is proportional to the light produced by the luciferin–luciferase reaction, is measured using a commercially available kit. An SI value for each treatment group is calculated as the ratio of the mean relative luminescence units for the treatment group compared to the mean relative luminescence units for the control group. The decision criterion for a positive result is an SI ≥ 1.8 [24,70]. A reduced protocol for the LLNA:DA, the rLLNA:DA, should be used in testing situations that do not require dose–response information. The same considerations for using the rLLNA as the initial test for ACD assessments should also be considered in using the rLLNA:DA as the initial test for ACD testing. The applicability domain for the LLNA:DA is the same as the LLNA, unless there are properties associated with a class of materials that may interfere with the test method's accuracy. For example, the use of the LLNA:DA might not be appropriate for testing substances that affect the ATP content of cells (e.g., substances that function as ATP inhibitors) [68].

LLNA:BrdU-ELISA Test Method

The LLNA:BrdU-ELISA (*BrdU* for bromodeoxyuridine and *ELISA* for enzyme-linked immunosorbent assay) is a nonradioactive LLNA method developed by Takeyoshi and colleagues [24,69,121]. The LLNA:BrdU-ELISA assesses lymphocyte proliferation in the draining auricular lymph nodes by measuring the incorporation of the thymidine analog, BrdU, into the DNA of dividing LNCs. The protocol of the LLNA:BrdU-ELISA is similar to that for the LLNA (Figure 21.5). It uses a minimum of four mice for each of three or more dose group and concurrent vehicle and positive control groups. Test and control substances (25 μL) are applied to the dorsum of each ear on days 1, 2, and 3. On day 5, 5 mg BrdU in a volume of 0.5 mL physiological saline is administered via intraperitoneal injection. The mice are

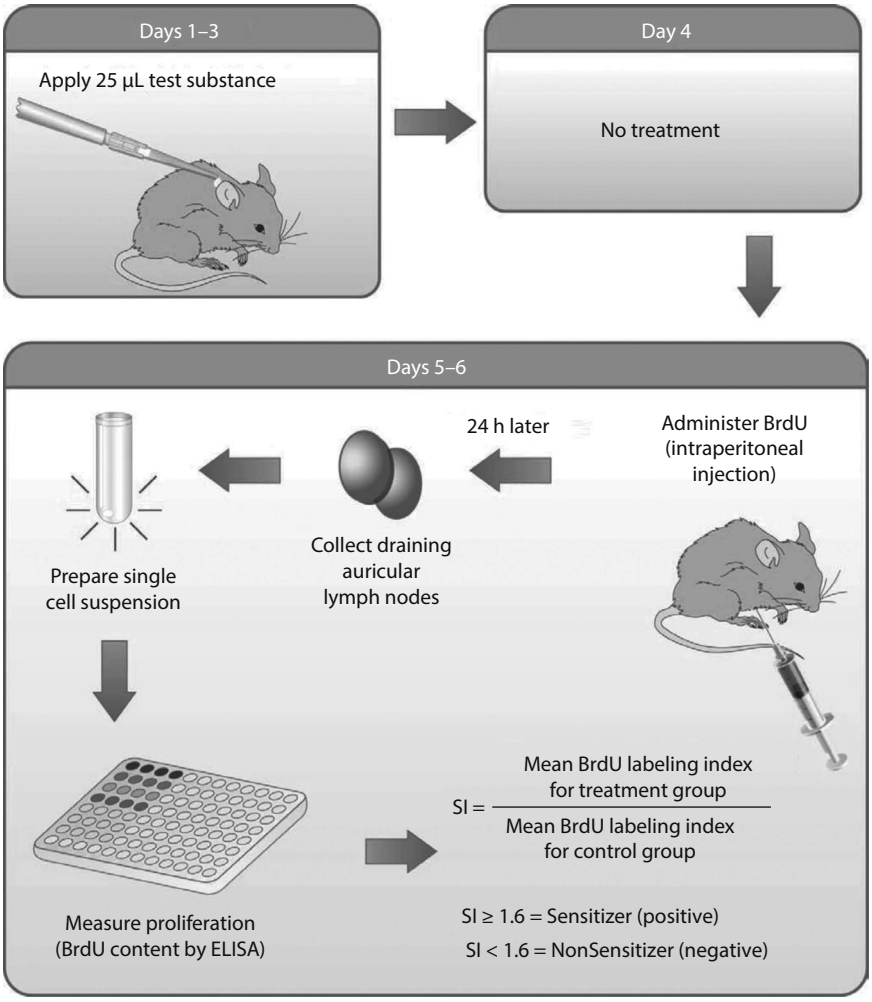

FIGURE 21.5 Testing procedure: Murine LLNA:BrdU-ELISA. (From Stokes, W.S. and Strickland, J., in Wilhelm, Z. and Maibach, H., eds., *Dermatotoxicology*, 8th edn. Informa Healthcare, London, U.K., 2012, pp. 473–485. With permission.)

humanely killed on day 6. The auricular lymph nodes are excised, and an LNC suspension is prepared individually for each mouse. A commercially available ELISA kit is used to assess BrdU incorporation, which is measured spectrophotometrically. An SI value is calculated for each treatment group as the ratio of the mean absorbance for the treatment group to the mean absorbance for the control group. A decision criteria of SI ≥ 1.6 is used to classify positive substances [24,71].

The LLNA:BrdU-ELISA also has a reduced protocol, the rLLNA:BrdU-ELISA, which requires only a single high-dose group, and that reduces the use of animals by 40% [24,69]. The same considerations for using the rLLNA as the initial test for ACD assessments should also be considered in using the rLLNA:BrdU-ELISA as the initial test for ACD testing. The applicability domain for the LLNA:BrdU-ELISA is the same as the LLNA, unless there are properties associated with a class of materials that may interfere with the test method's accuracy. The LLNA:BrdU-ELISA may also be useful in testing nickel compounds based on their correct identification as sensitizers in the validation study [24,69].

ALTERNATIVE METHODS FOR SKIN CORROSION

Four in vitro test methods are available for assessing the dermal corrosivity hazard potentials of chemicals: EPISKIN, EpiDerm (EPI-200), the rat skin TERassay, and Corrositex [31–35]. Based on the available data and performance for each test method, these methods are recommended for use as screening assays for the identification of corrosive substances in a tiered testing strategy, such as that proposed by the OECD (Table 21.12) [42]. In this strategy, positive results can be used to classify and label a substance as a dermal corrosive. Negative results generally require consideration of additional information or testing to properly identify any corrosive substances that may have been falsely identified as noncorrosives and to determine whether the substance is a dermal irritant.

In Vitro Membrane Barrier Test Systems for Skin Corrosion

Validation studies have been completed for an in vitro membrane barrier test system commercially available as Corrositex [35]. Based on its scientific validity, this test method has been recommended for use as part of a tiered testing strategy for assessing the dermal corrosion hazard potential of chemicals, whereby any substance that qualifies for testing can be evaluated [34,35]. In addition, this test method may be used to make decisions on the corrosivity and noncorrosivity of specific classes of chemicals (e.g., organic and inorganic acids, acid derivatives,* and bases) for certain transport testing circumstances. The basis of this test system is that it detects membrane damage caused by corrosive test substances.

The test substance is first evaluated to determine if it is compatible with the test procedure (i.e., if it qualifies for testing). If compatible, the substance is evaluated for the category of acid or base (strong or weak) to determine the appropriate time scale for classifying the potential corrosivity of the test substance. Finally, a compatible substance is applied to the surface of the artificial membrane barrier. The time it takes for the test substance to penetrate through the membrane barrier to an underlying indicator solution determines the corrosivity classification of that test substance. Penetration of the membrane barrier (or breakthrough) is indicated by a color change in a pH indicator dye in the solution below the barrier.

Performance standards have been established for in vitro membrane barrier test systems for corrosivity, as discussed in an earlier part of this chapter [65]. Essential test method components include a description of physical components of the test method (e.g., membrane barrier, categorization solutions, indicator solution), the test substance categorization system, the processes for determining test substance compatibility and test substance categorization, assembly of the physical components of the test method, application of a test substance, the appropriate control substances (solvent controls, positive [corrosive] controls, negative [noncorrosive] controls, benchmark controls), measurement of membrane barrier penetration, interpretation of results, and classification of test substances with regard to corrosivity potential. The test report should include the following information, if relevant to the conduct of the study: test and control substances, justification of the test method and protocol used, test method integrity, criteria for an acceptable test, test conditions, results, description of other effects observed, discussion of the results, and conclusions.

In Vitro Human Skin Cell Culture Systems for Skin Corrosion

The ECVAM conducted validation studies on two in vitro test methods that use cultured human skin cell test systems for assessing skin corrosivity: EpiDerm (MatTek; Ashland, MA) and EPISKIN (EPISKIN SNC; Lyon, France) [122]. These two methods utilize a 3D tissue culture model of human skin composed of a reconstructed epidermis and a functional stratum corneum composed of human keratinocytes. These test methods have been recommended for the testing of all classes of chemicals and for inclusion in tiered testing strategies as part of a tiered or weight-of-evidence evaluation. Neither test method has been validated for categorizing the corrosive properties of chemicals across the three UN PG subcategories of corrosivity, although data are available for differentiating between PG 1 and the two less severe PGs. The methods have been accepted by the European Commission and have been recommended by the ICCVAM for use as screening assays in a tiered testing strategy [31]. An international test guideline is also now available [33].

* *Acid derivative* is a nonspecific class designation and is broadly defined as an acid produced from a chemical substance either directly or by modification or partial substitution. This class includes anhydrides, haloacids, salts, and other types of chemicals.

TABLE 21.12
Testing and Evaluation Strategy for Dermal Irritation/Corrosion

Activity	Finding	Conclusion
1. Review existing human and/or animal data showing effects on skin or mucous membranes.	Corrosive	Apical endpoint—considered corrosive; no testing is necessary.
	Irritating	Apical endpoint—considered an irritant; no testing is necessary.
	Not corrosive/not irritating	Apical endpoint—considered not corrosive or irritating; no testing is necessary.
↓		
No information is available, or available information is not conclusive.		
↓		
2. Perform SAR evaluation for skin corrosion/irritation.	Predict severe damage to skin	Considered corrosive; no testing is necessary.
	Predict irritation to skin	Considered an irritant; no testing is necessary.
↓		
No predictions can be made, or predictions are not conclusive or negative.		
↓		
3. Measure pH (consider buffering capacity, if relevant).	pH ≤ 2 or ≥ 11.5 (with high buffering capacity, if relevant)	Assume corrosivity; no testing is necessary.
↓		
2 < pH < 11.5, or pH ≤ 2.0 or ≥ 11.5 with low or no buffering capacity, if relevant.		
↓		
4. Evaluate systemic toxicity data via dermal route.[a]	Highly toxic	No further testing is necessary.
	Not corrosive or irritating when tested to limit dose of 2000 mg/kg body weight or higher, using rabbits	Assumed not to be corrosive or irritating; no further testing is necessary.
↓		
Such information is not available or is not conclusive.		
↓		
5. Perform validated and accepted in vitro or ex vivo test for skin corrosion.	Corrosive response	Assume corrosivity in vivo; no further testing is necessary.
↓		
Substance is not corrosive, or internationally validated in vitro *and* ex vivo *testing methods for skin corrosion are not yet available.*		
↓		
6. Perform validated and accepted in vitro or ex vivo test for skin irritation.	Irritant response	Assume irritancy in vivo; no further testing is necessary.
↓		
Substance is not an irritant, or internationally validated in vitro *and* ex vivo *testing methods for skin irritation are not yet available.*		
↓		
7. Perform initial in vivo rabbit test using one animal.	Severe damage to skin	Considered corrosive; no further testing is necessary.
↓		
No severe damage.		
↓		
8. Perform confirmatory test using one or two additional animals.	Corrosive or irritating	Considered corrosive or irritating; no further testing is necessary.
	Not corrosive or irritating	Considered not corrosive or irritating; no further testing is necessary.

[a] Can be considered before steps 2 and 3.

The test material is applied topically to a 3D human keratinocyte culture model, composed of at least a reconstructed epidermis with a functional stratum corneum. Corrosive substances are identified by their ability to induce a decrease in cell viability below defined threshold levels at specified exposure periods. The principle of the human skin model assay is based on the premise that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion and are cytotoxic to the keratinocytes in the underlying layers. The use of test systems that include human-derived cells or tissue should be in accordance with applicable national and international laws, regulations, and policies.

Investigators using a similar in vitro human skin cell culture model system for skin corrosion must be able to demonstrate that the assay is valid for its intended use. This includes demonstrating that different preparations are consistent in barrier properties (i.e., capable of maintaining a barrier to noncorrosive substances, able to respond appropriately to weak and strong corrosive substances) and that any modification to the existing validated reference test method does not adversely affect its performance characteristics. Performance standards are now available that can be used for this purpose, as described earlier in this chapter [65]. Essential test method components are also available for in vitro human skin model test methods for skin corrosivity [65]. The components are essentially the same as those described for Corrositex with the addition of components unique to in vitro human skin model systems. Human skin models can be obtained commercially (e.g., EPISKIN, EpiDerm [EPI-200]) or they can be developed or constructed in the testing laboratory.

In Vitro Skin Transcutaneous Electrical Resistance Tests for Skin Corrosion

The ECVAM also conducted validation studies on the rat skin TER assay, another in vitro test method for assessing skin corrosivity [122]. The rat skin TER assay measures the extent to which a chemical alters the TER of a skin disc during a defined exposure period. Based on its scientific validity, this test method has been recommended for the testing of all classes of chemicals and for inclusion in tiered testing strategies as part of a tiered or weight-of-evidence evaluation [31,32].

The test substance is applied for up to 24 h to the epidermal surface of skin discs in a two-compartment test system in which the skin discs function as the separation between the compartments. The skin discs are prepared from humanely killed 28- to 30-day-old rats. Corrosive substances are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the TER below a specified level. For rat skin TER, a cutoff value of 5 kΩ has been selected based on extensive data for a wide range of substances where the majority of values were either clearly well above or well below this value. Generally, substances that are noncorrosive in animals but are irritating do not reduce the TER below this cutoff value; however, the use of other skin preparations or other equipment to measure resistance may require the use of a different cutoff value. In such situations, more extensive validation would be required. A dye-binding step is incorporated into the test procedure to confirm positive results. The dye-binding step determines if the increase in ionic permeability is due to physical destruction of the stratum corneum.

Investigators using an in vitro skin TER corrosivity test that is different from the validated test method protocol must be able to demonstrate that the assay is valid for its intended use. This includes demonstrating that different preparations are consistent in barrier properties (i.e., capable of maintaining a barrier to noncorrosive substances, able to respond appropriately to weak and strong corrosive substances) and that any modification to the existing validated reference test method does not adversely affect its performance characteristics. Performance standards are now available to accomplish the necessary validation of test method modifications of the TER [65].

FUTURE PROGRESS

Significant progress and consensus have been made in recent years on the scientific principles and processes for adequate validation of in vitro and other test methods proposed for regulatory applications. Governments, regulatory authorities, and research organizations continue to increase committed resources and personnel to the effort of identifying new and alternative test methods with potential regulatory applicability that are as good as or better than traditionally employed test methods. Regulatory authorities have also communicated the criteria that they will use as the basis for making decisions on the regulatory acceptability of new, revised, and alternative methods. The ICCVAM now provides an efficient process for the interagency evaluation of new, revised, and alternative methods of multiagency interest, thereby limiting or eliminating duplicative efforts by independent agencies. These established criteria and processes will facilitate the validation and regulatory acceptance of proposed test methods that incorporate new science and technology. Similarly, the recently formed International Cooperation on Alternative Methods will continue to provide an efficient means of coordinating international validation studies, reviews, and development of harmonized recommendations that will facilitate more rapid international and global acceptance of alternative test methods. Increased application of new science and technology to safety assessment methods will result in more accurate and efficient tools for the safety assessment process. Future progress in the development, validation, and adoption of improved testing methods based on sound science can be expected to support enhanced protection of public health, animal health, and the environment. Adoption of scientifically valid alternative methods will also support improved animal welfare by reducing, replacing, and providing for more humane use of laboratory animals.

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QUESTIONS

21.1 What are the basic requirements for adequate validation of a proposed new test method?

21.2 What are the considerations used by regulatory authorities in determining the acceptability of a new test method?

21.3 What are the three components of test method performance standards, and how can performance standards be used to more efficiently validate new test methods?

21.4 What are the three advantages provided in using the murine LLNA for ACD testing instead of the guinea pig maximization or Buehler test?

KEYWORDS

Alternative methods, Safety testing, Validation, Regulatory acceptance, Allergic contact dermatitis testing, Dermal corrosivity testing

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22 Acute Toxicity and Eye Irritancy

Ping Kwong (Peter) Chan and A. Wallace Hayes

CONTENTS

The methods and principles of evaluating two categories of hazards, acute systemic toxicity and eye irritation, both resulting from a single or very short-term exposure, are described in this chapter. In recent years, economics and concerns over animal welfare have raised many issues in animal testing. Alternate methods for acute toxicity and eye irritation are being developed and, in some cases, accepted by regulatory agencies for hazard assessment purposes. This chapter describes the classical and/or currently accepted methods for evaluating a test article's potential for acute systemic toxicity and eye irritation, and gives an overview of the current regulatory testing requirements in the United States, European Union, and Japan. Chapter 20 reviews the different nonanimal alternative models for assessing toxicity and irritation in greater detail.

PRINCIPLES OF ACUTE TOXICOLOGY

Acute toxicity testing began nearly a century ago when physicians and pharmacologists were concerned with potent poisons and drugs. In 1927, Trevan [196] introduced the concept of a median lethal dose (LD_{50}) for the standardization of digitalis extracts, insulin, and diphtheria toxin. He recognized that the precision of the LD_{50} value was dependent on many factors such as seasonal variation and the number of animals used in a test. High precision LD_{50} values can only be established with a large number of animals.

The list of extraneous factors that affect the precision of the LD_{50} includes, among other factors, sex, species, strain, age, diet, nutritional status, general health conditions, animal husbandry, experimental procedures, route of administration, stress, dosage formulation (vehicle), and intra- and interlaboratory variations. In spite of the many variables affecting the LD_{50} determination, many governmental agencies still regard the LD_{50} as the sole measurement of the acute toxicity of all materials; however, recent research has resulted in the development and gradual acceptance of alternative methods to assess the acute oral toxicity potential of test materials.

It is important to determine accurately the killing power of highly toxic substances, since a small difference in exposure can distinguish a safe from a lethal exposure. However,

a precise LD_{50} is not necessary when evaluating less toxic materials such as pesticides and consumer products. While the need to evaluate the safety of products with a low-to-moderate toxic potential is still desirable, an approximate measurement of their toxic potential is sufficient for the purposes of hazard and risk and exposure assessment. Furthermore, it may not be scientifically sound to determine a precise LD_{50} value for substances with a low-to-moderate potential for toxicity. This is because extraneous variables, some of which cannot be controlled by the experimenter and errors inherent in the determination of LD_{50} values, can have a significant impact on the study results. Many methods have been developed over the years to calculate the LD_{50} and evaluate the acute toxicity potential of chemicals with a small number of animals. Some of these methods are discussed later in this chapter.

Many scientists have advocated changes in the emphasis of acute toxicity testing. To date, there is a general consensus among toxicologists in academia, industry, and government that the emphasis of acute toxicity testing needs to change [6,16,58,71,110,117,140,184,188]. The value of a precise LD_{50}, except for highly toxic substances, should be de-emphasized and that the focus should be on obtaining as much information as possible on the toxic manifestation and mechanism using the fewest number of animals. Alternative methods for assessing the oral [159–161], dermal [148], and inhalation [149,152] toxic potential of test material have been proposed or adopted by various regulatory agencies. Undoubtedly, such information will be more useful than the LD_{50} to physicians in treating overexposure.

Even though the emphasis of acute toxicity testing is changing, the principles of dose–response and development of signs of toxicity remain the basis of the science of toxicology. It is the objective of this section to refresh the experienced and introduce the novice to these general concepts.

Definition of Acute Toxicity

Toxicity is defined as the harmful effect of a chemical or a drug on a living organism. Various expert groups have defined acute and subchronic toxicities. The Organization for Economic Cooperation and Development (OECD) [157] defines acute toxicity as "the adverse effects occurring within a short time of (oral) administration of a single dose of a substance or multiple doses given within 24 hours." In terms of human exposure, this definition of acute toxicity refers to life-threatening crises such as accidental catastrophes, overdoses, and suicide attempts.

Dose–Response Relationship

Toxicologists often obtain two types of data, quantal and graded. The quantal response is called the *all-or-none* response; it either happens or it does not happen. On the other hand, the graded response can be determined quantitatively and it is continuous. Mortality and incidences of pharmacotoxic signs are examples of quantal data, whereas enzyme activity, protein concentration, body weight, feed consumption, and electrolyte concentration are quantitative parameters. However, many apparently quantal responses are quantitative. If technical measurements permit, they may be graded. For example, the severity of a pharmacotoxic sign can be graded if detection methods are available.

At the molecular level, the graded dose–response relationship often can be explained by the receptor, a relatively old concept but still a valid one. Let "S" be a particular substance that produces a specific response by interacting with a target protein molecule, the receptor (R), in the body to form a substance–receptor (SR) complex. Assuming the reaction is reversible and there is only one binding site on every target receptor molecule, this process can be described by the following expression:

$$S + R \underset{K_2}{\overset{K_1}{\rightleftharpoons}} SR$$

and the mass equation for this reversible process is

$$\frac{k_2}{k_1} = K_d = \frac{[S][R]}{[SR]} \tag{22.1}$$

where

[S], [R], and [SR] are the concentrations of the substance, the receptor, and the SR complex at any particular time, respectively

K_d is the dissociation constant of the process

Let $[R]_0$ be the initial concentration of the receptor, which is usually very small and constant in number when compared with the concentration of the substance. Then

$$[R]_0 = [R] = [SR]$$

thus

$$[R] = [R]_0 - [SR]$$

Substituting the given equation into the mass equation (Equation 22.1) and rearranging

$$[SR]K_d = [S]\left([R]_0 - [SR]\right)$$

or

$$[SR]\left(K_d + [S]\right) = [R]_0[S]$$

which can be rearranged to

$$\frac{[SR]}{[R]_0} = \frac{[S]}{K_d + [S]} \tag{22.2}$$

SR/[R]0 is the fraction of receptor that has reacted with the substance to form the SR complex. If we assume that the response (E) resulting from the interaction of the substance with the receptor is dependent on the fraction of total receptor concentration that has reacted with the substance, then

$$E = \frac{[S]}{K_d + [S]} \tag{22.3}$$

Equation 22.3 is a hyperbolic function; therefore, the response (E) is related to the concentration of the substance in a hyperbolic function relationship. If the concentration of the substance at the receptor site is dependent on the dose, then the response is dependent on the dose administered. This phenomenon is perhaps the simplest version of the receptor kinetic concept relating the dose of the chemical to a biological response. The kinetics of the receptor–substrate interaction may be more complicated, and different dose–response relationships could be drawn based on these complicated kinetics. Readers who are interested in different receptor–substance kinetics are referred to Ferdinard [72].

The quantal dose–response relationship often is difficult to conceptualize based on the receptor theory. However, quantal response also can be viewed as a graded response if the whole population is considered as an individual. This relationship can best be explained in terms of a probability distribution. For a particular response, members of a population, for example, all the rats in the world, respond differently to a particular stimulus such as exposure to a chemical. Some rats will be highly sensitive whereas others will be very resistant. If these different responses are distributed normally within the population (i.e., with most members of the population being neither extremely sensitive nor resistant), the well-known bell-shaped population distribution curve results. If the probability of dose–response is expressed in terms of cumulative response, a sigmoidal curve can be obtained as shown in Figure 22.1. However, most biological response distributions are not exactly normal and tend to be skewed to the higher dose, that is, extreme resistors have a larger *range of dose* to response than the extremely sensitive portion of the population. In general, a logarithmic dose transformation can normalize the distribution (i.e., convert the skewed distribution to a normal distribution; Figure 22.2). After this logarithmic dose transformation, if the probability of the log dose–response is expressed cumulatively, the sigmoidal response curve is obtained (Figure 22.2). How is this lognormal transformation related to a regular dose–response curve? Is there justification or basis for a log dose transformation? To answer these questions, let us again look at Equation 22.3. This equation can be arranged to

$$E = \frac{[S]}{k_2/k_1 + [S]}$$

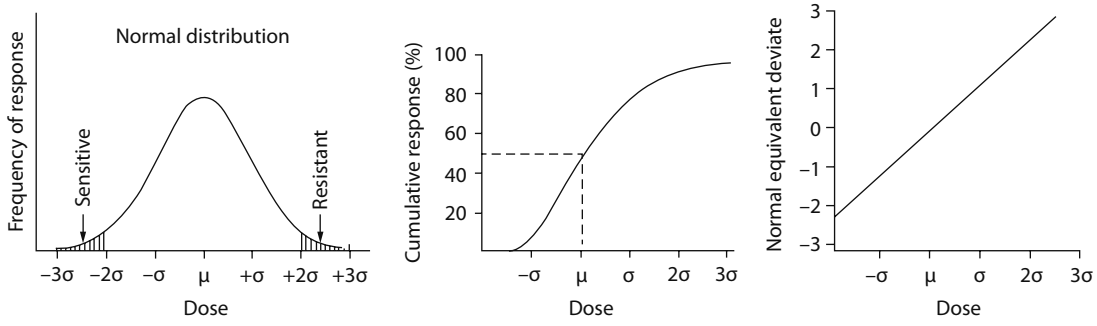

FIGURE 22.1 Normal distribution of dose–response relationships: frequency of response, cumulative response, and cumulative response in terms of NED.

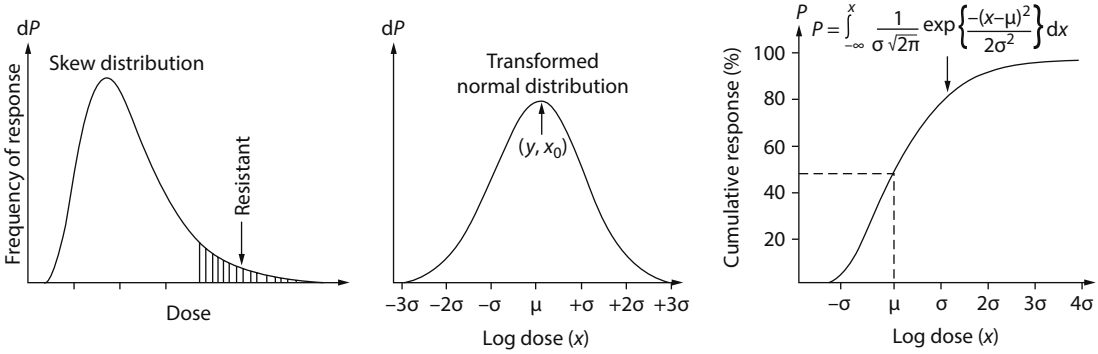

FIGURE 22.2 Skew of dose–response can be normalized by log dose transformation.

which can be rearranged to

$$E = \frac{k_1[S]}{k_2 + k_1[S]} \quad (22.4)$$

Over a certain concentration range, Equation 22.4 will produce a curve very similar to the logarithmic function $E = K_1 \log (k_2 [S] + 1)$ [34]. Therefore, there may be justification for the log transformation besides simply a mathematical convenience.

Since a sigmoidal curve is more difficult to analyze than a straight line, many experts feel that further transformation of the log dose–response hyperbolic function is necessary to obtain a *straight-line* function curve. Perhaps, the most widely used transformation is the normal equivalent deviate (NED) or the similar probit transformation [12,35,38,44,50,75,130]. This technique involves the log dose transformation and the transformation of the cumulative response probability to the NED or probit. After both the probability and the dose are transformed, their transformed values are directly related to each other. A brief derivation of the straight-line direct function relationship between the log dose and NED or probit will be presented later in this chapter.

LD_{50} and Its Determination

Definition

The LD_{50} in its simplest form is the dose of a compound that causes 50% mortality in a population. A more precise definition has been provided by the OECD panel of experts as the "statistically derived single dose of a substance that can be expected to cause death in 50% of the animals" [157].

In other words, an LD_{50} of a compound is not a constant, as it has been treated by many; rather, it is a statistical term designed to describe the lethal response of a compound in a particular population under a discrete set of experimental conditions.

The significance of the LD_{50} has been examined by many scientists [6,58,71,110,117,140,184,188] who have concluded that it is an imprecise value and not a biological constant; furthermore, its importance should be de-emphasized for most types of materials. For most materials, an approximate LD_{50} value is sufficient and that more emphasis should be placed on characterizing the signs of toxicity, identifying the target organs and elucidating the material's mechanism of action.

The numeric value of the LD_{50} has been widely used to classify and compare the toxic potential of chemicals; the importance placed on the LD_{50} and how it is used in a safety evaluation has almost reached a level of abuse. Although determining the LD_{50} under a set of experimental conditions can provide valuable information about the toxicity of a compound, the numeric LD_{50} per se is not equivalent to acute toxicity. It should always be remembered that lethality is only one of many reference points used to characterize acute toxicity. When evaluating the acute toxic potential of a material, the slope (response/dose) of the dose–response curve, time to death, pharmacotoxic signs, and pathological findings all need to be considered, as they are critical endpoints. Therefore, defining acute toxicity based only on the numeric value of an LD_{50} is inappropriate.

As pointed out in a previous paragraph, lethality is a quantal response and the probability of a cumulative response is related to dose in a hyperbolic (sigmoidal) function. The cumulative probability of response is directly related to the standard deviates of a log dose population (Figure 22.1). Therefore, the slope of the log dose–response curve will indicate the relationship between the range of dose and the lethal response. This relationship is more important in risk assessment than the numeric value of the LD_{50} because more insight is available about the intrinsic toxic characteristics of a compound. Sometimes, the slope can give a clue to the mechanism of toxicity. For example, a steep slope may indicate rapid onset of action or faster absorption. A large margin of safety is predicted when a compound has a flat slope, that is, only a small increase in response with a large increase in dose. With the slope, it often is possible to extrapolate the response to a low dose (e.g., LD_0, LD_1) or even to a no-observed-effect level (NOEL). It is especially important to know the slope when comparing a set of compounds. Two compounds may have identical LD_{50} values but different slopes and thus have quite different toxicological characteristics depending on the range of doses. Parallel dose–response curves may indicate a similar mechanism of toxicity, kinetic pattern, and probably similar prognosis. Neither the LD_{50} nor the slope can reveal absolutely a specific mechanism, but with pharmacokinetic and other biochemical studies elucidation of the mechanism of toxicity may begin to be possible.

Determination of LD_{50}

Many methods are available for the determination of the LD_{50}. They can be grouped into two categories, the *normal population assumption* and the *normal population assumption-free* methods. The former usually can be analyzed by graphic procedures.

The *normal population assumption-free* methods are represented by Thompson's moving average interpolation [194] and the *up-and-down* method [19–22,34,46]. The former method is widely accepted, and convenient tables [50,203] are available for estimation of the value of the LD_{50} with confidence limits when either 0% or 100% mortality incidences are observed. However, there are some restrictions on the use of the Thompson method, that is, four doses must be at equal log dose intervals and the number of animals per dose level must be equal. The up-and-down or *pyramid* method is designed to estimate the LD_{50} with a small number of samples. It has an economical advantage because fewer animals are needed, but the test can be time consuming and requires a larger amount of test article. Because it has the advantage of using only a few animals, the up-and-down method is popular when a study has to be conducted in large animals such as cows or sheep or expensive animals such as monkeys. A study comparing LD_{50}'s obtained using the up-and-down method and other methods revealed an excellent

agreement [20]. Two apparent shortcomings of the up-and-down method is that it is not adequate for estimating the incidence of delayed deaths and a dose–response of mortality or signs of toxicity cannot easily be obtained. However, Weil [204] has adapted the up-and-down method to calculate the slope of acute toxicity response.

The *normal population assumption* method is represented by the probit analysis approach, which can be performed either by graphic means [75] or by mathematical calculation [76]. Since the probit analysis is used widely in evaluating acute toxicity data, the principles in performing this analysis will be discussed briefly. This method involves the transformation of both the cumulative response probability and dosage data.

When the dose is transformed into a log dose (x), the frequency of response versus log doses follows a normal distribution (Figure 22.2), which can be expressed mathematically as

$$dP = \frac{1}{\sigma\sqrt{2\pi}} \exp\left(\frac{-(x-u)^2}{2\sigma^2}\right) \tag{22.5}$$

where

- σ^2 and u are the variance and the mean of the population, respectively
- P is the probability corresponding to each value of x (Figure 22.2)

The LD_{50} is defined as the log dose that can produce 50% mortality in a population (i.e., $P = 0.5$ or 50% cumulative response). Let x be the log LD_{50}; then $P = 0.5$ will correspond to the area under the lognormal distribution curve from $-\infty$ to x_o; or $P = 0.5$ will correspond to the integration of Equation 22.5 from $-\infty$ to x_o. That is,

$$P = 0.5 = \int_{-\infty}^{x_o} \frac{1}{\sigma\sqrt{2\pi}} \exp\left(\frac{-(x-u)^2}{2\sigma^2}\right) dx \tag{22.6}$$

The solution of Equation 22.6 is $x = u$, the true mean or the median of the lognormal distribution. One way to solve this equation is by a graphic method. The integration of Equation 22.5 from $x = -\infty$ to $+\infty$ can be represented graphically by a sigmoidal curve as illustrated in Figure 22.2. Analysis of the sigmoidal curve is more difficult than a straight line. One way to transform the sigmoidal curve to a straight line is by NED analysis or similarly by probit analysis. For a detailed description of this analysis, the reader should consult Finney's text [75]. A brief derivation of the straight-line function between log dose and the transformed probability of response is described in the following.

Probability (P) is normally expressed in terms of percentage or with values between 0 and 1; but Gaddum [81] has proposed to measure the probability of response on a transformed scale called the NED, or the standard deviation of a normal distribution, which can be described mathematically by Equation 22.5. In a particular case, the normal distribution of response with mean equal to 0 and the standard deviation equal to 1, Equation 22.5 can be written as

$$dP = \frac{1}{\sqrt{2\pi}} \exp\left(\frac{-x^2}{2}\right) dx$$

Similarly, if this distribution of response is plotted on the y axis (Figure 22.3), then

$$dP = \frac{1}{\sqrt{2\pi}} \exp\left(\frac{-y^2}{2}\right) dx \tag{22.7}$$

The probability in such a case is defined by a value on the y axis of Figure 22.3, that is, the integration of Equation 22.7 from $-\infty$ to y:

$$P = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{y} \exp\frac{-y}{2}\, dy \tag{22.8}$$

In other words, for each value of y (from $-\infty$ to $+\infty$) expressed in terms of the standard deviation of a normal distribution with the mean equal to 0 and the standard deviation equal to 1, there is a corresponding value of probability (P) expressed in terms of percentage or having a value ranging from 0 to 1. Thus, equivalent values on the y axis can be used to define the value of P or vice versa; y and P define each other. This relationship is illustrated in Figure 22.3.

The particular probability of response to a particular log dose value x, as described in Equation 22.6, will be

$$P = \int_{-y}^{-x} \frac{1}{\sigma\sqrt{2\pi}} \exp\left(\frac{-(x-u)^2}{2\sigma^2}\right) dx \tag{22.9}$$

where u and σ are the mean and standard deviation of the log dose, respectively.

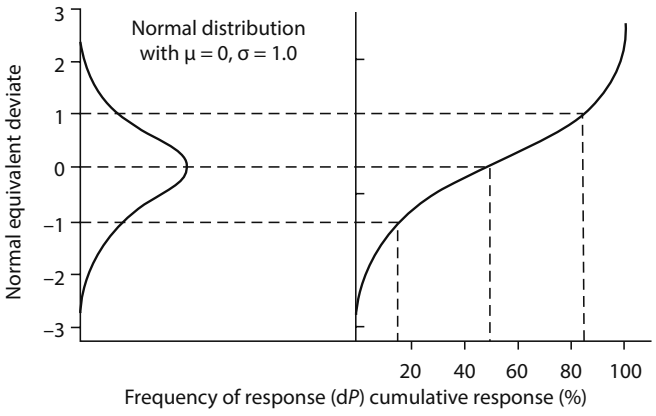

FIGURE 22.3 Probability of response can be expressed in terms of the percentage of population or the NED of a normal distribution with mean = 0 and standard deviation = 1.

If P is expressed by a value of y on the y axis (standard deviations), then

$$P = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{y} \exp\left(\frac{-y^2}{2}\right) dy$$

$$= \int_{-\infty}^{x} \frac{1}{\sigma\sqrt{2\pi}} \exp\left(\frac{-(x-u)^2}{2\sigma^2}\right) dx$$

The solution of this equation is $x = u + \sigma y$ or

$$y = \frac{(x-u)}{\sigma} = \frac{1}{\sigma}x - \frac{u}{\sigma} \qquad (22.10)$$

Therefore, the probability when expressed in terms of y (the NED scale) is related linearly to x, the log dose. If x is plotted against the corresponding y, a straight line with slope = $1/\sigma$ will be obtained. To further facilitate calculation, Bliss [11] suggested a slightly different NED unit called the probit, such that the new y value is equal to $[(x - u)/\sigma] + 5$. This procedure eliminates the negative values of NED when P has a value of less than 50%. Therefore, the probit is equal to the NED plus 5. The linear relationship between probits and log dose is similar to the relationship between NED and log dose. Thus, when $y = 5$, from Equation 22.10

$$5 = \frac{(x-u)}{\sigma} + 5$$

and $x = \mu$ (i.e., the median log dose that has a probability of response of 50%).

Estimation of LD_{50} by Probit Analysis

The basic linear equation for the probit analysis as described in the previous section is

$$y = 5 + \frac{1}{\sigma}(x-u)$$

where y is the probit (σ is the standard deviation of a log-normal distribution with mean u, and x is the log dose). This equation is linear with respect to y and x often can be expressed as a linear equation, for example, $y = \alpha + \beta x$, where $\beta = 1/\sigma$ = slope, and $\alpha = 5 - (u/\sigma)$. When $y = 5$, $(x - u)/\sigma = 0$; thus $x = \mu$ (the median log dose). Furthermore, y is related to P (the probability of response that has a value of 0–1) by the following equation:

$$P = \frac{1}{\sqrt{2\pi}} \int_{-x}^{y} \exp\left(\frac{-y}{2}\right) dy$$

The reader should bear in mind that both the u and x are in log dose scale.

The following steps should be taken for graphic estimation of LD_{50} by probit analysis.

1. Convert response probabilities to probit units by a probit transformation table (see Ref. [45, pp. 54–55]).
2. Convert all doses into log dose units (e.g., $\log_{10}$ dose = x). (Steps 1 and 2 may be eliminated if probit-log graphic paper is available.)
3. Using the probit as the abscissa and $\log_{10}$ dose as the ordinate, plot the response probit units against the $\log_{10}$ dose.
4. Draw a straight line such that the vertical deviations of points (the probits) at each x value are as small as possible. Extreme probits, for example, those outside the range of probits 7 and 1, carry little weight in the fitting of the probit–log dose–response line and thus should be excluded.
5. From the regression of the probit–log dose line, extrapolate the log dose corresponding to probit units of 5, which also correspond to the $P = 0.5$. Thus, this extrapolated dose should be the median lethal *log dose*, and the LD_{50} value would be the *antilog* of this log dose value.
6. Calculate the slope of the probit–log dose line. This slope, $\beta = 1/\sigma$, is defined as the number of increases in probit units for a unit increase in log dose. The slope defined by Litchfield and Wilcoxon [116] is equal to

$$\frac{1}{2}\left(\frac{LD_{84}}{LD_{50}} + \frac{LD_{50}}{LD_{16}}\right) = \sigma$$

   This slope is different but related to the slope described here, thus the larger the slope value, the steeper the probit–log dose response. The opposite is true in the Litchfield and Wilcoxon definition.
7. An χ^2 test should be conducted to determine if the fitted line is adequate. A small value of χ^2 statistic (within the limits of random variation) may indicate satisfactory agreement between the theoretically expected line and the fitted line. A significantly large χ^2 statistic may indicate either that the animals do not respond independently or that the fitted line (probit–log dose) does not adequately describe the dose–response relationship of the test substance. If the latter is true, forms of the dose–response curve other than the probit–log dose linearity may exist, and further transformation may be needed [75]. If the former is the case, then precision of the line is reduced.
8. Determination of precision is by weighting the coefficient. The standard deviation of a binomial distribution is $\sqrt{PQ/n}$, where P and Q are the mean probabilities, P equals $(1 - Q)$, and n is the number of test subjects. Thus the variance is PQ/n, the square of the standard deviation. It is obvious that the variance (i.e., the spread of a distribution) is

inversely related to n. This relationship means that the larger the number of test subjects, the smaller the variance and the better the precision. The reciprocal of the variance is invariance, which measures the weight, nW. Here W (weighting coefficient) = Z^2/PQ, where $Z = (1/\sqrt{2\pi})\exp(-y^2/2)$ and is related to the normal frequency function corresponding to the NED. A table of weighting coefficients (see Ref. [45, p. 55]) corresponding to probits (y) is available [76]. The standard error for the log LD_{50} is given by

$$\frac{\sigma}{\sqrt{\Sigma nW}}$$

if the estimated log LD_{50} does not greatly differ from the true mean log LD_{50}, because this estimation does not take into consideration the error in the estimation of α for the probit–log dose–response line. A better equation for the estimation of the variance of the estimated log LD_{50} is given by

$$V(m) = \sigma^2\left(\frac{1}{\Sigma nW} + \frac{(m-\bar{x})^2}{\Sigma nW(x-\bar{x})^2}\right)$$

where

$V(m)$ is the variance of LD_{50}
$\bar{x}$ is the weighted mean log dose
m is the median log dose
x is the log dose
$1/\sigma = 1/\Sigma nW(x-\bar{x})$

If the χ^2 is large, indicating that the test subjects do not respond independently to the dose, the estimation of variance of log LD_{50} may not apply, and adjustment due to the sampling variation of the slope ($1/\sigma$) of the probit–log dose line may have to be made [75]. For a quick estimation of the LD_{50}, this adjustment may be dropped, and the SE would be the square root of the variance, that is, $\sqrt{V(m)}$.

One must remember that the dose is expressed in log dose; therefore, the estimation of the SE for the LD_{50} in the original dose unit (e.g., mg/kg) is impossible. However, an approximation is given:

$$\text{SE}(LD_{50}) = (10^m)\cdot([\log_e(10)\cdot(S_m)]$$

where S_m (which equals $\sigma/\sqrt{\Sigma nW}$ or $\sqrt{V(m)}$) is the estimated SE for the median log dose m (i.e., m = log LD_{50} or $10^m = LD_{50}$).

A more rapid approximation of the SE of log LD_{50} was given by Litchfield and Wilcoxon [116] as

$$S_m = \frac{S}{N'/2}$$

where

S is the difference between two log doses of expected effects (as indicated by the probiting dose line) that differ by one unit of probit
N' is the total number of animals between the log dose limits, corresponding to the expected probit 4.0–6.0 (i.e., the 16% and 80% responses)

9. *Fiducial limits.* The concept of fiducial limit is similar to the confidence limit. The value of the two may be the same, but they are not always identical. The fiducial probability F (e.g., 95%) can be defined as the situation when the true value of a parameter lies between the calculated upper and lower limits, which would not be contradicted by a significance test at the 1/2 $(1 - F)$ probability level. These higher and lower limits are called the fiducial limits. For rapid analysis, the fiducial limits at the F = 95% level can be estimated by log $LD_{50} \pm 1.96$ (S_m). A more detailed estimation can be obtained by the maximum likelihood estimation [75].

   Another simple approximation of the fiducial limits is given by Litchfield and Wilcoxon [116] as LD_{50}/fLD_{50} and $LD_{50} \times fLD_{50}$ for the lower and upper limits, respectively, where LD_{50} is defined as the LD_{50} factor equal to $(s)\left(2.77/\sqrt{N'}\right)$.

   Here s is the slope, which is defined as

$$\frac{1}{2}\left(\frac{LD_{54}}{LD_{50}} + \frac{LD_{50}}{LD_{16}}\right) = \frac{1}{2}(3.55 + 3.55) = 3.55$$

   In this example, and N' is the total number of animals used between response probabilities 16% and 84% (i.e., probit 4 and 6, equal to 30 in this example). Then fLD_{50} equals 1.896. Therefore, the lower fiducial limit is equal to 8.91/1.896 = 4.70, and the upper fiducial limit is equal to 8.96 × 1.896 = 16.90.

Logistic Transformation

Waud [202] suggested a logistic approach to calculate the LD_{50}. Thus

$$P = \frac{D^E}{(D^E + K^E)}$$

where

P is the probability of response
D is the dose
E and K are scale and location parameters, respectively
K corresponds to the LD_{50}

With the procedure of iteration, K and E can be estimated with a range of confidence. The derivation of this equation is beyond the scope of this chapter, and interested readers are referred to the original article by Waud [202].

Nonlethal Parameters

Although the LD_{50} and the slope of the dose–response curve can provide valuable information on the toxicity of a compound, the LD_{50} *is not equivalent to toxicity.* Chemicals can induce damage to the physiological, biochemical, immunological, neurological, or anatomical systems not characterized by the LD_{50}. Depending on the severity and the extent of the disturbance of the normal biological functions, the animal may survive the toxic response even though some irreversible tissue damage may have occurred. Nonlethal, adverse effects are as undesirable as lethality and certainly should be taken into consideration during the risk assessment of a chemical.

A major problem in analyzing nonlethal responses is that in many cases the data are not quantal. For example, dermal toxicity ranges from slight to severe. These polychotomous data may be handled by RIDIT *analysis*, which was designed to analyze quantal responses with more than two outcomes [1,18,92].

While toxic effects may contribute to lethality, any attempt to correlate a particular nonlethal response to mortality may be irrational [189] unless that response is the only one responsible for the eventual death of the animal. Identification of the response or responses related to mortality is not often a straightforward matter. Nonlethal responses that affect the general well-being of an animal should be considered in the risk assessment of a compound.

If nonlethal responses can be viewed as true quantal data, the *median effective dose* (ED_{50}) and the corresponding dose–response curve may apply. The ED_{50}, which often is used in the standardization of biologically active compounds such as a drug, has a meaning similar to the LD_{50} except that it is designated to examine nonlethal parameters such as pharmacological responses and other nonlethal adverse effects. The ED_{50} is defined as a statistically derived single dose of a substance that can be expected to cause a particular effect to occur in 50% of the animal population. The therapeutic index (TI), defined by the ratio of LD_{50}/ED_{50} or LD_1/ED_{99}, has been applied to establish the safety margin of some biologically active drugs. The higher the index, the greater the margin of safety with the drug, that is, a large difference exists between the amount of compound predicted to kill 50% of the animals and the amount of compound predicted to elicit a particular response in 50% of the animals. The TI gives an even greater estimate of safety when the LD_1 is compared to the ED_{99}.

Reversibility of Nonlethal Parameters

In general, reversible responses are those that diminish with elimination of the chemical from the body. A true reversible response will cause no residual effects when the chemical is completely eliminated from the body. Such responses are commonly seen in drugs used at therapeutic dose levels. As the amount of drug in the body increases, the magnitude of the effect also increases. If it is truly reversible, the effect will wear off when the drug is completely eliminated.

The reversibility of a particular response is dependent on the organ or system involved, intrinsic toxicity of the chemical, length of exposure, total amount of the chemical in the body at a specific time, and the age and general health of the animal. If the amount of chemical in the body is high enough, the intensity of the response may overwhelm a particular organ. Effects indicated through hormonal imbalance such as thyroid effects generally are reversible unless the threshold is surpassed. Damage in rapidly regenerating organs, such as liver, is usually more likely to be reversible than damage in nonregenerating tissues, such as nerves. A good example is the delayed onset of neuropathy caused by many organophosphate insecticides. The chemical may be completely eliminated from the body before the effect manifests itself. Animals with renal or liver diseases are often more susceptible to damage (reversible or irreversible) by a chemical insult because of decreased ability to eliminate the chemical. Exposure to a chemical at an early age may induce irreversible damage more easily than at an older age because of the limited development of the kidneys and/or functional capacity of other organs, such as the liver.

In risk assessment, it is important to know whether a toxic effect is reversible. Irreversible effects seen in animals obviously are weighted more heavily in reaching a conclusion on the toxicity and hazard a chemical may pose for humans.

ACUTE TOXICITY TESTING

The objectives of acute toxicity testing are to define the intrinsic toxicity of the chemical, predict hazard to nontarget species or toxicity to target species, determine the most susceptible species, identify target organs, provide information for risk assessment of acute exposure to the chemical, provide information for the design and selection of dose levels for prolonged studies, and, the most important and practical of all, provide valuable information for clinicians in the prediction, diagnosis, and treatment for acute overexposure (poisoning) to chemicals. Acute studies often are called the "first line of defense" in the absence of data from long-term studies. These data help industrial, governmental, and academic institutions formulate safety measures for their researchers and for limited segments of their worker population during the early stage of the development of a chemical. From a regulatory standpoint, acute toxicity data are essential in the classification, labeling, and transportation of a chemical. From an academic standpoint, a carefully designed acute toxicity study can often provide important clues on the mechanism of toxicity and the structure–activity relationship for a particular class of chemicals.

Many acute toxicity studies have been conducted solely for the purpose of determining the LD_{50} of a chemical. However, the reader is reminded that acute toxicity is not equivalent to the LD_{50}, and that the LD_{50} is not an absolute biological constant to be equated, as some investigators have, with such chemical constants as pH, pK_a, melting point, and solubility. The LD_{50} is only one of many indices used in defining acute toxicity. A well-designed acute toxicity study should include

consideration of the dose–response relationship of both lethal and nonlethal parameters, as discussed earlier. Sometimes, biochemical measurements in an acute test can aid in elucidating the mechanism of toxic actions. Histopathology of organs may be helpful in determining the cause of death and identifying the target organs.

The use of animals in acute toxicity studies has been widely debated for many years. Aside from valid scientific concerns on the usefulness of classic LD_{50} values (e.g., uncertainty in species extrapolation, seldom needed for potent drug standardization), there are broader issues on animal testing, some are political in nature and others economically based. The cost of animal testing has been increasing at a skyrocketing rate over the last decades, and even without animal rights activism, the scientific community will need less costly alternatives to cope with the increasing demand for safety evaluation of a vast number of existing and new chemicals. While reduction in the number of animals used and refinement of existing testing methods to minimize pain and suffering of animals represent the short-term objective, replacement of animal testing with nonanimal-based methods is the ultimate goal. Currently, genuine, validated, and regulatory-accepted nonanimal alternative methods to replace whole animal acute toxicity testing are still more of a goal than a reality, even though the concept has been widely accepted by scientists from industry, professional societies, and certain regulatory bodies [4,128,187,188].

Types of Acute Testing

Since acute toxicity data may provide the first line of defense, a battery of tests under different conditions and exposure routes should be considered. In general, these tests should include oral, dermal, and inhalation toxicities, and skin and eye irritation studies. Other tests such as acute preneonatal and neonatal exposure, dermal contact sensitization, and phototoxicity should also be considered. Depending on sound scientific factors, which may vary from one chemical to another, the number and kind of acute tests needed to establish the initial toxicity database may not be the same. For example, inhalation testing may not be conducted when inhalation exposure is not expected to occur because of the physical properties of the chemical (e.g., respirable particles cannot be generated). Generally, oral, dermal, and inhalation toxicities along with eye irritation tests should be considered as part of an initial acute investigation. These tests are often used for the regulatory purposes of labeling and classifying the hazard potential of a chemical or formulation, although increasing concerns also are placed on skin sensitization studies. This chapter is concerned only with acute oral, dermal, and inhalation toxicities and eye irritation testing.

Acute Oral Toxicity

Classical oral toxicity tests, which use a large number of animals and precisely determine the LD_{50}, continue to be required by some regulatory bodies for the purpose of classification and labeling of chemicals. However, many studies have shown that adequate acute toxicity and lethality information can be obtained by using fewer animals than classical LD_{50} studies require. DePass [42], Lipnick et al. [115], and Gribaldo et al. [87] have reviewed several modified LD_{50} tests. Although the main endpoint remains lethality, these tests generally fulfill the goal of reducing the number and suffering of animals and, in some cases, provide adequate information for hazard classification and labeling.

One example of a modified LD_{50} test is the *approximate lethal dose method*; this method involves sequential dosing until the lowest lethal dose is obtained. Initially an arbitrary dose is given to an animal. If the animal survives, a second animal is given 1.5 times the initial dose; and sequentially several animals are given increasing doses in the same manner until a lethal dose is achieved. The lethal dose is the approximate lethal dose (ALD). In general, only 6–10 animals are required to achieve the ALD. Comparison of classical LD_{50} values and the ALD indicates that the ALD can be used to closely predict the LD_{50} (Table 22.1).

Recently developed alternative methods, such as the limit test [80], the British Society of Toxicology (BST) method [17], the up-and-down method [20–22], fixed-dose procedure [198], and acute toxic class method [177], do reduce the number of animals needed to assess the oral toxic potential of chemicals. A number of these alternative methods have been endorsed by scientific researchers, regulatory authorities, and animal advocates [4,128,187,188], with some having been adopted as regulatory guidelines [159–161]. As use and general acceptance of these alternative methods increases, regulatory bodies will likely need to redefine their approach to how chemicals are classified and labeled.

Classical Method

With regulatory acceptance of the *fixed-dose*, *acute toxic class*, and *up-and-down procedure* methods [153], use of the classical method to assess the toxic potential of chemicals is no longer accepted in the European Union [147].

Principle

The test material, undiluted or diluted with the appropriate solvent or suspending vehicle, is given to several groups of animals by gavage with a feeding needle or by gastric intubation. A vehicle control group is included if needed, but generally this group is not necessary if the toxicity of the vehicle is known. Clinical signs, morbidity, and mortality are observed at specific intervals. Animals that die or become extremely moribund during the study are subjected to necropsies. At the conclusion of the study's observation period, surviving animals are killed and necropsied. Tissues may be saved for histopathological examination to facilitate the understanding of the acute toxicity of the compound. In order to increase the reproducibility of the study, all experimental conditions and procedures should be standardized, and the study should be conducted according to generally recognized good laboratory practices (GLP) outlined by the Environmental Protection Agency (EPA) and the OECD [56,57,162].

TABLE 22.1
Comparison of Acute Oral Toxicity in the Rat Using ALD versus Classical (Conventional) Method

Chemical	Classical Method		Approximate Method	
	n	LD_{50} (mg/kg)	*n*	ALD (mg/kg)[a]
Tetraethyl lead	36	20	5	26
Methomyl	53	40	5	26
Hexachlorophene	46	165	11	90
Adiponitrile	65	301	7	300
Caffeine	40	483	8	450
N-butylhexamethylene diamine	35	536	7	1000
Hexamethylene diamine	92	1127	5	1500
Bromobenzene	35	3591	8	3400
Carbon tetrachloride	105	10,054	5	7500

Source: Kennedy, G.L. Jr. et al., *J. Appl. Toxicol*., 6, 145, 1986.
[a] The lowest dose that caused death.

Animals

Responses elicited by a compound often vary greatly among species. Ideally, toxicity tests should be conducted with an animal that will elicit compound-related toxic responses similar to those that occur in man, that is, an animal that metabolizes the compound identically to man and that has the same susceptible organ system(s). Under such conditions, the animal data may be extrapolated to man. Unfortunately, finding such an ideal animal is a difficult if not impossible task.

A less ideal but more manageable approach is to conduct acute toxicity studies in a variety of animal species under the assumption that if the toxicity of a compound is consistent in all the species tested, and then a greater chance exists that such a response may also occur in man. Even though the response in different species is not consistent, it generally is considered better to err on the safe side with the risk assessment being based on the most sensitive species, unless there is justification that such responses are less likely to occur in humans. An example of when a sensitive species would not be considered would be when it is known there is a dissimilarity in test article metabolism between a more sensitive animal species and man. While these are logical assumptions and generally quite reliable, there is the danger that the results in the animals may underestimate or overestimate the response in humans. Therefore, there is no absolute criterion for selecting a particular animal species. However, priority should be given to species with metabolism or other physiological and biochemical parameters similar to man. Animal species also should be selected on the basis of convenience, economical factors, and the existing database for the animal. Rats, mice, rabbits, and guinea pigs are most commonly chosen for acute toxicity studies.

Acute toxicity, even within a particular species, can vary with health conditions; age; sex; genetic makeup; body weight; differences in absorption, distribution, metabolism, and excretion of the compound; and the influence of hormones [48]. For example, immature animals may lack an effective drug-metabolizing enzyme system; this may contribute to higher toxicity of the compound in an immature animal if the enzyme is responsible for the detoxification of the compound. On the other hand, if the enzyme responsible for generating a toxic metabolite is inactive then it is possible that the toxic potential of the material will be underestimated. Obesity may affect the distribution and storage of a compound, especially if it is highly lipophilic. Sex hormones may be the target, or sex hormones may modify a particular toxic response, which then may account for different toxic responses between sexes. Liver and renal diseases associated with old age may contribute to higher toxicity. Variations in genetic makeup among different strains may alter metabolism or other parameters, which may affect the toxicity of a particular compound. It is therefore important to document all data on animals: age, sex, body weight, strain, general health condition, and source. In general, healthy, young adults should be used.

Number and Sex

The precision of the acute test is dependent to a large extent on the number of animals employed per dose level. Historically, 10 rats (5/sex) have been recommended in most regulatory guidelines [51,61,66,157,186], although more recently modified protocols are acceptable using as few as three animals per dose level [160,161]. The degree of precision needed and in turn the number of animals per dose group needed depend on the purpose of the study. In screening tests or tests designed to define the range of toxicity, fewer animals per dose level or fewer numbers of dose levels may be considered. In rare situations where a fairly precise LD_{50} is needed, the number of dose levels (at least three dose levels) and animals per dose group may need to be increased. Literature surveys have shown that when there are sensitivity differences between the sexes, females, in general, are more sensitive [115].

Grouping, Preparation of Animals, and Randomization

Animals not previously treated with test substances in other studies should be identified individually by coded marks, metal ear tags, or tattoos. The animals then should be quarantined for at least a week prior to dosing to acclimatize them to the conditions of the animal room. The animals should be fasted prior to administration of the test substance if the route of administration is oral. The purpose of fasting the animal is to eliminate feed in the gastrointestinal (GI) tract, which may complicate absorption of the test substance. Rats usually are fasted overnight. Because mice have a higher metabolic rate, withholding feed for 3–4 h may be adequate. Over-fasting small animals with a high metabolic rate may induce undesirable effects. The animals should be randomly assigned to dose groups. Randomization ensures a homogeneous population and can minimize errors due to sampling bias. All animals with body weights and health conditions out of the normal range should be eliminated prior to the randomization procedure.

Dose Levels

In general, the dose levels should be sufficient in number to allow a clear demonstration of a dose–response relationship and to permit an acceptable determination of the LD_{50}, if required. Three dose levels generally are sufficient. The selected dose levels should bracket the expected LD_{50} value with at least one dose level higher than the expected LD_{50} but not causing 100% mortality, and one dose level below the expected LD_{50} value but not causing 0% mortality, when the probit analysis method is applied to estimate the LD_{50}. However, with a method such as the moving average under some specific conditions (at least four dose levels with equal logarithmic intervals between each dose level, and with equal numbers of animals in each dose group), the LD_{50} can be estimated even with 0% mortality at the lower-dose levels and 100% mortality at the two higher dose levels. In any event, three or more dose levels with a wide range of toxicity responses are recommended if no other toxicity data are available.

Dosages

If necessary, the test substance should be dissolved or suspended in a suitable vehicle, preferably in water, saline, or an aqueous suspension such as 0.5% methylcellulose in water. If a test substance cannot be dissolved or suspended in an aqueous medium to form a homogeneous dosage preparation, corn oil or another solvent can be used. If the toxicity of the vehicle is not known, a vehicle control group should be included in the test. The animals in the vehicle control group should receive the same volume of vehicle given to animals in the highest dose group.

The test substance can be administered to animals at a constant concentration across all dose levels (i.e., varying the dose volume) or at a constant dose volume (i.e., varying the dose concentration). However, the investigator should be aware that the toxicity observed by administration in a constant concentration may be different from that observed when given in a constant dose volume. The maximum dose volume in rodents should not exceed 10 mL/kg body weight for nonaqueous vehicles or 20 mL/kg body weight for aqueous solution or suspension. In any event, for scientific and humane reasons the dose volume should be as small as possible.

Observations

The emphasis in acute toxicity studies is on the determination of the dose–response and the onset of toxic signs. The observation period should be flexible depending on the purpose of the study. This period should be based on the onset of signs, the nature of the toxicity, time to death, and the rate of recovery. For most highly toxic substances, the onset of toxic signs and the time to death may be very short, and prolonged observation may not be necessary. The slope of the dose–response curve for such test substances is usually very steep, and the treated animals either die or survive within a very short time. The observation period also should be long enough for the determination of reversibility or the recovery of an adverse effect. Under specific circumstances, the observation period might be longer, but it normally does not exceed 14 days.

Clinical examination, observation, and mortality checks should be made shortly after dosing, at frequent intervals over the next 4 h, and at least once daily thereafter. The intervals and frequency of observation should be flexible enough to determine the onset of signs, onset of recovery, and the time to death. The mortality checks should be frequent enough to minimize unnecessary loss of animals due to autolysis or cannibalism. Cage side observations should include any changes in the skin, fur, eyes, mucus membranes, circulatory system, autonomic and central nervous systems, somatomotor activities, behavior, etc. Any pharmacotoxic signs such as tremor, convulsions, salivation, diarrhea, lethargy, sleepiness, morbidity, fasciculation, mydriasis, miosis, droppings, discharges, or hypotonia should be recorded. The most common pharmacotoxic signs are listed in Tables 22.2 through 22.4. Individual body weights should be determined just prior to dosing, once weekly, and at death or at termination. Necropsies should be performed on animals that are moribund, found dead, and killed at the conclusion of the study. All changes in the size, color, or texture of any organ should be recorded. Any gross change observed at necropsy should be described according to the size, color, and position of the lesion. While a complete microscopic examination of tissues and organs is ideal and would be helpful in defining acute toxicity, economic and time factors may preclude such a study. If the investigator feels that microscopic examination of a lesion is essential, tissues from these lesions should be preserved in an appropriate fixative such as 10% buffered formalin.

Fixed-Dose Procedure (Test Limit)

The traditional test limit for acute oral toxicity was considered to be 5.0 g/kg body weight, but more recently accepted

TABLE 22.2
Common Signs and Observations in Acute Toxicity Tests

Clinical Observation	Observed Signs	Organs, Tissues, or Systems Most Likely to Be Involved
I. Respiratory blockage in the nostril, changes in rate and depth of breathing, changes in color of body surface	A. Dyspnea: difficult or labored breathing, essentially gasping for air, respiration rate usually slow	
	1. Abdominal breathing: breathing by diaphragm, greater deflection of abdomen upon inspiration	Central nervous system (CNS) respiratory center, paralysis of costal muscles, cholinergic
	2. Gasping: deep labored inspiration, accompanied by a wheezing sound	CNS respiratory center, pulmonary edema, secretion accumulation in airways, increased cholinergic
	B. Apnea: a transient cessation of breathing following a forced respiration	CNS respiratory center, pulmonary cardiac insufficiency
	C. Cyanosis: bluish appearance of tail, mouth, foot pads	Pulmonary–cardiac insufficiency, pulmonary edema
	D. Tachypnea: quick and usually shallow respiration	Stimulation of respiratory center, pulmonary–cardiac insufficiency
	E. Nostril discharges: red or colorless	Pulmonary edema, hemorrhage
II. Motor activities: changes in frequency and nature of movements	A. Decrease or increase in spontaneous motor activities, curiosity, preening, or locomotions	Somatomotor, CNS
	B. Somnolence: animal appears drowsy, but can be aroused by prodding and resumes normal activities	CNS sleep center
	C. Loss of righting reflex, loss of reflex to maintain normal upright posture when placed on the back	CNS, sensory, neuromuscular
	D. Anesthesia: loss of righting reflex and pain response (animal will not respond to tail and toe pinch)	CNS, sensory
	E. Catalepsy: animal tends to remain in any position in which it is placed	CNS, sensory, neuromuscular, autonomic
	F. Ataxia: inability to control and coordinate movement while animal is walking with no spasticity, epraxia, paresis, or rigidity	CNS, sensory, autonomic
	G. Unusual locomotion: spastic, toe walking, pedaling, hopping, and low body posture	CNS, sensory, neuromuscular
	H. Prostration: immobile and rests on belly	CNS, sensory, neuromuscular
	I. Tremors: involving trembling and quivering of the limbs or entire body	Neuromuscular, CNS
	J. Fasciculation: involving movements of muscles, seen on the back, shoulders, hind limbs, and digits of the paws	Neuromuscular, CNS, autonomic
III. Convulsion (seizure): marked involuntary contraction or seizures of contraction of voluntary muscle	A. Clonic convulsion: convulsive alternating contraction and relaxation of muscles	CNS, respiratory failure, neuromuscular, autonomic
	B. Tonic convulsion: persistent contraction of muscles, attended by rigid extension of hindlimbs	CNS, respiratory failure, neuromuscular, autonomic
	C. Tonic–clonic convulsion: both types may appear consecutively	CNS, respiratory failure, neuromuscular, autonomic
	D. Asphyxial convulsion: usually of clonic type but accompanied by gasping and cyanosis	CNS, respiratory failure, neuromuscular, autonomic
	E. Opisthotonos: tetanic spasm in which the back is arched and the head is pulled toward the dorsal position	CNS, respiratory failure, neuromuscular, autonomic
IV. Reflexes	A. Corneal eyelid closure: touching of the cornea causes eyelids to close	Sensory, neuromuscular
	B. Primal: twitch of external ear elicited by light stroking of the inside surface of ear	Sensory, neuromuscular
	C. Righting: ability of animal to recover when placed dorsal side down	CNS, sensory, neuromuscular
	D. Myotact: ability of animal to retract its hindlimb when limb is pulled down over the edge of a surface	Sensory, neuromuscular
	E. Light (pupillary): constriction of pupil in the presence of light	Sensory, neuromuscular, autonomic
	F. Startle reflex: response to external stimuli such as touch and noise	Sensory, neuromuscular

(*continued*)

TABLE 22.2 (continued)
Common Signs and Observations in Acute Toxicity Tests

Clinical Observation	Observed Signs	Organs, Tissues, or Systems Most Likely to Be Involved
V. Ocular signs	A. Lacrimation: excessive tearing, clear or colored	Autonomic
	B. Miosis: constriction of pupil regardless of the presence or absence of light	Autonomic
	C. Mydriasis: dilation of pupils regardless of the presence or absence of light	Autonomic
	D. Exophthalmos: abnormal protrusion of eye in orbit	Autonomic
	E. Ptosis: dropping of upper eyelids, not reversed by prodding animal	Autonomic
	F. Chromodacryorrhea: red lacrimation	Autonomic, hemorrhage, infection
	G. Relaxation of nictitating membrane	Autonomic
	H. Corneal opacity, iritis, conjunctivitis	Irritation of the eye
VI. Cardiovascular signs	A. Bradycardia: decreased heart rate	Autonomic, pulmonary–cardiac insufficiency
	B. Tachycardia: increased heart rate	Autonomic, pulmonary–cardiac insufficiency
	C. Vasodilation: redness of skin, tail, tongue, ear, foot pad, conjunctivae, and sac, and warm body	Autonomic, CNS, increased cardiac output, hot environment
	D. Vasoconstriction: blanching or whitening of skin, cold body	Autonomic, CNS, decreased cardiac output, cold environment
	E. Arrhythmia: abnormal cardiac rhythm	CNS, autonomic, pulmonary–cardiac insufficiency, myocardiac infarction
VII. Salivation	A. Excessive secretion of saliva: hair around mouth becomes wet	Autonomic
VIII. Piloerection	A. Contraction of erectile tissue of hair follicles resulting in rough hair	Autonomic
IX. Analgesia	A. Decrease in reaction to induce pain (e.g., hot plate)	Sensory, CNS
X. Muscle tone	A. Hypotonia: generalized decrease in muscle tone	Autonomic
	B. Hypertonia: generalized increase in muscle tension	Autonomic
XI. GI signs:		
Droppings (feces)	A. Solid, dried, and scant	Autonomic, constipation, GI motility
	B. Loss of fluid, watery stool	Autonomic, diarrhea, GI motility
Emesis	A. Vomiting and retching	Sensory, CNS, autonomic (in rat, emesis absent)
Diuresis	A. Red urine	Damage in kidney
	B. Involuntary urination	Autonomic sensory
XII. Skin	A. Edema: swelling of tissue filled with fluid	Irritation, renal failure, tissue damage, long-term immobility
	B. Erythema: redness of skin	Irritation, inflammation, sensitization

TABLE 22.3
Autonomic Signs

Sympathomimetic	Piloerection
	Partial mydriasis
Sympathetic block	Ptosis
	Diagnostic if associated with sedation
Parasympathomimetic	Salivation (examined by holding blotting paper)
	Miosis
	Diarrhea
	Chromodacryorrhea in rats
Parasympathomimetic block	Mydriasis (maximal)
	Excessive dryness of mouth (detect with blotting paper)

TABLE 22.4
Toxic Signs of Acetylcholinesterase Inhibition

Muscarinic Effects[a]	Nicotinic Effects[b]	CNS Effects[c]
Bronchoconstriction	Muscular twitching	Giddiness
Increased bronchoconstriction	Fasciculation	Anxiety
Nausea and vomiting (absent in rats)	Cramping	Insomnia
Diarrhea	Muscular weakness	Nightmares
Bradycardia		Headache
Hypotension		Apathy
Miosis		Depression
Urinary incontinence		Drowsiness
		Confusion
		Ataxia
		Coma
		Depressed reflex
		Seizure
		Respiratory depression

[a] Blocked by atropine.
[b] Not blocked by atropine.
[c] Atropine might block early signs.

protocols for acute toxicity [159–161] have a test limit of 2.0 g/kg body weight. Protocol used is a modification of the protocol developed by the BST as described by Van den Heuvel et al. [199]. Basically, this procedure calls for dosing animals in a stepwise fashion using fixed doses of 5, 50, 500, and 2000 mg/kg. The initial dose level selected (discriminating dose) would be a dose expected to produce some signs of toxicity and should be nonlethal, nonpainful, and nonstressful. The dose could be selected by using available information, or by conducting a *sighting study* using three or four animals. If no mortality was observed at the highest dose level, a higher dose level was generally not necessary. The focus of the test should not be limited to mortality (found dead or killed for humane reasons), but should include other toxicity endpoints such as time course of signs of toxicity and necropsy findings. These data and the *discriminating dose* should provide adequate data for hazard assessment, comparative reference, and labeling classification (Table 22.5).

A multinational validation study in 33 laboratories with 20 materials using the fixed-dose approach produced consistent results on the time course of signs of toxicity, which was adequate for acute toxicity risk assessment and acute toxicity classification based on the European Economic Community (EEC) criteria. Compared to the classical method, fewer animals were used and less stress occurred [199]. This test has been adopted by the OECD as an alternative acute oral toxicity method [159].

Acute Toxic Class Method

This method has been described by Roll et al. [177] and is based on the assumption that using a minimum number of animals in a stepwise procedure will provide enough information on the acute toxicity of a substance to allow classification according to the most commonly used classification schemes. Three animals of one sex are used for each step; while normally females are used (considered to be generally slightly more sensitive, see Ref. [115]), either sex can be used. The initial dose is selected from one of the following fixed-dose levels: 5, 50, 300, or 2000 mg/kg body weight and should be chosen to produce some mortality. If existing information suggests that mortality is unlikely at the 2000 mg/kg dose, then a limit test at that level may be conducted with three animals of each sex. If deaths occur, further testing at the lower-dose levels may be necessary. This method was evaluated in national and international validation studies [182,183], and has been adopted by the OECD as an alternative acute oral toxicity method [160]. Like the fixed-dose method (see preceding section), this method will enable a judgment with respect to classifying the test material in one of a series of toxicity classes in accordance with the Globally Harmonized System (GHS) for the classification and labeling of chemicals [154].

Up-and-Down Procedure

The up-and-down procedure [20–22,161], which is one of the more modern methods of estimating the LD_{50}, is based on the maximum likelihood method. Like the *acute toxic class* method animals are dosed following a stepwise procedure. However, animals are dosed one at a time at a minimum of 48 h intervals, with the first animal receiving a dose just below the estimated LD_{50}. If the first animal survives, the next one receives a higher dose. If the first animal dies, the next one receives a lower dose. The spacing of doses generally is adjusted by a factor of 3.2 (default factor corresponding to a dose progression of one half log unit) up or down depending on the outcome of the previous animal. Comparison of classical LD_{50} values to the up-and-down derived LD_{50} shows close agreement (Table 22.6). This test has been adopted by the OECD as an alternative to the more traditional methods of LD_{50} determination [161]. The OECD test guideline also contains the provision for a limit test that uses a maximum

TABLE 22.5
Investigation of Acute Oral Toxicity Using the Fixed-Dose Method of Interpretation of Results

Fixed Dose (mg/kg)	Results	Interpretation
5[a]	Less than 100% survival[b]	Compounds that may be very toxic if swallowed
	100% survival but evident toxicity	Compounds that may be toxic if swallowed
	100% survival; no evident toxicity	Retested at 50 mg/kg if not already tested at that level
50	Less than 100% survival[b]	Compounds that may be toxic or very toxic if swallowed; retested at 5 mg/kg if not already tested at that level
	100% survival but evident toxicity	Compounds that may be harmful if swallowed
	100% survival; no evident toxicity	Retested at 500 mg/kg if not already tested at that level
500	Less than 100% survival[b]	Compounds that may be toxic or harmful if swallowed; retested at 50 mg/kg if not already tested at that level
	100% survival but evident toxicity	Compounds that do not present a significant acute toxic risk if swallowed
	100% survival; no evident toxicity	Retested at 2000 mg/kg if not already tested at that level
2000[c]	Less than 100% survival[b]	Compounds that may be harmful if swallowed; retested at 500 mg/kg if not already tested at that level
	100% survival with or without evident toxicity	Compounds that do not present a significant acute toxic risk if swallowed

Source: Adapted from Van den Heuvel, M.J. et al., *Food Chem. Toxicol*., 28, 469, 1990.

[a] Where a dose of 5 mg/kg produces significant mortality, or where a sighting study suggests that mortality will result at that dose level, the substance should be investigated at a lower dose level. The level chosen should be one that is likely to produce evident toxicity but no mortality.

[b] Includes compound-related mortality and humane kills but not accidental deaths.

[c] It should be noted that testing mortality at this dose level is carried out primarily for risk assessment purposes. However, where no evident toxicity is seen at 500 mg/kg its results are relevant to classification if there is greater than 50% mortality (including humane kills).

TABLE 22.6
Comparison of Rat Oral LD_{50} Using the Up-and-Down Method versus Classical (Conventional) Method

	Classical Method		Up-and-Down Method	
Chemical No.	*n*	LD_{50} (g/kg)	*n*	LD_{50} (g/kg)
1	50	0.273	6	0.388
2	40	0.344	9	0.421
3	40	3.490	8	4.120
4	40	3.520	6	4.020
5	40	4.040	6	3.520
6	40	5.560	6	5.700
7	40	9.280	6	8.770
8	20	>10.00	3	>10.10
9	50	10.11	7	11.09
10	10	>20.00	8	22.40

Source: Bruce, R.D., *Fundam. Appl. Toxicol*., 5, 151, 1985.

of five animals. This test is used when there is information suggesting that the test material has a low potential to be toxic, in this study a test dose of 2000 mg/kg or, as required, 5000 mg/kg is used. Animals are dosed in a sequential manner with the second animal receiving the dose only if the first animal survives the limit dose.

Data from this method can be analyzed using either SAS [181] or BMDP [47] computer program packages, which are available to many toxicology laboratories. Other examples of programming for the estimation of the LD_{50} with a small computer have been reported [113,178].

Acute Dermal Toxicity

Dermal exposure is an important route of exposure. The objective of conducting an acute dermal toxicity study is the same as an acute oral toxicity study, to assess the adverse effects resulting from a single dermal application of a test substance. The acute dermal test also provides the initial toxicity data for regulatory purposes, labeling, classification, transportation, and subsequent subchronic and chronic dermal toxicity studies. In addition, results from this type of test could provide information on dermal absorption and a test material's potential mode of toxic action. Comparison of acute toxicity by the oral and dermal routes may provide evidence of the relative penetration of a test material.

While the general experimental design and principles of acute dermal toxicity testing are similar to those of acute oral testing, there are differences. These differences include selection of the animal species, the number of animals per dose level, preparation of animals, dosage, and administration of the test substance. Only differences in the acute dermal test are described in this section.

Recently, an OECD test method similar in principle to the acute oral toxicity fixed-dose procedure [198] has

been developed and is currently under review [148]; when approved, this method would reduce the number of animals needed to assess the dermal toxic potential of chemicals. Because this method has not been finalized and formally approved, it will not be discussed here.

Principle

The test material is applied dermally, undiluted or diluted with the appropriate solvent, in graduated doses to several groups of animals. A vehicle control group is included if needed, but generally this group is not necessary if the toxicity of the vehicle is known. Clinical signs, morbidity, and mortality are observed at specific time intervals. Animals that die or become extremely moribund during the study are necropsied. At the conclusion of the study's observation period, surviving animals are killed and necropsied. Tissues may be saved for histopathological examination to facilitate the understanding of the acute toxicity of the compound. In order to increase the reproducibility of the study, all experimental conditions and procedures should be standardized, and the study should be conducted according to generally recognized GLP outlined by the EPA and the OECD [56,57,162].

Animals

The three most commonly used animal species for this type of test are rabbits, rats, and guinea pigs. However, other species can be used for this type of test. At the start of the study, healthy animals between 8 and 12 weeks old with a range of weight variation not exceeding ±20% of the appropriate mean value should be used. Variables, such as species used, age and health of the animal, body weight, sex, and housing environment, can affect the outcome of an acute dermal toxicity test. The animals should be housed individually in a controlled environment. Quarantine, acclimatization, and randomization are as described in earlier text for acute oral toxicity studies. The back of the animal or a band around the trunk should be clipped free of hair. When clipping the hair, care must be taken not to abrade the skin. If abraded skin is called for, a needle may be used, but care must be taken not to damage the dermis. Increasingly, investigators have come to question the value of conducting tests on abraded skin, and many consider such tests to be irrelevant. To date, almost all testing guidelines call for conducting the dermal test only on intact skin [51,62,67,151,157,186]. In contrast to the acute oral toxicity test method, fasting animals overnight is not necessary. Generally, 10 animals per dose level (5/sex) are sufficient to allow for an acceptable estimation of the dermal LD_{50}. However, depending on the nature of the test substance and available safety information, smaller numbers of animals can be used. Females used in the study should be nulliparous and not pregnant.

Dose Levels

Dose selection is similar to the acute oral toxicity test. Higher doses do not need to be tested when a test substance at 2000 mg/kg, considered the limit dose, has not produced test substance-related mortality. This is because administration of additional test substance would only be applied on top of the test substance layer already present. This layering may form a physical barrier to prevent further absorption of the test substance from the application site.

While a control group generally is not needed, a vehicle control group should be included in the study if the toxicity of the vehicle is not known. Its influence on dermal penetration of the test substance should be fully established prior to the study.

Preparation of Dosage and Dosing Procedure

The test substance should be applied uniformly to approximately 10% of the body surface of the animal (e.g., 4 cm × 5 cm for rats, 12 cm × 14 cm for rabbits, 7 cm × 10 cm for guinea pigs). Under certain conditions, the area of application may vary; for example, the area of application for highly toxic substances may be small because a lower volume is applied.

Liquid test substances generally are applied undiluted. If the test substance is a solid, it should be pulverized, weighed, placed on a plastic sheet or porous gauze dressing, moistened so as to form a paste with normal saline (one part test substance for one part saline) or an appropriate solvent, and then spread evenly on the closely clipped skin to ensure uniform contact with the skin. Grinding of the solid test substances may not be needed under some conditions. For example, when a granular formulation is tested, it may be more relevant to test the substance in its formulation state than to destroy the formulation by grinding.

The test substance can be applied under semiocclusive, occlusive, or nonocclusive (open) conditions; choice of the application method depends on what the most likely exposure pattern is in humans. The application method with the highest potential for skin irritation is occlusive, followed by semiocclusive and nonocclusive exposures. It should be noted that skin irritation may not only cause stress to the animal but can also increase dermal penetration of the test substance.

For nonocclusive application, the application site remains uncovered but the volume of liquid test material that can be applied to the skin may be limited depending on the volatility of the liquid. It may be necessary to immobilize the animal or use a device such as an *Elizabethan* collar so as to prevent the animal from ingesting the test material as a result of licking the application site. For occlusive application, the application site is covered with an impervious material such as a plastic sheet. For semiocclusive application the application site is covered with a porous gauze dressing as described in the following paragraph. The volume that can be applied with the occlusive or semiocclusive patch generally is larger than that of the nonocclusive method.

Dosing Procedures for Liquid Test Substances

The dosing procedure for the rabbit is detailed because rabbits are the most widely used species for this type of testing. Rabbits are clipped free of hair with an electric animal hair clipper. The rabbit may have to be restrained by tightening the

hind legs to a secured post and holding the nape of the neck during clipping. When using the occlusive method, a plastic cuff in a cylindrical shape (approximately 12–15 in. long and 10 in. in diameter) open at both ends can be used. The cuff is put onto the trunk of the rabbits, covering the application site. With the help of another investigator, the plastic cuff is folded around the trunk and secured at the thorax and flank of the rabbit with surgical adhesive tapes. Care should be exercised so that the cuff is sufficiently secured but not too tight to affect breathing. Using a long feeding needle, the correct amount of the liquid test substance is drawn into a syringe of appropriate size. The needle then is placed under the cuff and half of the dose is delivered evenly on each side of the vertebral column. After withdrawal of the needle, the test substance is evenly distributed over the application site by gently rubbing the top of the plastic cuff. A piece of cloth of appropriate size is then wrapped around the plastic cuff and taped in place to absorb any test substance that may spill off the cuff. After dosing, the investigator should observe the animal for a moment to see if breathing is affected, prior to putting the animal back into the cage. In the semiocclusive method, a porous gauze dressing replaces the plastic cuff. In nonocclusive exposure, the test substance is applied uniformly over the skin: care must be taken to minimize run off from the skin, especially for aqueous dosing solutions. Applying the test substance in small amounts at a time may help.

Dosing Procedure for Solid Test Substances

If the test substance is a solid, it should be ground with a mortar and pestle unless there is justification not to pulverize. The correct dose of the ground solid is weighed, placed in the center of a plastic sheet of appropriate size, and moistened with sufficient normal saline or another appropriate vehicle. If a vehicle other than saline or water is used, the effect of the vehicle on the skin penetration of the test substance should be considered, and its toxicity should be known. The type of vehicle selected should be based on the expected mode of exposure of the test substance and should be mixed into a paste. The paste then is spread evenly around the center of the plastic sheet. With one person holding the rabbit by grasping it at the back, another person moistens its belly and its back with paper towels soaked with saline. Then the rabbit is placed with its belly on the test substance paste on the plastic sheet, and another investigator wraps the sheet around the trunk of the rabbit. The plastic cuff is secured in place with surgical tape at the thorax and the flank. A piece of cloth of appropriate size then is wrapped around the plastic cuff and secured in place in the same manner. In the semiocclusive method, a porous gauze dressing replaces the plastic sheet.

Dosing Procedures for Rats and Guinea Pigs

Method of dosing rats and guinea pigs is similar to that of the rabbit. Liquid samples should be placed on the back instead of belly or on the lateral trunk. If nonocclusive exposure is called for in rats, the test substance should be applied to the skin as near to the head as possible to prevent ingestion by preening of the application site. A plastic collar may be used to further limit access to the treatment site. Generally, the plastic collar produces more stress in the rat, as indicated by chromodacryorrhea (red stain around the eyes), than in the rabbit. To minimize stress in rats, small collars can be hand made from light cardboard. The collar is lined with cut rubber tubing around the neck area and stapled in place. The cardboard collar is lighter and easier to place on small animals. It can readily be replaced if needed (the collar placed on the neck usually will last about 3 days), and it is more economical than the commercially available plastic collars.

Exposure Period and Removal of Cuff

Almost all testing guidelines [51,61,66,151,157,186] call for 24 h continuous exposure. Upon completion of the exposure, the cuff is removed and the application site is gently wiped with a paper towel soaked with saline, water, or any appropriate solvent to remove residual test substance remaining on the application site.

Observation Period

As in the acute oral toxicity test, the recommended minimum observation period is 14 days. However, the duration and intervals of observation should be flexible enough to establish onset of signs, time to death, and time to recovery, but should be frequent enough such that the loss of animals due to autolysis and cannibalizing is minimal. In addition, skin irritation should be assessed according to a scoring system such as the one described by Draize et al. [49].

Acute Inhalation Toxicity

Inhalation exposure is an important route of exposure. The objective of conducting an acute inhalation toxicity study is to evaluate the toxic potential of a test material that may be inhaled, such as a gas, a volatile substance, or an aerosol. Such testing may provide information on the adverse effects resulting from exposure to inhalation application of a single dose of a test substance. The acute inhalation test provides the initial toxicity data for regulatory purposes, labeling, classification, transportation, and subsequent subchronic and chronic dermal toxicity studies. Comparison of acute toxicity by the oral and inhalation routes may provide evidence of the relative penetration and bioavailability of a test material.

While the general experimental design and principles of acute inhalation toxicity testing are similar to those of acute oral testing, there are differences. These differences include selection of the animal species, the number of animals per dose level, preparation of animals, dosage, and, most importantly, administration of the test substance.

Recently, OECD test methods similar in principle to the acute oral toxicity's fixed-dose [198] and acute toxic class [160] methods have been developed and are currently under review [149,152]; when approved, these methods would reduce the number of animals needed to assess the inhalation toxic potential of chemicals. Because these methods have not been finalized and formally approved they will not be discussed here.

Principle

Several groups of animals are exposed to a fixed concentration of test material by inhalation for a short period of time, one concentration per group. While whole body exposure inhalation data are accepted by regulatory agencies, it is recommended that nose-only or head-only exposure be used as this minimizes oral exposure from animals licking the compound off their fur. When a vehicle is used to help attain an appropriate concentration of test material, a vehicle control group should be included in the study. Clinical signs, morbidity, and mortality are observed at specific time intervals. Animals that die or become extremely moribund during the study are subjected to necropsies. At the conclusion of the study's observation period, surviving animals are killed and necropsied. Tissues may be saved for histopathological examination to facilitate the understanding of the acute toxicity of the compound.

Animals

While several mammalian species have been used, the preferred species is the rat. In selecting a test species, priority should be given to the species with metabolism or other physiological and biochemical parameters similar to man. At the start of the study, healthy animals between 8 and 12 weeks old with a range of weight variation within and between test groups should not exceed ±20% of the mean weight. The animals should be housed individually in a controlled environment. Quarantine, acclimatization, and randomization are as described in earlier text for acute oral toxicity studies.

At least 10 animals (5/sex) per concentration level are recommended in most regulatory guidelines [63,64,68,150,186] giving sufficient numbers to allow for an acceptable estimation of the inhalation LC_{50}. However, depending on the nature of the test substance and available safety information, smaller numbers of animals can be used. Females, if used, should be nulliparous and not pregnant.

Environmental Conditions

Inhalation equipment used should be able to sustain a dynamic airflow of 12–15 air changes per hour, ensure adequate oxygen content of 19% and an evenly distributed exposure atmosphere [63,150]. If a whole body chamber is used, individual housing must be used and the total volume of test animals should not exceed 5% of the volume of the test chamber. Temperature and relative humidity need to be monitored continuously and should be maintained at 22°C ± 2°C and 30%–70%, respectively.

Dose Levels

In general, three concentration levels should be used and spaced to produce a concentration–response curve in order to permit an acceptable determination of the LC_{50}. Animals are usually exposed for a period of 4 h. Dose range–finding studies using single animals may help determine the doses to be used in the main study when the toxic potential of the test material is unknown. In selecting the dose levels to be used, particle size analysis should be performed to determine the consistency of particle size distribution. While not specifically required by OECD, U.S. EPA guidelines recommend that the mass median aerodynamic diameter (MMAD) particle size range should be between 1 and 4 μm (particle size distribution that permits deposition throughout the respiratory tract). When a vehicle is used to attain the desired test material concentration, a vehicle control group should be included in the test.

Observations

The recommended minimum observation period is 14 days. However, the duration and intervals of observation should be flexible enough to establish onset of signs, time to death, and time to recovery, but should be frequent enough such that the loss of animals due to autolysis and cannibalizing is minimal.

Clinical examination, observation, and mortality checks should be made shortly after dosing, at frequent intervals over the next 4 h, and at least once daily thereafter. Cage side observations should include any changes in the skin, fur, eyes, mucus membranes, circulatory system, autonomic and central nervous systems, somatomotor activities, behavior, etc. Individual body weights should be determined just prior to dosing, once weekly, and at death or at termination. Gross necropsies should be performed on animals that are moribund, found dead, and killed at the conclusion of the study with particular attention paid to any changes in the respiratory tract.

Test Limit

If no test substance–related mortality is observed at an exposure of 5 mg/L or the maximum attainable concentration for 4 h, then it is not necessary to conduct a full study [64,150].

ASSESSMENT OF EYE IRRITATION INDUCED BY CHEMICALS

The eye captures visible energy and converts the energy to neurosignals, which are transmitted to the intricate central nervous system where they form neuroimages (vision). The importance of having this ability to perceive the external environment through vision is a giant step in the evolution process. In humans, vision along with hearing is vital for the development of speech, learning, and intelligence. Loss of vision can greatly curtail normal living.

There are three basic components of vision: optics, photoreceptors, and conducting nerves. All three components must function properly to form a clear and sharp neuroimage in the visual cortex. The optics of the eye (cornea, aqueous humor, iris, lens, and vitreous humor) must remain transparent and be able to refract and focus light on the right position on the photoreceptors. The photoreceptors (the cones and rods) of the retina must be able to undergo photolysis and convert light energy to neuropotential impulses. The optic nerves must be able to carry these neuroimpulses to the visual cortex.

Because the eye is constantly exposed to the external environment, the cornea must be protected from drying, dust, and microorganisms. The eyelids, the lacrimal system, and the somatosensory response of the cornea all work together to protect this outermost structure of the eye. Like other organs, the major portion of the eye is nourished by blood vessels.

The retinal, circumcorneal, and uveal vessels also nourish and help maintain the eye. These vessels are so arranged and constructed that they normally do not alter the transparency of the ocular optics. Nutrients reach the transparent tissues of the eye via tears, the aqueous humor, and vitreous fluids.

Normal ocular functions are in delicate balance and are interdependent. Any traumatic insult, chemical or physical, can upset one or many of these ocular functions, thus creating a disturbance in vision. Depending on the extent of the traumatic injury (ranging from drying of the tear film to corneal ulceration or optic nerve damage), partial or complete loss of vision can result. Ocular injury not only can result from accidental physical trauma, but also radiation and chemicals.

Chemicals can cause ocular damage locally by accidental exposure to the eye, or systemically by ingestion of chemicals such as food contaminants and drugs. Because many chemicals can produce ocular damage either locally or systemically [85,95,129,176], it is important to test products for ocular effects before exposing workers during manufacturing and, ultimately, before subjecting consumers to products on the market. Ocular effects resulting from systemic exposure are beyond the scope of this chapter. This section focuses on eye irritation resulting from direct ocular contact.

Conducting ocular tests in humans is not only impractical but also unethical. Consequently, many methods and techniques have been developed over the years for testing ocular effects in animals. This section describes the animal methods for detecting potential eye irritants and discusses their limitations. In recent years, in vitro methods intended to replace eye irritancy tests in animals have evolved; an overview of some of these methods is discussed in Chapter 20.

Testing for potential eye irritancy is required for labeling and classification of chemicals by most regulatory agencies worldwide. The test protocol, interpretation of results, and classification scheme vary among countries. The differences among major industrial countries also are discussed.

Definition of Chemically Induced Eye Irritation and Corrosion

Irritation can be defined as reversible inflammatory changes in the eye and its surrounding mucus membranes following direct exposure to a material on the surface of anterior portion of the eye. Corrosion is irreversible ocular tissue damage following exposure to a material. From a practical point of view, the distinction between reversible and irreversible changes sometimes is limited by the length of the observation period. Therefore, the term *eye corrosion* should be reserved for gross tissue destruction of the eye, which generally occurs rapidly following exposure. When interpreting results from an eye irritation study, one must take into consideration the biological nature and significance of the ocular changes. For example, conjunctival redness is considered a mild ocular effect.

Normal Physiology and Anatomy of the Eye

A brief description of the normal physiology and anatomy of the eye is essential for understanding the development of eye irritation. Details can be found in a variety of textbooks and reviews [74,126,172].

Functionally, the eye can be divided into three basic parts (Figure 22.4). From posterior to anterior, they are as follows.

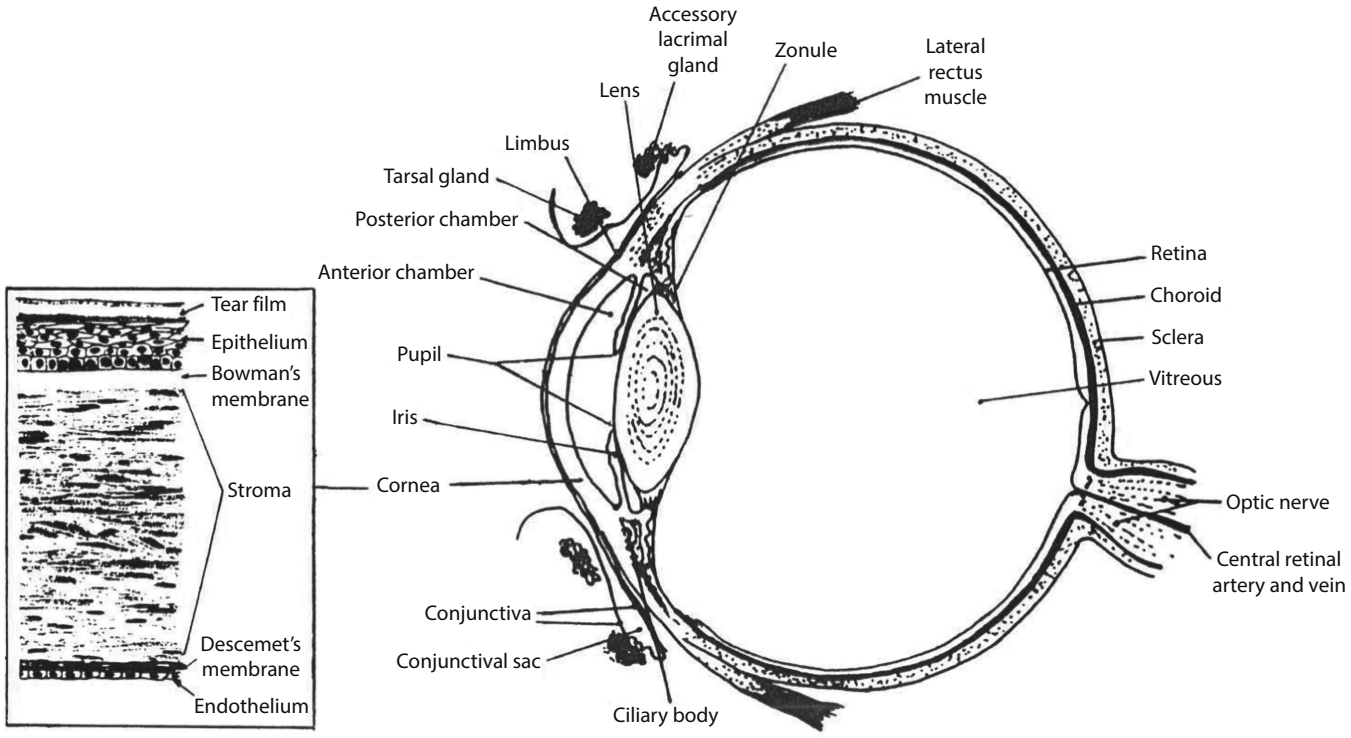

FIGURE 22.4 Schematic illustration of the eye.

Photoreceptors (*retina*): The part of the eye that connects to the central nervous system via the optic nerve.

Optics: Structure that focuses visible light (image) onto the retina; it includes (from anterior to posterior) the cornea, iris, aqueous humor in the anterior chamber, the lens, and its related organelles such as the zonules and ciliary body (muscles), and the vitreous in the posterior chamber.

Protective, lubricating, and nutritional structures: These include the anterior eyelids and conjunctiva and associated secretory glands, the sclera and its outside layer (the fibrous tunic) and inside layer (uvea–vascular), and the ciliary body (secretory).

For chemically induced eye irritation, the main concern is generally on the directly exposed organelles such as the cornea, conjunctiva, and the iris. Effects on these structures can easily be detected by gross observation. If the chemical can penetrate deeper into the eye, other organelles also can be affected. Detection of the effects on these deeper structures requires special aids.

Cornea

The cornea is composed of, from anterior to posterior, the epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium. The epithelium is about five cells deep in the transitional zones at the periphery. The basal cells are columnar, the other cells are squamous, and the cells between the two layers are polygonal (wing cells). Bowman's membrane (12 μm) is an acellular layer of collagen and ground substance, which provides a functional interface between the stroma and epithelium. An intact Bowman's membrane and the epithelial basal cell layer are vital to the regeneration of damaged epithelium. Damage to Bowman's membrane may predispose the cornea to fibrosis. The stroma consists of lamellae of collagen fibrils and fibroblasts supported by ground substances. The stroma forms most (nine-tenths) of the cornea and is limited on its inner surface by Descemet's membrane.

In addition to the organization of sheets of fibrils, other unique features such as proper hydration also contribute to corneal transparency. Descemet's membrane (5–10 μm), like Bowman's membrane, is an acellular layer, which is the basement membrane of the endothelium. The endothelium is a single layer of cells, which completely covers the posterior surface of the cornea. The cells are hexagonal with large nuclei. This layer of the cornea is particularly rich in the active transport enzyme adenine triphosphatase (ATPase). The maintenance of proper hydration of the cornea has been attributed to the activity of this enzyme, which catalyzes an active sodium–potassium pump [15,108,118]. The limbus is a transitional region between the cornea and the sclera. This region, rich in vascularization, is the source of fluid and infiltration cells during corneal injury.

The epithelium and the overlying tear film provide the intrinsic protection for the cornea. Other layers have almost no intrinsic resistance to injury. Penetration into deeper layers of the cornea and other structures of the eye is limited by the chemicals' solubility and lipophilicity. Chemicals that are lipophilic and water-soluble penetrate more rapidly and probably deeper into the eye than other chemicals.

The cornea is always covered with a film of tears, which consists of several oily and aqueous layers. Proper tear formation and drainage as well as the stability of the precorneal tear film are important for a normal precorneal optical surface, proper lubrication, nutrition for the cornea, removal of bacteria and debris from the cornea, and activity on the cornea. Reduction of tear formation can lead to a dry eye, mechanical friction, irritation, or infection. A discussion on the assessment of tear film formation, stability, and drainage is available [31].

The cornea is a powerful refractive biological optic. Its refractive power is dependent on its being transparent and on proper hydration. Maintenance of proper transparency and hydration is dependent on many mechanisms, for example, proper tear flow, absence of deposits and blood vessels, proper arrangement of collagen fibrils, unimpaired nutritional supply for the metabolic active pump (Na^+–K^+), and proper intraocular pressure. Decreased transparency or hydration can be a result of corneal scars (decreased corneal thickness) or corneal edema (increased corneal thickness). Corneal edema can be caused by epithelial damage, endothelial damage, increased intraocular pressure, lack of oxygen, or inhibition of the electrolyte balance pump (Na^+–K^+-activated ATPase), which is located mainly in the endothelial membrane, but also is found in the epithelium. Methods for measuring corneal curvature, corneal thickness, intraocular pressure, blood/aqueous humor barrier, and corneal endothelium damages have been reviewed [31].

Conjunctiva

The conjunctiva is part of the eyelid. It is the delicate membrane that lines the eyelid (palpebral conjunctiva) and covers the exposed surface of the eyeball (bulbar conjunctiva). Histologically, the conjunctiva is an aqueous, nonkeratinized epithelium with numerous mucus-secreting cells. Accessory lacrimal glands are present in the conjunctiva, which contribute to the aqueous layer of a precorneal tear film. The Meibomian gland, a specialized sebaceous gland in the eyelid, secretes the outer oily layer of the tear film.

The main function of the eyelid is to protect the eye, especially the cornea, from external trauma through proper blinking reflexes and secretion of tears. Normal secretory and excretory functions of the tear are also important for normal optical function of the eye. The precorneal tear film can form an optically uniform layer over the microscopically irregular surface of the corneal epithelial cells. The tear flow continuously flushes cellular debris or foreign bodies from the eye, lubricates the corneal surface from mechanical friction caused by blinking, provides nutrients to the cornea, and induces antibacterial activities by proteolytic enzymes and immunoglobulin. All of these functions are important to

maintain an optically intact corneal surface. Substances that affect the stability of the precorneal tear film by interfering with the secretory/excretory functions or with the blinking mechanism can cause serious damage to the cornea and may even cause corneal ulceration.

The nictitating membrane or the third eyelid is an important and prominent structure in many species of animals including the rabbit but is not as important in humans and nonhuman primates. It aids in protecting the conjunctiva and the cornea when the eyeball is retracted. The nictitating membrane, like the conjunctiva, also contains lacrimal glands and its secretion contributes to the aqueous layer of the precorneal tear film. In addition, the nictitating membrane helps to support the position of low eyelids and forms the lacrimal lake in the medial canthus. Vascularization in the conjunctiva generally consists of superficial and deep groups, mainly in the bulbar conjunctiva.

Three endpoints generally are associated with irritation in the conjunctiva: redness, chemosis, and discharge. In response to an irritant, the eyelids blink, the tear secretion increases, and the conjunctiva vessels dilate. Blinking and tearing (discharge) aid in removing the irritant from the eye, and tear flow also may reduce the acidity or basicity of the irritant. Vessel dilation may be triggered by histamine, prostaglandins, or other inflammatory mediators, resulting in an apparent increase in vascularity (redness) in the conjunctiva. If irritation is severe, the dilation of the vessel increases and vascular fluid and proteins leak into the conjunctiva resulting in edema (chemosis). If the edema is severe, bulging may hinder normal functioning of the eyelids.

Iris

The iris forms the pupil and functions in regulating the amount of light that may reach the retina. High-intensity light causes constriction of the diameter of the pupil whereas low-intensity light dilates it. It does so by two sets of muscles acting opposite each other to control the diameter of the pupil. These muscles, circulatory and radiating, are innervated by both the autonomic and sympathetic nervous systems. The set of muscles forms the distinct characteristic of iridic furrows of the iris.

The iris is anatomically located posterior to the cornea, and is a very vascular structure made of loose connective tissues, muscle, and pigmented cells. The amount of pigment in the iris varies. Heavily pigmented cells are found in most species except albinos. Only a small amount of pigment is found in the albino rabbit eye. This is an advantage in ocular studies because it allows easier and better examination of the iridal vessels, lens, and retina.

The observation endpoints of local iridic injury are increased vascularity, edema (increased thickness of the stroma/swelling), reaction to light, aqueous flare, and gross destruction of tissue. These are the manifestations of an inflammatory process (iritis) responding to an irritant. Like the conjunctival vessels, the iridic vessels dilate and leak vascular fluid in response to irritants. Dilation of vessels and leakage cause edema and apparent changes in vascularity such as injection of iridic vessels (hyperemia). Aqueous flare is a result of protein leaking from the iridic vessels into the aqueous humor of the anterior chamber. Protein leakage into the anterior chamber alters the refractive index of the aqueous humors. Light beams entering the anterior chamber are scattered, giving the anterior chamber a cloudy appearance which contrasts with a clear appearance in normal eyes as a light beam passes through the pupil and the anterior chamber, for example, during examination with a slit lamp. This is called the aqueous flare or Tyndall phenomena, which is usually not noted during routine gross examination of the eye. In a more severe form of iritis, tissue destruction may result and nerve innervation may be disrupted, causing the pupil to be unresponsive to light. Failure to react to light, from a practical standpoint, is the most reliable observation of a severe iridic reaction since severe iritis is usually accompanied by severe opacity in the cornea, which may obscure the visible detection of changes on the iris.

DRAIZE TEST

The Draize test was developed in 1944 by Draize et al. to study eye irritation [49]. The test was based on the original work of Friedenwald et al. [79]. For years, the Draize test has been used as the animal test to identify human eye irritants. It is a simple and generalized test. It is easy to conduct and requires no special instruments. While simplicity is probably the main reason for its popularity, it is also the limitation of the test per se. Undeniably, the Draize test can adequately identify most of the moderate-to-severe human eye irritants, but the test may fail to detect mild or subtle ocular irritation even with proper modification.

In the original Draize test, a standard 0.1 mL or 0.1 g of test substance is applied to the conjunctival sac of an albino rabbit's eye. The eyelid is held together for a few seconds and then released. The degree or extent of opacity on the cornea, the redness on the iris, and the chemosis and discharge on the conjunctiva are scored subjectively according to an arbitrary scale at preselected intervals (1, 24, 48, and 96 h) after exposure. Scoring is based on the degree of effects caused by the testing substance. More emphasis is placed on the opacity of the cornea, which has a maximum score of 80, whereas emphasis is progressively less with other effects: conjunctival changes (maximum score of 20) and iritis (maximum score of 10) [24,84,121].

The Draize test has been a subject of controversy among animal rights groups [93,179] and even in the scientific community [9,24,84,88,96,121,174,205]. This test has been criticized on the dose volume, use of animals as models, methods of exposure, irrigation, number of animals, observation and scoring including laboratory procedure variability, and interpretation of results, all of which are discussed in the following.

Dose Volume

The 0.1 mL dose volume used in the original test was based on the volume used earlier by Friedenwald et al. [79] to study

the mechanism of acid- and base-induced ocular damage. This dose volume was selected arbitrarily as a standard volume for intraocular injection. Draize et al. [49] adopted it solely for convenience, which unfortunately has set a seemingly unchangeable doctrine for years even though the 0.1 mL dose volume lacks a scientific basis and, in conjunction with the conjunctival dosing method, often over-predicts the eye irritancy of a chemical.

Proponents of the 0.1 mL dose volume argue that this dose is a maximized test for the worst case and that it can better predict human eye irritants. While the purpose of the Draize test is to predict what would happen to human eyes within the expected range of exposure, the 0.1 mL dose is out of the range of human exposure. Even though the intent is to maximize the dose that is applied to the rabbit eye, use of 0.1 mL is excessive when you consider the fact that the maximal volume the cul-de-sac of a rabbit's eye can hold is only 30–50 μL [134]. Thus, because the cul-de-sac will not retain more than 50 μL, the remaining amount of test material will simply fall from the eye. Furthermore, the worst case is not necessarily the best case. Constantly estimating the eye irritation potential of test materials will have a desensitizing effect on consumers' and workers' awareness of potential eye irritation, thereby defeating the purpose of testing for eye irritancy in order to protect consumers and workers.

There are no data to substantiate the argument that the 0.1 mL dose can better predict human eye irritants. On the contrary, in at least one survey, there was little correlation between human accidental exposure experience and data generated by the traditional 0.1 mL maximal dose. The survey did not support the general presumption that rabbit eyes are more sensitive than human eyes [28]. Simply reducing the dose volume has produced data closer to eye irritation experienced in humans. For example, comparison of human eye irritation resulting from accidental exposure to many consumer products has revealed that lower-dose volume (0.01 mL) predicts the eye irritancy potential much better than the 0.1 mL dose volume [78,88]. In one of the studies, the time needed for recovery from eye irritation in humans was compared with animal tests in monkeys and rabbits [78]. Results of this study clearly demonstrated that the modified Draize test (Federal Hazardous Substances Act [FHSA] protocol) with a dose volume of 0.1 mL was the poorest predicting test. While all three animal tests over-predicted the eye irritancy experienced in humans, the low dose volume and monkey tests were better than the standard Draize test.

In 1977, a panel on eye irritancy test of the National Academy of Sciences (NAS), formed at the request of the Consumer Product Safety Commission (CPSC), recommended lowering the dose volume [144]. Subsequently, dose volumes ranging from 0.003 to 0.03 mL were proposed because they appeared to predict human eye irritants accurately, cause less pain to animals, and were able to discriminate slight-to-moderate eye irritants [88,90,210]. Williams et al. [210] showed that direct corneal application in a dose volume of 0.01 mL increased the response on the cornea when compared with the standard 0.1 mL dose but did not change the response on the conjunctiva. These results in the absence of compounding effects of a high-dose volume suggest that the lower-dose volume is just as sensitive a method for eye irritancy testing as the higher-dose volume.

Animal Models

As with other toxicological tests in animals, the primary reason for assessing the ocular irritation potential of test articles in animals is their predictability for humans. Recognizing that there are anatomical, physiological, and biochemical differences between human and animal eyes, researchers are confronted with the difficult task of selecting the appropriate animal model and suitable test conditions to identify potential human eye irritants. The corneal thickness of dogs and rhesus monkeys is similar to that of humans (approximately 0.5 mm) [121,125,135], whereas rabbit corneal thickness is somewhat thinner (0.37 mm) [121]. There is a lack of a recognizable Bowman's membrane in rabbits, but they have a well-developed nictitating membrane (an additional target tissue). Rabbits have thick fur around the eyes, loose eyelids susceptible to mild irritants, an ineffective tear drainage system, and a poorly developed blinking mechanism [144]. There are also species differences in biochemistry (e.g., variation in enzyme content [112]), and different penetration rates of various substances [120].

Even though there are shortcomings and exceptions in predictability, the rabbit has been the preferred species for eye irritancy studies. Advantages of using the rabbit include a large established database, relatively inexpensive animal to use, availability, ease of handling, and large, unpigmented eyes suitable for various ophthalmological examinations. With some exceptions [86,174], the rabbit eye is generally more sensitive to irritating materials than human or monkey eyes [7,25]. Thus there are built-in safety factors for making extrapolation and assessment of hazard to humans.

In addition to rabbits, dogs and primates sometimes are used for ocular testing. Eye irritancy in primates generally is more closely correlated with the exposure experience in humans, although dogs also have been shown to be suitable under certain circumstances [10]. Because they are more expensive and less available, dogs and primates are only used occasionally to assess eye irritancy.

Regardless of which animal is used, the investigator should always have a good understanding of the animal eye being observed. Background ocular findings, if not observed prior to exposure, can be recorded falsely as chemically induced damage.

Methods of Exposure

Basically, there are two ways of administering a test article to the eye: (1) instilling the test material into the cul-de-sac of the conjunctiva or (2) applying it directly onto the cornea. Of these methods, the conjunctival exposure procedure has been more frequently used historically because of the ease of application and has been perceived as an accurate method

of dosing. However, some studies [10,88] have shown that conjunctival instillation of the test article is inappropriate under many circumstances, especially when the test article is a solid powder. The possibility exists that a solid test article can become trapped in the conjunctival sac, producing some undesirable mechanical effects that make it difficult to interpret the observed ocular irritation results. It is also known that a considerable amount of the standard 0.1 mL or 0.1 g dose (especially as a solid powder) either falls or is blinked from the eye once the animal's eyelids are released. Based on this evidence, the claim that conjunctival dosing is more accurate than direct application to the cornea may not be valid.

The corneal exposure method, on the other hand, mimics more closely the actual accidental exposure experience in humans. When assessing the hazard of most chemical accidents, this method should be considered except when the chemical is intended for pharmaceutical use [144]. When applicators that had been developed for the corneal exposure method [7,25] were used, a more uniform corneal lesion was observed, resulting in less observation variability [7]. For a study as specific as corneal wound healing, it is recommended that a corneal applicator be used [139]. However, for hazard assessment, it is desirable to apply the test substance directly onto the cornea while the lids of the test eye are gently held open. Immediately afterwards, the eyelids are closed for a second and then released to allow blinking; this action more closely mimics actual human exposure [88].

Irrigation

Washing the eye is a typical emergency remedy after accidental exposure to chemical substances. In experimental studies, the treated eye usually is irrigated 20–30 s after exposure to the test substance. Water is rapidly but gently squeezed from a plastic bottle to produce a constant gentle stream of water irrigating the entire treated eye. Irrigation should last for at least 1 min.

The effect of irrigation on the interpretation of test results has been the subject of many studies [7–9,14,40,77,86,89,165,185]. While irrigation of the treated eye right after exposure can prevent or minimize eye irritation in rabbits, the effectiveness of irrigation is dependent on the chemical, the concentration, the time lag between exposure and initiation of the irrigation, and the volume of irrigation. Early washing (less than 1 min after test article application) generally is recommended to reduce irritation [40,77,89,185]; however, in some cases, irrigation has been shown to increase ocular irritation [82,185]. In other cases, ocular damage was almost instantaneous if irrigation were not initiated within a few seconds [40].

Number of Animals

As a rule the precision of a study increases with the number of animals used. Sometimes, the desired precision may be offset by animal-to-animal variabilities. Economic considerations also are important in determining the number of animals used in a test group. A balance between economic considerations and reliability of test results should determine the number of animals tested in a study.

For eye irritation studies, a group size of nine rabbits was recommended in the original Draize test, and group sizes of at least six, three, three, and four rabbits have been recommended by the FHSA [73], Interagency Regulatory Liaison Group (IRLG) [101], OECD [158], and NAS [144], respectively. The relationship of variability, classification, and group size is addressed in the literature [8,89,206]. With larger group size, smaller variability has been noted [206], whereas with a decreased group size, lesser differentiation of irritancy has been suggested [8]. Recognizing these facts, Guillot et al. [89] suggested that with three rabbits in an initial study, there was a 96% chance that a positive or negative eye irritation result would be obtained. A similar conclusion was reached in another study in which the ocular irritation potential of 67 petroleum products was evaluated using six rabbits per product [43]. The eye irritation scores for the petroleum products based on all six rabbits were compared statistically with the scores using two, three, four, or five animals. The comparison showed that a subsample size of two, three, four, and five rabbits correctly classified (compared with the original six rabbits/test classification) the chemicals at 88%, 93%, 95%, and 96% accuracy, respectively.

Observations and Scoring

Reversibility and severity are the two major criteria used to measure eye irritancy in the Draize test. Reversibility refers to the time needed for the ocular effects to disappear and for the eye to return to its normal state. To determine this reference time, treated eyes are examined periodically at 24 h intervals, on day 7 after exposure, or at longer intervals if needed to establish reversibility [49]. The observation period varies for different guidelines. For example, the FHSA uses 24, 48, and 72 h time spans [73]; the OECD uses 1, 24, 48, and 72 h, and, if needed, extended observations [158]; and the NAS recommends 1, 3, 7, 14, and 21 days [144]. The observation period should be flexible so that one can confidently assess the persistence of ocular effects and fully characterize the degree of involvement, since the onset and healing of ocular effects often are unpredictable [86].

Assessing the severity of different ocular effects is subjective. This subjective evaluation is the major source of error for intra- and interlaboratory variations [205]. Therefore, to minimize at least the intralaboratory variability in scoring, uniformity in scoring techniques must exist among investigators regardless of which scoring system is followed. Pictorial references such as those prepared by the Food and Drug Administration (FDA) [70] and the CPSC [39] can be extremely helpful in the standardization of scoring eye irritation.

The types of ocular effects observed in the Draize test involve the cornea, iris, nictitating membrane, and

TABLE 22.7
Scale of Weighted Scores for Grading the Severity of Ocular Lesions

Lesion	Score[a]
I. Cornea	
A. Opacity—degree of density (area that is most dense is taken for reading)	
Scattered or diffuse area—details of iris clearly visible	1
Easily discernible translucent areas, details of iris clearly visible	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4
B. Area of cornea involved	
One-quarter (or less) but not zero	1
Greater than one-quarter—less than one-half	2
Greater than one-half—less than three-quarters	3
Greater than three-quarters—up to whole area	4
Corneal score equals A × B × 5; total maximum = 80	
II. Iris	
A. Values	
Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)	1
No reaction to light; hemorrhage, gross destruction (any one or all of these)	2
Score equals A × 5; total maximum = 10	
III. Conjunctivae	
A. Redness (refers to palpebral conjunctivae only)	
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
B. Chemosis	
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of the lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
C. Discharge	
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3
Score equals (A + B + C) × 2; total maximum = 20	

Source: Buehler, E.V., Testing to predict potential ocular hazards of household chemical, in *Toxicology Annual*, Winek, C.L., Ed., Marcel Dekker, New York, p. 53, 1974. With permission.

[a] The maximum total score is the sum of all the scores obtained for the cornea, iris, and conjunctivae.

conjunctiva. A system for grading ocular responses (Table 22.7) was originally proposed by Draize et al. [49], subsequently a number of modifications were proposed [39,70,144]. In the Draize system, the intensity and area of involvement on the cornea are graded separately on a scale of 0–4. The product of the two scores is multiplied by 5 to obtain a weighted corneal score. The congestion, swelling, circumcorneal injection, hemorrhage, and iridic failure of reactions to light are graded collectively on a scale of 0–2, and the score is multiplied by 5 to obtain a weighted iridic score. The redness, chemosis, and discharge of the conjunctivae are graded on a scale of 0–3, 0–4, and 0–3, respectively. The sum of the conjunctival scores is then multiplied by 2 to obtain a weighted conjunctival score. Other lesions also are recorded, such as pannus (corneal neovascularization), phlyctena, and rupture of the eyeball.

In the guidelines set forth by the EPA, CPSC, FHSA, OECD, EEC, and Japan's Ministry of Agriculture, Forestry and Food (MAFF) [39,51,61,73,186], only the degree (intensity of cornea damage, iritis, and redness and chemosis [swelling]) of the conjunctivitis is scored (Table 22.8). The area involved on the cornea as well as the discharge of the conjunctiva are not taken into consideration in scoring. Various aids are used at times to facilitate or increase the resolution power of these observations. These aids include fluorescein staining and ophthalmoscopic or slit lamp microscopic examinations. A scoring system has been developed for the slit lamp and fluorescein staining examination [144] (Table 22.9).

TABLE 22.8
Grades for Ocular Lesions

Lesion	Grades
Cornea	
No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible	1[a]
Easily discernible translucent areas, details of iris slightly obscured	2
Nacreous areas, no details of iris visible, size of pupil barely discernible	3
Complete corneal opacity, iris not discernible	4
Iris	
Normal	0
Markedly deepened folds, congestion, swelling, moderate circumcorneal injection (any of these separately or combined); iris still reacting to light sluggish reaction is positive	1[a]
No reaction to light, hemorrhage, gross destruction (any or all of these)	2
Conjunctivae	
Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)	
Vessels normal	0
Some vessels definitely injected	1
Diffuse, crimson red, individual vessels not easily discernible	2[a]
Diffuse, beefy red	3
Chemosis	
No swelling	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2[a]
Swelling of lids about half closed	3
Swelling of lids more than half closed	4

[a] The lowest grade considered positive.

TABLE 22.9
Scoring Criteria for Ocular Effects Observed in Slit Lamp Microscopy

Location of Observations	Grades
Corneal observations	
Intensity	
Only epithelial edema (with only slight stromal edema or without stromal edema)	1
Corneal thickness 1.5× normal	
Corneal thickness 2× normal	2
Cornea entirely opaque so that corneal thickness cannot be determined	3
Area involved	4
≤25% of total corneal surface	
>25% but ≤50%	
>50% but ≤75%	1
>75%	2
Fluorescein staining	3
≤25% of total corneal surface	4
>25% but ≤50%	
>50% but ≤75%	1
>75%	2
Neovascularization and pigment	3
migration	4
≤25% of total corneal surface	
>25% but ≤50%	1
>50% but ≤75%	2
>75%	3
Perforation	4
Maximal corneal score	4
	20
Iridal observations	
Cells in aqueous chamber	
A few	
A moderate number	
Many	1
	2
	3
Iridal observations (continued)	
Aqueous flare (Tyndall effect)	
Slight	1
Moderate	2
Marked	3
Iris hyperemia	
Slight	1
Moderate	2
Marked	3
Pupillary reflex	
Sluggish	1
Absent	2
Maximal iridal score	11
Conjunctival observations	
Hyperemia	
Slight	1
Moderate	2
Marked	3
Chemosis	
Slight	1
Moderate	2
Marked	3
Fluorescein staining	
Slight	1
Moderate	2
Marked	3
Ulceration	
Slight	1
Moderate	2
Marked	3
Maximal conjunctival score	12

Other scoring systems have been proposed for lacrimation, blepharitis, chemosis, injection of conjunctival blood vessels, iritis, kerectasis, and corneal neovascularization [3].

Interpretation of Results

There are essentially four categories of data generated by the Draize test to be considered when interpreting the results of ocular testing: (1) type of ocular effects, (2) severity, (3) reversibility, and (4) rate of incidence. Weighting the scores in the original Draize test has to some extent take the first category into consideration, yet it biases toward the cornea, one of the most critical ocular tissues. Severity is measured according to a graded scoring system, whereas reversibility is expressed as the time needed for the affected ocular tissue to return to the normal state. Incidence is the number of animals that show some kind of ocular effect during the study. Interpretation of the data is a multiple and factorial undertaking. All four categories of data are somewhat interrelated; the individual scores do not represent an absolute standard for the irritancy of a material [156].

In one study, how the eye irritation was interpreted was not considered to be the major factor contributing to interlaboratory variability [205]. This finding is not surprising, if one assumes that everyone adheres to the same interpretation criteria. However, the question is what are the appropriate criteria for interpreting eye irritation results that would have an impact on placing eye irritants into different categories? The individual tissue scores do not represent an absolute standard for the irritancy of a material [157].

Many classification systems for eye irritants have been proposed. Some have been published in the literature [86,89,107,144] and in various testing guidelines [51,73,186], yet many others are used in individual laboratories. There is general agreement among investigators on how to classify test substances when no irritation is observed or when severe irritation or corrosion is seen, but there is little agreement on how to classify irritancy that falls between these two extremes. The manner in which data are evaluated directly affects the conclusions reached.

Because of the complexity of eye irritancy data and their interdependence, some investigators have chosen to simplify the interpretation to a pass-or-fail approach. For example, in the FHSA guideline [73], if four or more of the six test rabbits show ocular effects within 72 h after a conjunctival sac exposure (0.1 mL or 0.1 g of the test material), the test material is considered to be a positive eye irritant. The ocular effects in consideration are "ulceration of the cornea (other than a fine stippling), corneal opacity (other than a slight deepening of the normal luster), inflammation of the iris (other than deepening of folds), an obvious swelling with partial eversion of the lids, or a diffuse crimson red with individual vessels but not easily discernible." If only one of the six animals tested shows ocular effects within 72 h, the test is considered negative. If two or three of the six animals tested shows ocular effects, the test is repeated. The test substance is considered to be a positive irritant if three or more animals show ocular effects in the repeated test; otherwise, the test is repeated. Any positive ocular effect observed in the third test automatically classifies the test substance as an irritant. A similar approach has been adopted in the IRLG guideline [101], but an option is given that declares a test positive when two or three of six rabbits tested show a positive ocular effect and the test is not repeated. The pass-or-fail interpretation is too simplistic, however, and it does not separate eye irritants, especially those that fall between the two extreme irritancy categories (from nonirritating to severely irritating). Gradation of potential eye irritation is important to denote an anticipated hazard and to convey to consumers or workers that a specific degree of precaution should be exercised whenever a potential exposure to the substance exists.

Green et al. [86] used a different approach in which eye irritancy was classified into four easily recognizable categories based on the most severe responder in a group. The four categories are as follows.

Nonirritation: Exposure of the eye to the test article under the specified conditions causes no significant ocular changes. No tissue staining with fluorescein was observed. Any changes that did occur cleared within 24 h and were no greater than those caused by normal saline under the same conditions.

Irritation: Exposure of the eye to the test article under the specified conditions causes minor, superficial, and transient changes of the cornea, iris, or conjunctiva as determined by external or slit lamp examination with fluorescein staining. The appearance at any grading interval of any of the following changes was sufficient to characterize a response as an irritation: opacity of the cornea (other than a slight dulling of the normal luster), hyperemia of the iris, or swelling of the conjunctiva. Any changes cleared within 7 days.

Harmfulness: Exposure of the eye to the test article under specified conditions causes significant injury to the eye, such as loss of the corneal epithelium, corneal opacity, iritis (other than a slight infection), conjunctivitis, pannus, or bullae. The effect healed or cleared within 21 days.

Corrosion: Exposure of the eye to the test article under specified conditions results in the types of injury described in the previous category and also results in significant tissue destruction (necrosis) or injuries that adversely affect the visual process. Injuries persisted for 21 days or more.

This classification system took into consideration the nature of ocular effects, reversibility of those effects, and, to a certain extent, the qualitative severity, but not the incidence. The NAS committee that revised NAS publication 1138 [144] proposed a system of classification similar to that of Green et al. [86] even though the categories were named differently: inconsequential or complete lack of irritation, moderate irritation, substantial irritation, and severe or

corrosive irritation. The NAS classification was also based on the most severe responder, and incidence was not considered. A provision for repeating the test was given as an option to increase the confidence level in making a judgment in some borderline cases. This eye irritancy classification system has been widely adopted. One shortcoming of the NAS system was that too wide a spectrum was created for moderate irritancy, which may lead to overutilization of the cautionary term *moderate*. Many investigators have experienced problems in interpreting results from fluorescein staining of the cornea when the NAS gradation system is used. The confusion arises mainly from the occasional artifacts inherent in fluorescein staining. Experience and sound scientific judgment are needed to properly interpret the fluorescein staining results (see the discussion on ophthalmological techniques).

Griffith et al. [88] disagreed with using the most severe responder for classification of eye irritancy, claiming that there was no epidemiological evidence to suggest that the most severe rabbit responder would correlate with the worst possible case of human exposure. Instead, these investigators used the median time for recovery for classification according to the same temporal criteria as in the NAS system. The underlying logic is that the incidence of responders is being considered indirectly.

The classification systems of Green et al. [86], Griffith et al. [88], and NAS [144] have not taken into account the severity of irritancy, although there is a perception of a direct relationship between severity and reversibility. Examining the data of Griffith et al. [88] supports the conclusion that there is a direct correlation between median time to recovery and the severity of irritancy.

Kay and Calandra [107] proposed yet another rating system based on the Draize scores, taking into account the extent and persistence of irritation and the overall consistency of the data. The Kay and Calandra system has not been verified for correlation to human exposure experience, nor has it been compared with other classification systems.

Guillot et al. [89] proposed a scoring system in which the greatest mean irritation score within an observation period is identified. On the basis of this score, the test substance is classified into six categories, ranging from nonirritating to maximum or extremely irritating. In order to maintain this initial rating, the data also must meet the arbitrary criteria for reversibility and frequency of occurrence; otherwise, the rating is upgraded one category. Guillot et al. [89] did attempt to compare their rating with the OECD protocol and claimed that one-third of the 56 materials tested could be classified into a lower category by the OECD protocol.

The most current modification of the OECD protocol [158] is an effort to minimize the number of animals used to produce data suitable for hazard classification. In this simplified scheme, a Draize eye test is conducted using one animal if severe effects are expected, or three animals if no severe eye irritation is anticipated. Scoring is based on ocular lesions that occur within 72 h of exposure and results are expressed in terms of the lesions and their reversibility (eye irritation) or irreversibility (eye corrosion). The EPA has revised its health effects test guidelines for acute eye irritation [65] to be more consistent with the OECD protocol. A revised EEC directive, based on the OECD approach, provides hazard classification corresponding to risk phrases (R36—Irritating to eyes and R41—Risk of serious damage to eyes). These risk phrases are assigned to the label of a chemical when two or more of the three animals exhibit scores within certain arbitrary numbers [52].

A summary of the current international classification systems and major features for eye irritancy testing is shown in Table 22.10. Despite such a range of classification schemes, there is little difference in the actual scoring system (basically adhering to the original Draize) [49].

SPECIAL OPHTHALMOLOGICAL TECHNIQUES

The Draize test is a generalized test concentrating on the effects of the material on the cornea, iris, and conjunctiva. Examination usually is performed using a hand light. Accurate observations are limited by the experience and training of the investigator and it is possible for subtle ocular changes to be missed. If subtle ocular changes are to be detected and ambiguous gross observations resolved, or if internal tissues (e.g., the lens and the retina) are to be examined, the investigator must rely on special techniques. Many such techniques have been developed over the years, most of which are more objective than the gross examination itself. A few comments on the fluorescein staining technique and several of the more objective methods are presented.

Fluorescein Staining for Corneal Damage

Fluorescein is a weak organic acid (Figure 22.5) that is only slightly soluble in water, but its sodium salt is moderately soluble in water. It is very efficient in absorbing ultraviolet light and emitting fluorescent light. The maximum absorption of fluorescein is 490 nm (excitation) in the violet region and its maximum emission is 520 nm in the green region of the spectrum. Its nonionized form is less fluorescent than its ionized form. At pH 7.4, fluorescein does not appear to bind to tissue and is nontoxic in animals, making it an ideal marker for an ocular fluid dynamics study. Because fluorescein is a deeply colored and highly fluorescent chemical, it can be detected at very low concentrations in biological tissues or fluid; however, its detection sensitivity often is limited by the background fluorescence of biological tissues.

Because sodium fluorescein is a polar molecule, it can easily diffuse into aqueous medium and does not readily traverse lipophilic membranes. For example, if ulceration occurs on the cornea, the lipophilic membrane barrier is compromised and the fluorescein diffuses freely through the ulcerated area of the cornea and either is dissolved or suspended in the aqueous medium of the stroma. More detailed information

TABLE 22.10
Major Features of Eye Irritation Tests and International Classification Schemes

Methodology	FHSA (CPSC FDA OSHA)	OECD	EPA (Modified OECD)	Canada (Modified OECD)	European Union (EEC)
Initial considerations					
Screen for pH (<2 or >11.5)	NS	Yes	Same as OECD	Same as OECD	Same as OECD
Results from skin irritation	NS	Yes	Same as OECD	Same as OECD	Same as OECD
Number of animals:					
Screen for severe effects	NS	1	Same as OECD	Same as OECD	Same as OECD
Main test	≥6	≥3	Same as OECD	Same as OECD	3
Volume administered	0.1 mL or 100 mg	0.1 mL or ≤100 mg	Same as OECD	Same as OECD	Same as OECD
Scoring times	1, 2, 3 days	1 h, 1, 2, 3 days (may be extended to assess reversibility)	1 h, 1, 2, 3 days (may be extended to assess reversibility ≤21 days)	1 h, 1, 2, 3 days (may be extended to assess reversibility)	1 h; 1, 2, 3 days
Minimal positive response:					
Corneal opacity	1	NS[a]	1	2.0[b]	≥2.0, <3.0[c]
Iritis	1	NS[a]	1	1.0[b]	≥1.0, <1.5[c]
Conjuctival					
Redness	2	NS[a]	2	2.5[b]	≥2.5[c]
Chemosis	2	NS[a]	2	2.5[b]	≥2.0[c]
Positive test	≥4 positive of 6 animals	NS	NS[a]		≥2 positive of 3 animals
Label categories:					
Irritant	Reversible inflammatory effect	Same as FHSA	Same as FHSA	Positive response requires labeling as a poisonous and infectious material	R 36
Severe irritant	NS	NS	NS		R 41
Corrosive	Visible destruction or irreversible alterations	Same as FHSA	Same as FHSA	NS	NS

Note: NS, not specified.
[a] Individual scores do not represent an absolute standard for the irritant properties of a material.
[b] Mean of at least three animals.
[c] Mean of three scoring intervals and scores representing two or more animals.

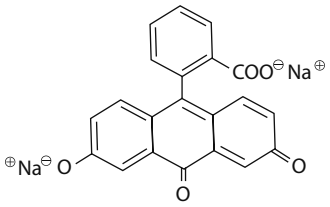

FIGURE 22.5 Structure of fluorescein.

on the chemical and biological properties of fluorescein is provided in two excellent reviews [124,136].

Since its first use in studying the origin of aqueous humor secretion more than a century ago [55], fluorescein has become an important aid in ophthalmology. It has been used as a marker in detecting obstructions in the nasolacrimal drainage systems, for studying changes in the flow dynamics of different ocular fluids, for demonstrating leakage of retinal vessels in angiography, for estimating permeability of the cornea and lens, and for identifying ulcerations on the cornea [124]. Among these uses, fluorescein's ability to detect subtle changes on the corneal epithelium [36,98] has been a routine procedure in animal eye irritation studies.

An intact corneal epithelium is a lipophilic barrier to sodium fluorescein, but when the barrier is damaged and an ulceration or change in membrane structure occurs, some of the fluorescein added to cornea will penetrate into the intercellular aqueous spaces of the stroma. When light is cast on the cornea, fluorescence is detected in the damaged area of the epithelium. Once fluorescein enters the stroma, it eventually passes through Descemet's membrane and the endothelium into the aqueous humor.

Staining is usually performed using either a prepared solution of fluorescein or fluorescein-impregnated paper strips. Commercially available solutions contain 0.25%, 1.0%, or 2.0% fluorescein sodium salt. These solutions will contain preservatives that act to minimize bacterial contamination [29]. A drop of the solution is instilled onto the eye

with excessive fluorescein being flushed immediately with a sufficient amount of water. The eye is then examined under a cobalt-filtered UV light for epithelial defects. Fluorescein-impregnated paper strips [111] are free of contamination and easy to use. Moistened with collyria (medicated eye lotion), a strip is touched lightly to the dorsal bulbar conjunctiva. The small amount of fluorescein should distribute uniformly on the cornea by either diffusion or blinking. Flushing is not usually necessary if the strips are applied properly. Nonetheless, if the strip touches the cornea, it becomes necessary for the cornea to be flushed with water before examination. Better results are obtained with the fluorescein-impregnated strip when examination is by slit lamp microscopy.

Fluorescein staining has two valuable applications in a routine eye irritation test: It can be used to screen eyes prior to the study so as to ensure that only healthy eyes are used and to evaluate the cornea's recovery from grossly observed damage. Slight epithelial effects still can be detected by fluorescein even though they are not visible during gross observation. While most of these subtle effects on the cornea will disappear in a relatively short period of time, prolonged effects detected by fluorescein staining, but not by gross examination, should raise a concern over the healing process. However, when no gross lesions are detected at any time during a study (except for a few incidences of minor fluorescein staining on the cornea), one should not be overly concerned. If there are any effects on the cornea, they must be extremely minimal ones on the superficial epithelium for eye irritation to rate as nonirritating or inconsequential. If the staining is not an artifact, the minimal ocular effects detected under such circumstances should be readily reversible.

Although fluorescein staining can detect very subtle corneal epithelial changes, significant background staining can alter the interpretation of the actual amount of damage present because of an increase in the number of artifacts present. Apparent staining of the cornea can also result from incomplete flushing of excessive fluorescein with water or even from reflected light. A strong jet of water during irrigation can cause mild damage to the cornea. Damage also can occur if the eye is not handled properly during gross examination. These changes are not related to the test article but may be detected with fluorescein staining. Sometimes, fluorescein staining can cause haziness on the cornea even though a clear cornea is seen prior to fluorescein staining. Whether the hazy appearance of the cornea is a reflection of mild change or artifact depends on several factors. If the hazy appearance also is visible under a cobalt filter and is preceded by grossly visible lesions, it generally is considered to be a residual effect of mild severity that will disappear within a short time. However, if the hazy appearance is seen intermittently or is not preceded by ocular effects, it is likely an artifact. Proper training and experience are necessary to recognize artifacts and to obtain reliable, reproducible, and consistent results from fluorescein staining. In general, it is not necessary to stain lesions that are obvious and grossly evident. It is when lesions would otherwise go undetected by gross examination that fluorescein staining is of value.

Slit Lamp Microscopy

The slit lamp biomicroscope is an important instrument for studying ocular tissues, especially the cornea. As its name suggests, a slit lamp consists of a microscope that views optical sections of different layers of the cornea made by an intense light beam acting as a surgical knife or microtome cutting through different layers of the eye. Many lesions that would remain undetected by gross examination can be observed with the slit lamp biomicroscope. Using recent models of slit lamp microscopes, one not only can observe the different layers of the cornea but also can examine other transparent parts of the eye such as the aqueous humor, lens, and vitreous body.

The slit lamp biomicroscope consists of an illuminating light source and a microscope. Both components are movable and adjustable, allowing the eye to be illuminated and observed from different angles and with different width and height adjustments of the slit light beam. An area of the cornea can simultaneously be illuminated and magnified by aligning the incidence of the light beam and the focus of the microscope. The light beam also can be directed at the area from different angles, providing several views of the same area.

Two types of slit images are used for illumination: parallelepiped and optical section [127]. For the parallelepiped slit image, a rectangular light beam (approximately 1–2 mm wide and 5–10 mm high) is projected onto the cornea. The shape of the illuminated area is similar to a parallelepiped prism where the outer and inner surfaces are bent because of the shape of the cornea. For the optical section slit image, the width (20 μm) of the light beam is narrowed to its minimum and is projected onto the cornea, providing a sagittal view that is similar to a thin histological section.

There are several basic illumination techniques (Figure 22.6): diffuse illumination, sclerotic scatter illumination, direct and indirect focal illumination, direct and indirect retroillumination, and specular reflection [135,190].

Diffuse Illumination

In diffuse illumination, a slightly out-of-focus wide beam is used to scan and localize any gross lesions of a large area of the eye. Usually, the first step in examining the eye under a microscope is for gross lesions and their extent of change. This technique is similar to observing the eye with a hand light, except that the observation is made under a microscope (Figure 22.6a).

Sclerotic Scatter Illumination

In sclerotic scatter illumination (Figure 22.6b), a narrow light beam is directed at the temporal limbus, and the microscope is focused centrally on the area of the cornea to be examined. The light reflected from the sclera will transmit within the cornea by total reflection. Under normal conditions nothing will be seen, but if even minor changes are present the reflected light will be obstructed and the damaged area (e.g., mild corneal edema) will be illuminated. This technique is useful for detecting minimal changes in the cornea.

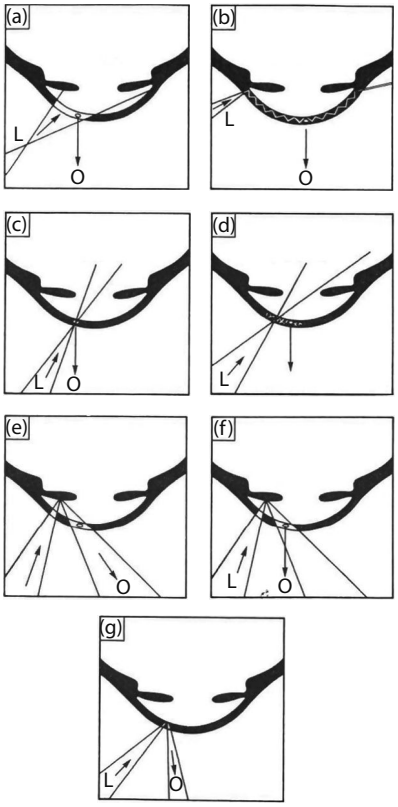

FIGURE 22.6 Seven basic methods of illumination in slit lamp microscopy: (a) diffuse, (b) sclerotic scatter, (c) direct focal, (d) indirect focal, (e) direct retroillumination, (f) indirect retroillumination, and (g) specular reflection. O = observer; L = illuminator light. (Modified from McDonald, T.O. et al., *J. Soc. Cosmet. Chem.*, 24, 163, 1973. With permission.)

Direct Focal Illumination

In direct focal illumination, the light beam and the microscope are focused sharply at the same point of interest in the same plane (Figure 22.6c). If a rectangular slit image is used for illumination and focused on the cornea, three general areas are seen when the parallelepiped is formed on the cornea: the epithelium (anterior bright line), the stroma (central clear marble-like area), and the endothelium (posterior thin bright line). If an optical section slit image is used for illumination, the corneal layers seen from anterior to posterior are a thin bridge layer, a thin dark layer, a granular layer, and another thin bright layer. These correspond to the tear film, the epithelium, the stroma, and the endothelium, respectively. Altering the angle of incidence of the light beam decreases or increases the reflection. This allows for the detection of depth of the lesion. Opacities on the different layers can be detected easily as obstructions of the incident light beam.

Indirect Focal Illumination

Indirect focal illumination (Figure 22.6d) is accomplished by a narrow beam of light directed at an opaque area of the cornea. For example, changes in blood vessels at the cornea adjacent to the opaque area are illuminated and can be detected by focusing the microscope at these areas.

Direct and Indirect Retroillumination

In direct (Figure 22.6e) and indirect (Figure 22.6f) retroillumination, the light beam is directed at tissues behind the cornea, for example, the iris or the fundus. The reflected light illuminates the area of interest of the corneal tissue and can be focused under the microscope. The microscope can be located directly on the path of the reflected light (direct retroillumination), thus permitting subtle changes to be observed against a contrasting background. Any optical obstruction by lesions such as scars, pigment, or vessels located along the reflection light path will appear as darker areas on a brighter background. Lesions such as corneal edema and precipitates that can scatter the reflection light will show up as a brighter area against a darker background. When the microscope is located off the reflection light path (indirect retroillumination), the corneal structure is observed against a dark background such as the pupil or iris. Indirect retroillumination is better for observing opaque structures, whereas direct illumination often is used to detect corneal edema and precipitates.

Specular Reflection Illumination

Specular reflection (Figure 22.6g) is most useful in studying the endothelium and precorneal tear film. This technique makes use of the difference in refractive properties between the corneal surface and the adjacent medium of the posterior and anterior surfaces of the cornea. The microscope is focused on the cornea adjacent to the path of the incident slit light beam. By alternating the angle of incidence, a point can be reached such that a total reflection is obtained on the junction between the aqueous medium and the most posterior corneal surface, thus illuminating endothelial cell patterns and Descemet's membrane. Similar techniques can be performed on the anterior corneal surface to visualize precorneal tear film.

Scoring System for Slit Lamp Examinations

By using slit lamp microscopic techniques, many subtle changes can be observed that would not otherwise be evident from the Draize test. A different scoring system must be developed to reflect such subtle changes. Baldwin et al. [2] proposed a scoring system for the cornea, anterior chamber, iris, and lens. Subsequently, the NAS [144] developed a scoring system for slit lamp examinations that is similar to the Draize system in which an emphasis was placed on changes occurring in the cornea, iris, and conjunctiva. In the NAS system, the intensity and area involved are the two main criteria for scoring. Using this scoring system, the investigator must have a good understanding of the physiology of the normal eye. Like the Draize score, the NAS system is based on corneal effects; total maximal corneal score is 20 as compared with 11 and 15 for iridic and conjunctival scores, respectively. A detailed scoring scale and criteria are listed in Table 22.9.

Corneal Pachymetry

Since corneal transparency is so important to vision (over 70% of the Draize score is derived from assessment of damage to the cornea), objective procedures to quantify corneal effects

are an important element in eliminating intra- and interlaboratory variabilities in assessing the effects of ocular exposure to exogenous agents. Corneal transparency, thickness, and hydration are related in a linear fashion [94]. Therefore, changes in corneal thickness can be used as an indicator of irritant affects, which may impair normal corneal hydration and transparency. When corneal thickness is measured appropriately, it can be used to objectively quantify swelling of the corneal stroma, which is a typical early irritant response. The measurement of corneal thickness is called pachymetry, which comes from the Greek words *pachys* (thick) and *metry* (the process of measuring). One method for measuring corneal thickness uses an optical pachometer attached to a slit lamp microscope. Optical pachometers provide essentially indirect measurements of apparent corneal thickness based on displacement of light beams bouncing off the endothelial and epithelial surfaces of the cornea. The principles of this method have been previously described [31].

Various investigators [27,37,109,138] have reported that corneal thickness is significantly correlated with the Draize corneal score using a variety of substances from different chemical classes. Moreover, Kennah et al. [109] clearly demonstrated a substantial reduction in the coefficient of variation when comparing corneal swelling to the Draize scores for various surfactants, alcohols, ketones, acetates, and aromatic chemicals.

Recent advances in human ophthalmological procedures to correct visual acuity (i.e., radial keratotomy, eximer-laser photorefractive keratectomy) have resulted in the development of improved devices to measure corneal thickness, which both guide the practitioner before and after the procedure, and provide a means to measure the effectiveness of the treatment. The ultrasonic pachymeter is such a device [13,142,193,200] and it may have useful application to in vivo ocular irritation testing.

The ultrasonic pachymeter is an instrument with a handheld probe that emits an ultrasonic signal of fixed velocity. The probe is placed directly on the anterior surface of the cornea and after signal emission; a sensor directly measures the time difference between echoes of signal pulses reflected from the front and back surfaces of the cornea. This time differential is directly proportional to the thickness of the cornea via a function that is computed as the product of the time delay between the two echoes (in seconds) and the velocity of sound in the corneal tissue (in meters/second). Whereas the optical pachometer indirectly equates displacement of incident light to corneal thickness, the ultrasonic pachymeter provides a direct measurement.

Comparative evaluations of the sources of variability in human corneal thickness measurements using optical and ultrasonic [83,97,114,166,180] or between different ultrasonic devices [207] have been reported and discussed. Salz et al. [180] found that sources of variation include intra- and intersession variations, interobserver variation, left/right eye variation, and variations due to alternate settings of ultrasonic sound frequency. They reported that the optical pachymeter had significant intersession variation, significant interobserver variation, and significant differences in left and right eye thickness measurements, whereas the ultrasonic pachymeter demonstrated high reproducibility, no interobserver variation, and no left/right eye variation.

The ultrasonic pachymeter has many desirable features such as relatively low cost, portability, ease of operation, and it requires less operator skill and training than the optical pachometer. When used in humans a topical anesthetic is employed since the tip of the measuring probe must be in contact with the corneal surface before a measurement can be taken. However, it has been reported that because of a lower corneal sensitivity in rabbits [33,131], an anesthetic is not necessary before taking corneal thickness measurements.

Since the velocity of sound can vary in different tissue, accurate readings for absolute corneal thickness require that the ultrasonic sound frequency of the instrument be matched to the tissue of interest. The velocity of sound in human corneal tissue has been variously reported as 1502 m/s [143], 1586 m/s [175], and 1610 m/s [146]. Salz et al. [180] in their human cornea comparison of optical to ultrasonic pachymetry used an approximate velocity of 1590 m/s and found good agreement between the two measurement methods. The velocity of sound in cat [114], rabbit [32], and bovine [164] corneal tissue was found to be 1590, 1580, and 1550 m/s, respectively. Empirical methods to determine the velocity of sound in corneal tissue have been described [114,164].

The utility of ultrasonic pachymetry in measuring corneal thickness changes in rabbits [119,141] and rats [119] after treatment with ocular irritants has been reported. The findings, albeit limited to a small number of chemicals, support the continued pursuit of this method as a relatively inexpensive, objective way to measure corneal irritant effects.

Confocal Microscopy

The confocal microscope is another instrument that can be used to measure corneal thickness, as well as provide high-resolution microscopic images to study the cellular structure within corneal tissue. The first confocal microscope was described by Minsky [132,133] in a 1957 patent application. This device had a pinhole and a lens (objective and condenser) located on either side of the specimen to be viewed. The intent of the design was to eliminate any scattered light that might pass through the specimen, thus concentrating all light at a point source that was the focal point. The term confocal originated because the objective lens and the condenser lens were focused on the same specimen point.

Whereas the image seen in a conventional light microscope includes the in-focus image in the x,y (horizontal) plane and the out-of-focus image above and below in the z (vertical) plane, the confocal microscope only focuses in the x,y plane. Indeed, de-focusing a confocal microscope makes the image totally disappear rather than appear blurred. Reducing the out-of-focus signal above and below the focal plane results in enhanced resolution. In contrast to the light microscope, which is focused by moving the objective, moving the specimen focuses the confocal microscope. This feature provides an

optical sectioning capability that allows thick tissue sections such as the cornea to be viewed in vivo or in vitro in both the horizontal and vertical planes. Because of the point source light illumination, however, scanning the specimen is necessary in order to produce a full field of view with the confocal microscope. Scanned images can be viewed through a video monitor on a real-time basis, imported into a videocassette recorder, or stored as a digital image [168] for later viewing and analysis. For a complete review of the principles and applications of the scanning confocal microscope, see Petroll et al. [169].

By successively scanning the cornea and capturing a series of optical sections, it is possible to reconstruct a three-dimensional (3D) image of the tissue. Methods for 3D imaging of rabbit cornea in vitro [104,122,170] and in vivo [69,104,137,167] have been described. These methods have been used to characterize the changes in area and depth of corneal injury of surfactant-induced eye irritation in the rabbit [123] and to examine the relationship between area and depth of injury to corneal cell death [103].

Mauer et al. [123] used in vivo scanning confocal microscopy to qualitatively and quantitatively characterize the initial changes occurring after treatment with surfactants known to produce slight, mild, moderate, and severe corneal irritation. Materials were applied directly to the corneas of six rabbits per group at a dose of 10 μL with macroscopic (Draize) and microscopic evaluations beginning at 3 h after treatment and continuing periodically through day 35. Microscopic 3D images were obtained from the surface epithelium to the endothelium and measurements were made for surface epithelial cell size, epithelial layer thickness, total corneal thickness, and depth of keratocyte necrosis. The average Draize scores at 3 h for the slight, mild, moderate, and severe irritants were 6.0, 39.3, 48.5, and 68.7, respectively. Confocal microscopic images at 3 h showed that corneal injury with the slight irritant was limited to the epithelium (cell size and thickness 59% and 82% of control). The mild irritant had removed the surface epithelium, increased the corneal thickness to 158% of control, and produced keratocyte necrosis to a depth of 4.3 μm. With the moderate irritant, the epithelium was markedly attenuated, the corneal thickness was 156% of controls, and keratocyte necrosis extended to a depth of 19 μm. For the severe irritant, the epithelium was significantly thinned, the corneal thickness was 166% of controls, and keratocyte necrosis extended to a depth of 391 μm.

The use of confocal microscopy in studies designed to provide semiquantitative information on the nature and depth of injury to the cornea after chemical treatment has the potential to serve as an important link to the development of physiologically relevant and mechanistically based in vitro alternatives to the Draize eye test [123].

LOCAL ANESTHETICS

For humane and scientific reasons, guidelines such as those established by the IRLG [101] and the OECD [158] provide options for using local anesthetics in eye irritation studies. Tetracaine, lidocaine, butacaine, proparacaine, and cocaine have all been tested for their eye irritation, with the results being mixed and inconclusive. While most of these anesthetics can alleviate pain, they also can inhibit or reduce the somatosensory area of the eye and the blinking reflex. Tear flow is reduced causing the test substance to be trapped and remain undiluted on the cornea instead of being blinked from the eye or diluted and flushed away by the tear flow. The blinking and tearing reflexes are important defense mechanisms, especially among higher primates, to accidental exposure to any substance [96]. Some local anesthetics can cause delay in corneal epithelial regeneration and loss of surface cells from the cornea [90]. Some local anesthetics such as procaine, lignocaine, piperocaine, amylocaine, amethocaine, and cinchocaine are cytotoxic to cultured human cells including conjunctival cells [41]. However, at least one study has shown that a 0.5% tetracaine solution apparently had no effect on corneal healing [171]. Further research is needed to reveal the interaction of local anesthetics and chemically induced ocular effects. Local anesthesia is sometimes useful to induce akinesia of the eyelid during eye examination.

Local anesthetics are desirable to alleviate pain, but one must be aware of the potential physical, chemical, physiological, and toxicological incompatibilities before considering the use of local anesthetics.

HISTOLOGICAL APPROACHES

Histological examination of the eyes has been included routinely in subchronic and chronic toxicity studies, but because it is time consuming and costly, it is performed only occasionally in eye irritation studies. Results may be no more informative than those from observations and measurements by other techniques. However, histological examination of ocular tissue can reveal the type of damage, tissues involved, and certain subtle changes in ocular tissue.

Both electron and light microscopic examinations have been used to evaluate local ocular injury [86,105,106,176,192,195,201, see Ref. 95 for review]. Although these methods sometimes can reveal morphological changes of different parts of the cornea, conjunctiva, lens, and retina, as well as visual nerve degeneration, there are shortcomings with electron and light microscopy. Issues with using histological techniques to examine damage include, being able to section the precise lesion, problems in slide preparation and subjective interpretation of observations. Another problem is that histological examination generally is made on dehydrated tissue [26], which makes some lesions, such as corneal edema, difficult to detect. However, histological examination of ocular tissues in local eye irritation studies has been considered an objective method because of its high sensitivity in detecting very mild ocular effects [96].

Protocol Refinement

Since it is generally agreed that in vitro techniques will not replace animal testing immediately, efforts should be made to reduce the number of animals used and to refine the study

design in order to minimize pain. Since precision of an eye irritancy test is a function of the number of animals used, the question arises as to whether it is justified to use a large number of animals to increase precision? The answer is no, since there is seldom an advantage to testing eye irritancy with more than three to six animals. The largest variable in an eye irritancy test is among animals, and the test itself is designed to be a bioassay. Therefore, to use a large number of animals in hope of achieving a higher level of precision is neither realistic nor scientifically sound. A statistical analysis of 155 Draize irritancy studies with six-rabbit scores has shown that reducing the number of animals to five-, four-, three-, or two-animal scores retains a 98%, 96%, 94%, or 91% agreement, respectively, with an irritant classification of these chemicals based on the six-rabbit scores [191]. The correlation coefficients for randomly selected subsets of five, four, three, or two scores were 0.998, 0.996, 0.992, and 0.984, respectively. The results of this study show that sufficient accuracy can be obtained by reducing the number of animals used in the Draize test. A combination of lower test substance dose volume (one-tenth the Draize test dose volume) and fewer animals (three) also has yielded good correlation with the standard Draize test [23].

Another proposal is to test only for skin irritation. If the material causes severe skin irritation, it is presumed to be severely irritating to the eye as well. Thus, the argument concludes, an eye irritation test is not needed. Extrapolation from skin to eye is not always valid. In at least one study of 60 severe skin irritants, only 39 also caused severe eye irritation, 15 caused mild or no ocular effects, and the other 6 caused moderate eye irritation [208,209]. Nonetheless, this approach has been proposed as one element of a tier system to prevent conducting an eye irritancy test when other potentially relevant information is available [102].

Many company guidelines specify that materials with extremely high or low pH do not need to be tested for eye irritancy. This approach is fully justified, especially for highly basic compounds. Alkali compounds generally have a higher potential of causing severe eye irritation than acidic compounds.

ALTERNATIVE ACUTE AND EYE IRRITANCY TESTS

The issue of pain and suffering of animals traditionally used in toxicity tests has long been recognized, and after decades of advocate by animal right organizations, both industry and regulatory authorities around the world have embraced the need for minimizing animal pain and suffering in their strategy for conducting toxicity tests, and regulating chemicals. The general strategy to minimize pain and suffering of animals used for toxicity testing is through (1) using a tier approach to guide the need for, and the way of, conducting animal toxicity studies; (2) refining test protocol and evaluation endpoints to minimize the pain and suffering of animals; (3) reducing the number of animals used in the test to achieve the same testing objectives; and (4) replacing whole animals tests with tests conducted with phylogenetically lower species of animals such as insects, or with nonanimals systems such as cell cultures, reconstructed tissues, or isolated animal or human tissues and organs. To be accepted for regulatory purposes, these alternative approaches and systems must be validated in terms of their scientific rationales, predictability, and consistency. Since the founding of the Fund for Replacement of Animals in Medical Experiments (FRAME) in the United Kingdom in 1969, significant advances have been accomplished toward achieving the "3 Rs" of replacement of animals; reduction in the numbers of animals used; and refinement of techniques to alleviate or minimize potential pain, distress, and/or suffering. Over the last several decades, a number of alternative methods have been validated and implemented by regulatory bodies. These advances have been made through the coordinated efforts of organizations such as the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) in the United States; the European Centre for the Validation of Alternative Methods (ECVAM), the ECVAM Scientific Advisory Committee (ESAC), and the German Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) in Europe; the Japanese Centre for the Validation of Alternative Methods (JaCVAM); the Korean Centre for the Validation of Alternative Methods (KCVAM); and by international consensus-driven bodies such as the OECD, ICH (*International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use*), VICH (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products), and International Cooperation on Alternative Test Methods (ICATM). A list of validated alternative methods for acute toxicity, eye corrosion/irritation, skin sensitization, and phototoxicity is found in Table 22.11.

This section discusses alternative toxicity test methods for acute toxicity and eye irritancy. Readers are referred to Chapter 20 for further discussions on other alternative test methods for other toxicity.

Alternative Test Methods for Acute Toxicity

Traditionally, acute toxicity has been expressed as the LD_{50}, a statistical estimate of the acute lethality of an agent. The objective of conducting such studies, however, should not be limited to establishing an LD_{50} value for the substance. The LD_{50} is a historically chosen reference point for defining the lethal poisoning potential of a substance. More importantly, acute toxicity studies should define the substance's dose–response relationship of all toxicities.

Nonetheless, the LD_{50} value provides a measure of the relative toxicity of an unknown agent compared with other agents administered by the same route to the same species, strain, age, and sex of the animal. The LD_{50} value, an indicator of lethal potency, is frequently the first safety test for a new chemical, the agent being administered via the route by which humans might be exposed, and animal mortality

TABLE 22.11
List of Validated and Regulatory Accepted Alternative Toxicity Tests (1998–2011)

Method/Test	United States	OECD TG/ISO/EU
Acute toxicity		
Up-and-down procedure for acute oral toxicity	OECD TG 425 accepted in 2003	TG 425 (2001)
Fixed-dose procedure for acute oral toxicity	OECD TG 420 accepted in 2001	TG 420 (2001)
Acute toxic class method for acute oral toxicity	OECD TG 423 accepted in 2001	TG 423 (2001)
Acute toxicity in vitro starting dose procedure, 3T3 cells	Accepted in 2008	GD 129 (2010)
Acute toxicity in vitro starting dose procedure, NHK cells	Accepted in 2008	GD 129 (2010)
Inhalation toxicity—acute toxic class method	OECD TG 436 accepted in 2009	TG 436 (2009)
Acute skin irritation/corrosion		
EPISKIN™ in vitro human skin model skin corrosivity test	OECD TG 431 accepted in 2004	TG 431 (2004)
EpiDerm™ in vitro human skin model skin corrosivity test	OECD TG 431 accepted in 2004	TG 431 (2004)
SkinEthic™ in vitro human skin model skin corrosivity test	OECD TG 431 (meets performance standards in 2006)	TG 431 (2004)
Rat transcutaneous electrical resistance (TER) test in vitro skin corrosivity test	OECD TG 430 accepted in 2004	TG 430 (2004)
EST-1000 method for skin corrosivity testing	OECD TG 431 (meets performance standards in 2009)	TG 431 (2004)
EPISKIN™ in vitro human skin model skin irritation test	OECD TG 439 accepted in 2010	TG 439 (2010)
EpiDerm™ in vitro human skin model skin irritation test	OECD TG 439 accepted in 2010	TG 439 (2010)
SkinEthic™ in vitro human skin model skin irritation test	OECD TG 439 accepted in 2010	TG 439 (2010)
Acute eye irritation/corrosion		
BCOP test method to identify severe eye irritants/corrosives	Accepted in 2008	TG 437 (2009)
ICE test method to identify severe eye irritants/corrosives	Accepted in 2008	TG 438 (2009)
Cytosensor Microphysiometer® test method for eye safety testing	Accepted in 2011	New TG public comment (2011)
Use of anesthetics, analgesics, and humane endpoints for in vivo ocular safety testing	Accepted in 2011	New TG public comment (2011)
FL test method for identifying ocular corrosives and severe irritants		New TG public comment (2011)
Skin sensitization test guidelines		
Murine local lymph node assay (LLNA) for skin sensitization	Accepted in 1999; as OECD TG 429 in 2002	TG 429 (2002); ISO (2002)
Updated LLNA protocol (requires 20% fewer animals)	Updated TG 429 accepted in 2010	TG 429 (2010)
Reduced LLNA protocol (requires 40% fewer animals by using only the high-dose group)	Updated TG 429 accepted in 2010	TG 429 (2010)
LLNA: DA for skin sensitization testing (a nonradioisotopic LLNA test method)	OECD TG 442A accepted in 2010	TG 442A (2010)
LLNA: BrdU-ELISA for skin sensitization testing (a nonradioisotopic LLNA test method)	OECD TG 442B accepted in 2010	TG 442B (2010)
Phototoxicity test guideline		
3T3 NRU phototoxicity test for skin photo-irritation	OECD TG 432 accepted in 2004	TG 432 (2004)
3T3 NRU phototoxicity test: application to UV filter chemicals	OECD TG 432 accepted in 2004	TG 432 (2004)

assessed in the 24 h period after treatment. Given that people might acquire the chemical by different routes, it may be necessary to carry out two experiments, choosing two of the three possible routes (ingestion, inhalation, and dermal) of administration in anticipation of quite different values. Although accurate determinations of the lethal potency are no longer required, some insight into the potency, even a rough estimate of the range of acute toxicity, can be important for certain types of ingredients. Regulatory agencies are still concerned about massive spills and the impact of these on the health of local populations. The Bhopal, India incident revealed just how little information was available on the toxicity associated with inhalation, dermal exposure, and ocular exposure to methyl isocyanate.

A properly designed study can yield much more information than just *a number*. While, by definition, 50% of the animals will die, close observation of these animals during the first 12 h period after treatment often reveals clues to possible means by which the toxicant may be causing an effect; clues that are valuable to the clinical toxicologist. Furthermore, 50% of the animals will survive the treatment and these survivors are a repository of biological effects elicited by the test agent. These animals are observed over the next 14-day period to assess the short or long duration of toxicity; the rapid

or slow recovery; the appearance of any additional, delayed, or secondary toxic effects; changes in hematology, serum biochemistry, and urinalysis; and changes in organ/tissue function measured by relatively noninvasive techniques. When the animals are euthanized at 14 days after treatment, organs/tissues are available for detailed microscopic examination to correlate observed untoward effects and/or injury with morphological changes. Thus, animals surviving the toxic insult are a veritable treasure trove of information concerning the mode(s) of the chemical-induced toxicity.

Alternative In Vivo Animal Acute Toxicity Test Methods

Traditional methods for assessing acute toxicity use death of the animals as an endpoint to establish the LD_{50}. Newer approaches avoid using death as the only endpoint, but rely instead on the observation of clear signs of toxicity, or their absence, at one of a series of fixed-dose levels administered to the animal in a stepwise manner. These newer methods have been validated and accepted by regulatory bodies worldwide (Tables 22.11 and 22.12).

The classical acute toxicity protocol, OECD TG 401, has been replaced by three reduction alternatives: the fixed-dose procedure (OECD TG 420), the acute toxic class method (OECD TG 423), and the up-and-down procedure (OECD TG 425). Each uses preset, defined, or hazard class cutoff doses administered to a single or small group of animals, in a stepwise manner. The starting dose is selected on the basis of a small range-finding study, cytotoxicity screen, or existing data. After each stepwise dosing, the animals are monitored for overt toxicological signs until death in the up-and-down procedure, or the absence or presence of signs of toxicity and/or death in the toxic class method, or clear sign of toxicity in the fixed-dose procedure. Details for each of these procedures can be found on the OECD website [163].

Alternative In Vitro Nonanimal Acute Toxicity Test Methods

Over the last couple of decades, researchers have been developing in vitro cytotoxicity data (IC_{50}) to predict the LD_{50} of

TABLE 22.12
List of Most Updated OECD Acute Toxicity Test Guidelines (Section 4: Health Effects)

TG No.	Title	Most Recently Updated
Acute oral toxicity test guidelines		
401	Acute oral toxicity	Deleted: December 20, 2002
420	Acute oral toxicity—fixed-dose procedure	December 17, 2001
423	Acute oral toxicity—acute toxic class method	December 17, 2001
425	Acute oral toxicity—up-and-down procedure	October 3, 2008
Acute dermal toxicity test guidelines		
402	Acute dermal toxicity: limit dose methods	February 24, 1987
434	Acute dermal toxicity: fixed-dose procedure	Draft
Acute inhalation toxicity test guidelines		
403	Acute inhalation toxicity	September 7, 2009
433	Acute inhalation toxicity: fixed concentration procedure	Draft
436	Acute inhalation toxicity—acute toxic class method	September 7, 2009
Acute dermal irritation/corrosion test guidelines		
404	Acute dermal irritation/corrosion	April 24, 2002
430	In vitro skin corrosion: TER test	April 13, 2004
431	In vitro skin corrosion: human skin model test	April 13, 2004
435	In vitro membrane barrier test method for skin corrosion	July 19, 2006
439	In vitro skin irritation: reconstructed human epidermis test method	July 22, 2010
Acute eye irritation/corrosion test guidelines		
405	Acute eye irritation/corrosion (adopted April 24, 2002)—revision date in 2011	Public comments
437	BCOP test method for identifying ocular corrosives and severe irritants	September 7, 2009
438	ICE test method for identifying ocular corrosives and severe irritants	September 7, 2009
Draft	Cytosensor Microphysiometer® test method: an in vitro method for identifying chemicals not classified as irritant, as well as ocular corrosive and severe irritant chemicals	Public comments
Draft	FL test method for identifying ocular corrosives and severe irritants	Public comments
Skin sensitization test guidelines		
406	Skin sensitization	July 17, 1992
429	Skin sensitization: LLNA radiolabeled	July 22, 2010
442A	Skin sensitization: LLNA: DA (nonradiolabeled)	July 22, 2010
442B	Skin sensitization: LLNA: BrdU-ELISA	July 22, 2010
Phototoxicity test guideline		
432	In vitro 3T3 NRU phototoxicity test	April 13, 2004

chemicals. These researchers have proposed the use of the in vitro toxicity data to predict the starting dose used in many reduction alternative acute toxicity studies, and as part of a tier strategy to eliminate or minimize the need for conducting in vivo animal studies. So far, two in vitro toxicity tests have been validated and accepted in the United States and the European Union for the purpose of predicting the starting dose: the neutral red uptake (NRU) test with rodent cells (3T3 NRU assay), and the NRU test with normal human keratocyte (NHK) cells (OECD Guideline Document 129; Table 22.11).

Alternative Test Methods for Eye Corrosion/Irritation

Damage to the eye is an all too common consequence of an accidental splashing of an industrial chemical, a home or health care product, a pesticide or a solvent, resulting in a painful, and frequently permanent, injury. The Draize eye test has been successfully used since first described in 1944. The thought, however, of placing a highly irritating agent in an animal's eye and causing pain is abhorrent. If the dermal irritancy test is positive, there is little scientific basis for carrying out the eye test, since that agent will almost certainly be positive in the eye. Hence, dermal irritancy tests can be used to screen out strong eye irritants. However, between these highly damaging, strong acids or bases and completely innocuous agents lie a wide variety of seemingly neutral, slightly acidic or basic soaps, detergents, shampoos, cosmetic creams, and lotions, all of which may show minimal effects on the skin but which may still be irritating if accidentally introduced into the eye.

Alternative In Vivo Test Methods: The Animal Eye Test with Reduced Number of Animals

The basic ocular irritation test in the rabbit has been reduced from six animals at each exposure level to two or, at most, three animals per dose without sacrificing much accuracy (Supplement to OCED TG 405). Many test series have shown 88%–91% accuracy with two animals per treatment group. The agent, instilled in the pouch formed by the lower eyelid, is held in place for 1 s and then released. The treated eye is not washed, allowing the animal's own tear secretions to flush out the material. The untreated eye serves as a control. Both eyes are examined at 1, 24, 48, and 72 h after treatment. The irritation (or damage) to the cornea, the conjunctiva, and the iris is scored numerically in a subjective manner. The test is open in that the experiment can be terminated at 72 h if there is no evidence of irritation, but observed effects can be assessed for a longer time period.

Tier Approach for Conducting Animal Eye Corrosion/Irritation Tests

A variety of tier testing strategies have been proposed to reduce the number of animals in eye irritation testing [5,91,99,102]. These strategies usually begin with a weight-of-evidence approach in an effort to review existing information that would allow classification and labeling a material as a severe ocular irritant without animal testing, or to conduct testing with a reduced number of animals. An example of this approach is shown in Table 22.13, a tier testing scheme adopted by the OECD in TG 405 for assessing eye corrosion/irritation. The tier approach ultimately enables conducting confirmatory test using one or two animals only.

Alternative In Vitro Nonanimal Eye Corrosion/Irritation Tests

A number of nonanimal in vitro eye corrosion/irritation test systems have been developed, using a variety of marker endpoints (Tables 22.14 and 22.15). The in vitro eye corrosion/irritation tests fall under four general categories: isolated eye assays, chicken egg membrane assays, reconstituted corneal systems, and acellular synthetic membrane models. The endpoints used include cytotoxicity/viability/proliferation histology/ultrastructure, barrier function, inflammatory mediator release or expression, and cellular metabolism. Some of these tests are briefly described in the following.

The Bovine corneal opacity and permeability (BCOP) assay: This test uses enucleated cow eyes that would otherwise be discarded at slaughterhouses. The cornea is isolated from the rest of the eye and maintained in a holder. A test substance is applied to this isolated cornea for a specified time, then removed and the test substance's effect on the permeability of the cornea to fluorescein dye and on the opacity of the corneal (the degree of transmission of light through the cornea) are determined. Histopathology often is included.

Isolated chicken eye (*ICE*): This test uses enucleated chicken eyes obtained from slaughterhouses. The eyes are placed in a holder, kept moist, and treated with the test substance. Three biological endpoints on the cornea are measured: corneal swelling, corneal opacity, and fluorescein retention. The irritation potential of a substance is calculated from the mean values of these measurements. Histopathology often is included.

EpiOcular assay: "MatTek's EpiOcular™ corneal model is a reconstructed multilayered of stratified, squamous epithelium equivalent to that found in human cornea. The reconstructed corneal epithelium equivalent is derived from normal, human-derived epidermal keratinocytes. EpiOcular has been used in nonanimal in vitro eye corrosion/irritation tests based on a number of biological endpoints including MTT, IL-1a, PGE2, lactate dehydrogenase (LDH), and sodium fluorescein permeability.

HCE-T TEP assay: The epithelial barrier used in this test is a stratified culture of human corneal epithelial cells (HCE-T cell line) similar to the human surface. It measures the transepithelial permeability (TEP) of fluorescein dye to assess the integrity of tight junction of epithelial barrier. The assay's endpoint is the concentration of a test material that causes fluorescein retention by the HCE-T cultures to decrease to 85% relative to the control cultures (FR85). It is possible that the TEP fluorescein permeability in the HCE-T model is not only regulated by the integrity of the tight junction, but it may also affected by other factors such as cell viability and desmosomal junction integrity. Other endpoints that have also been evaluated using the HCE-T model are, for example, lactate release, PGE2 release, various cytokines, and MTT dye uptake.

TABLE 22.13
Tier Strategy for Acute Eye Irritation/Corrosion Test in OECD Test Guideline (TG 405)

Activities	Finding and Conclusions
1.1. Existing human and/or animal data showing effects on eyes.	*Severe damage*: considered corrosive; no testing needed; *irritating*: considered irritant. No testing is needed *Not corrosive/not irritating*: considered noncorrosive and nonirritating; no testing is needed
1.2. Existing human and/or animal data showing corrosive effect on skin.	*Skin corrosive*: assume corrosive to eyes; no testing needed
1.3. Existing human and/or animal data showing severe irritant effects on skin. (*If no information available, or available information not conclusive, go to 2.)*	*Severe skin irritant*: Assume irritating to eyes; no testing needed
2.1. Perform structure activity relationship (SAR) evaluation for eye.	*Predict severe damage to eye*: assume corrosivity to eyes, no testing needed; *predict irritation to eyes*: assume irritating to eyes, no testing needed.
2.2. Perform SAR evaluation for skin. *(If no predictions can be made, or predictions are not conclusive or negative, go to 3.)*	*Predict corrosivity to skin*: assume corrosivity to eyes, no testing needed
3. Measure pH (consider buffering capacity, if relevant). (*If 2 < pH < 11.5, or pH ≤ 2.0 or ≥ 11.5 with low/no buffering capacity, if relevant, go to 4.)*	*pH ≤ 2 or ≥ 11.5* (*with high buffering capacity, if relevant*): assume corrosivity to eyes, no testing is needed
4. Evaluate systemic toxicity data via dermal route. (*If such information is not available or is not highly toxic, go to 5.)*	*Highly toxic at concentrations that would be tested in eye*: substance would be too toxic for testing, no testing needed
5. Perform validated and accepted in vitro or ex vivo test for eye corrosion. (*If substance is not corrosive, or internationally validated in vitro/ex vivo testing methods for eye corrosion are not yet available, go to 6.)*	*Corrosive response*: Assume corrosivity to eyes, no further testing needed
6. Perform validated and accepted in vitro or ex vivo test for eye corrosion. (*If substance is not an irritant, or internationally validated in vitro/ex vivo testing methods for eye irritation are not yet available, go to 7.)*	*Irritant response*: assume irritancy to eyes, no further testing needed
7. Experimentally assess in vivo skin irritation/corrosion potential (see OECD TG 404). (*If substance is not corrosive or severely irritating to skin, go to 8.*)	*Corrosive or severe damage response*: considered corrosive to eye, no further testing needed
8. Perform initial in vivo eye test using one rabbit. *(If no severe damage, or no response, go to 9.)*	*Severe damage to eyes*: considered corrosive to eye, no further testing needed
9. Perform confirmatory test using one or two additional animals.	*Corrosive or irritating*: considered corrosive or irritating to eye, no further testing needed *Not corrosive or irritating*: considered nonirritating and noncorrosive to eyes, no further testing is needed

TABLE 22.14
In Vitro Systems Used for Testing Eye Corrosion/Irritation

Systems	Examples
Isolated eye assays	BCOP; porcine corneal opacity and permeability (PCOP); ICE assay; isolated rabbit eye (IRE); isolated mouse eye; human cornea
Chicken egg membrane assays	Chorioallantoic membrane vascularization assay (CAMVA); hen's egg test—chorioallantoic membrane (HET-CAM) assay
Reconstituted corneal systems	
Cornea models	Human cornea models; rabbit cornea models (3D corneal tissue construct); bovine cornea models (epithelium and stroma)
3D human corneal epithelial cell models	HCE-T human corneal epithelial cell model; SkinEthic™ HCE model; Corneal epithelial cell line (CEPI); coty corneal epithelial model
3D epithelial cell models	EpiOcular; Madin Darby Canine kidney cell line (MDCK) FL
3D conjunctival epithelial cell models	Human conjunctival model
Monolayer epithelial cell	Human corneal epithelial cells; rabbit corneal cells; rabbit corneal cell line; various epithelial and fibroblast cell lines
Acellular models	EYETEX™/irritection; hemoglobin denaturation

TABLE 22.15
Biological Endpoints Used for In Vitro Testing for Eye Corrosion/Irritation

Systems	Examples
Cytotoxicity/viability/proliferation	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (CAS 298-93-1); Thiazolyl blue tetrazolium bromide (MTT) (tetrazolium salt; mitochondrial dehydrogenase dye reduction); LDH; lactate release
	Dye uptake/exclusion/penetration: neutral red release (NRR); NRU; Akamar blue™; Methylene blue; WST-1 (tetrazolium salt that yields water-soluble cleavage products)
Opacity/histology/ultrastructure	Histology for opacity in BCOP model; transmission electron microscopy (TEM) in cell models
	Chemical-induced damage causing changes in light transmission through the cornea—an increase signifying corneal cell loss and a decrease indicating opacity
Barrier function (cell integrity and membrane damage)	Fluorescein dye uptake assessing cell damage: fluorescein permeability in isolated eye assays (BCOP, etc.); FL/MDCK cells; fluorescein transepithelial permeability (TEP);
	Transepithelial electrical resistance (TER or TEER) and other measures of tight junctions
Inflammatory mediator release or expression	Inflammatory response releasing chemotactic factors and then reacted with neutrophils; inflammatory response releasing specific cytokines mediators (histamine, serotonin, prostaglandins, leukotrienes, thromboxanes) that can be collected in the bath medium and quantitated by chemical assay; Arachidonic acid metabolites
	Fertile chicken egg chorioallantoic membrane (CAM) assay: scoring for vascular changes in the membrane blood vessels with fluorescein dye as well necrotic damage
Cellular metabolism	Lactate release/glucose uptake; pH/Cytosensor Microphysiometer®
Other	Total glutathione content; ATP content; methionine incorporation; cell migration; cell differentiation; cytoskeletal changes

MDCK fluorescein leakage (FL) assay: The FL assay used a monolayer of Madin Darby Canine kidney cell line (MDCK) cells cultured on permeable membrane culture inserts. It measures the rate of fluorescein penetration across the membrane to assess any damage caused by the test substance on the integrity of tight junctions of the MDCK cell layer membrane.

Regulatory Acceptance of Alternative In Vitro Nonanimal Eye Corrosion/Irritation Tests

Four in vitro methods have been validated, and either have been accepted or are in the process of being accepted by the OECD (Table 22.11) as part of a tier strategy to identify strong to moderate irritants and nonirritants, leaving those agents showing suspicious or equivocal results to be tested in animals. The four in vitro eye tests are BCOP test method for identifying ocular corrosives and severe irritants (OECD TG 437); ICE test method for identifying ocular corrosives and severe irritants (OCED TG 437); the Cytosensor Microphysiometer® test method for identifying chemicals not classified as irritants, as well as ocular corrosive and severe irritants (OECD TG draft); and the FL test method for identifying ocular corrosives and severe irritants (OECD TG draft). The draft for the revision of OECD TG 405 has taken into consideration the performance of the validated and accepted in vitro tests as part of testing strategy for eye corrosion/irritation.

BCOP test method for identifying ocular corrosives and severe irritants (*OECD TG 437*): The BCOP test method is an organotypic model that provides short-term maintenance of normal physiological and biochemical functions of the bovine cornea in vitro. In this test method, damage by the test substance is assessed by quantitative measurements of changes in corneal opacity and permeability with an opacitometer and a visible light spectrophotometer, respectively. Both measurements are used to calculate an *in vitro* irritation score (IVIS), which is used to assign an in vitro irritancy hazard classification category for prediction of the in vivo ocular irritation potential of a test substance. The BCOP test method uses isolated corneas from the eyes of freshly slaughtered cattle. Corneal opacity is measured quantitatively as the amount of light transmission through the cornea. Permeability is measured quantitatively as the amount of sodium fluorescein dye that passes across the full thickness of the cornea, as detected in the medium in the posterior chamber. Test substances are applied to the epithelial surface of the cornea by addition to the anterior chamber of the corneal holder.

ICE test method for identifying ocular corrosives and severe irritants (*OECD TG 438*): The ICE test method is an organotypic model that provides short-term maintenance of the chicken eye in vitro. In this test method, damage by the test substance is assessed by determination of corneal swelling, opacity, and fluorescein retention. While the latter two parameters involve a qualitative assessment, analysis of corneal swelling provides for a quantitative assessment. Each measurement is either converted into a quantitative score used to calculate an overall Irritation Index, or assigned a qualitative categorization that is used to assign an in vitro ocular corrosivity and severe irritancy classification. Either of these outcomes can then be used to predict the in vivo ocular corrosivity and severe irritation potential of a test substance.

REGULATORY STATUS

The purpose of conducting safety testing is to obtain information that enables the toxicologist to evaluate the hazard potential of a test material in order to if and how it can be used safely. Information pertaining to a test material's hazard potential is also used by Regulatory Agencies for the purposes of classification and labeling when a *new* or *existing* chemical

is being registered or *notified*. Ultimately, the goal of safety testing is to be able to predict the probability of risk to human health under conditions of intended or accidental exposure. Understanding a chemical's or product's potential for toxicity enables one to manage the risk associated with the use of the material through communication (e.g., labeling, use instructions, material safety data sheets [MSDS]), package design (e.g., use of a child resistant closure), availability (e.g., institutional vs. consumer product), and medical management following an accidental exposure. The following sections will summarize the primary test guidelines; international chemical inventories that require the conduct of acute toxicity and ocular irritation testing; and classification schemes used by regulators in the United States, European Union, and other countries or region, for labeling or other hazard communication purposes.

Chemical Inventories

Since the establishment of the toxic substance inventory by the U.S. EPA under Toxic Substances Control Act (TSCA) in the 1970s, many countries around the world now regulate their chemical substances by establishing similar chemical inventories—lists of chemicals that are allowed to be manufactured, imported, used, and put on the market in that country. The producer, importers, or user of chemical can put the chemical on the list by satisfying the regulatory requirements, sometimes including toxicity testing, and often need to put (or include) a new chemical onto the chemical inventory list through processes such as notification, registration, etc. In general, *existing* chemicals are defined as chemicals that were in use or in the market at the time the legislation creating the inventory went into effect. *New* chemicals are those chemicals that are not included in the inventory list. The legislative differentiation between *existing* and *new* mainly impacts on a chemical's regulatory priority. Many countries place high regulatory priority on the new chemicals, but in European Union and United States, high priorities have also been given to certain categories of *existing* chemicals, for example, PBT (persistent, bioaccumulative, and toxic) chemicals as well as HPVC (high production, volume chemicals); and others under the EU Council Regulation (EEC) No. 793/93 also known as Existing Substances Regulation (ESR).

While notification/registration requirements vary from country to country, most do require, at a minimum, that data from some acute toxicity testing be provided. In some cases, irritation testing (ocular and dermal) is also required. Table 22.16 lists some of the chemical inventories of existing and new chemicals, which require data from acute toxicity and irritation studies. The chemical inventories listed in Table 22.16 include chemicals and chemical mixtures, but exclude agrochemicals, cosmetics, food additives, pesticides, pharmaceuticals, and radionucleotides, which are regulated by separate legislations not discussed here.

Within the last decade, many new legislations around the world, especially in European Union, have been or are being implemented to expand and update existing inventories [30,145], to address an existing data gap [60,155] or to unify multiple databases [173]. The EU Registration, Evaluation, and Authorization of Chemicals (REACH) entered into force on June 1, 2007, and into operation on June 1, 2008. The REACH has a major impact on the regulation of chemicals in the EU Communities as well as other countries and regions; it has replaced the European Inventory of Existing Commercial Chemical Substances (EINECS) and European List of Notified Chemical Substances (ELINCS) chemical inventories in the European Union, with a single system managed by the European Chemicals Agency (ECHA). Because of its far reaching impact on regulating chemicals in European Union as well as other parts of the world, REACH is discussed in more details here.

EU Registration, Evaluation, Authorization, and Restriction of Chemicals

In 2007, the EU REACH came into force and implementation started in 2008. This new EU chemical control legislation is complex and comprehensive, aiming to address many critical issues in controlling the risks associated with the uses of chemicals, their mixtures as well as articles treated with chemicals in European Union. REACH replaces all pervious chemical legislations with patchy goals and inefficient processes; addresses issues of data deficiency on many existing chemicals; shifts the burden of collecting information/data, risk assessment, risk management, and risk communication to the manufacturers and importers; promotes broader involvement in the decision making processes and implementation (manufacturers, importers, downstream users, Member States competent authorities, EU Commission, EU Council, and the public at large); installs mechanisms for random or selected checks for compliance; prioritizes the evaluation of chemicals of high concerns; restricts or bans the uses of chemicals with unreasonable risks to health and environment; rationalizes animal testing; and promotes cost sharing.

The legislation base for EU REACH is Regulation (EC) No. 1907/2006 of the European Parliament and of the Council of December 18, 2006 concerning the REACH; establishing a ECHA; amending Directive 1999/45/EC; and repealing Council Regulation (EEC) No. 793/93 and Commission Regulation (EC) No. 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC, and 2000/21/EC. Because of the inefficiency of older EU legislations to address the vast number of chemicals in use or in the market that have little or no toxicity data on the potential hazards to the human health and the environment, the REACH legislation was enacted to unify all the patchy EU legislation on *existing* and *new* chemicals enacted over the last decades. The primary aim for REACH is, over a period of 11 years, to assess the potential hazards of all the chemicals of high concern or high production volume, and through the regulatory process to either register or restrict the use of these chemicals. To increase the efficiency, one of the main purposes of REACH is to shift the burden of testing and risk assessment of many existing and new chemicals, from regulatory authorities to manufacturers and importers. REACH is a complex, comprehensive legislation covering

TABLE 22.16
Chemical Inventories with an Acute Toxicity Data Requirement

Country	Inventory	Regulatory Authority
Australia	Australian Inventory of Chemical Substances (AICS).	National Occupational Health & Safety Commission (Worksafe Australia).
Canada	Domestic Substances List (DSL). Non-Domestic Substances List (NDSL). New Substances Notification Regulations (NSNR).	Environment Canada.
China	Inventory of Existing Chemical Substances in China (IECSC).	State Environmental Protection Administration.
European Union	ECHA central data base of chemicals registered under REACH. Annexes IV and V: list of chemicals exempted from registration, mainly based on the previous EINECS and ELINCS. Annex XIV: list of substances of high concerns (CMR, accumulative, etc.); once a substances is included on this list, authorization is required for commerce and use, by demonstrating safety and no risk for the specific use. Annex XVII: list of use restrictions for some dangerous substances, mixtures, or articles.	ECHA. *Note*: The EINECS and ELINCS previously managed by European Chemicals Bureau have been transferred to ECHA under REACH. REACH creates a single system for both *existing* and *new* substances; non-phase-in substances (i.e., those not produced or marketed prior to the entry into force of REACH) and phase-in substances (those substances listed in the EINECS, or those that have been manufactured in the Community, but not placed on the Community market, in the last 15 years or the so-called no longer polymers of Directive 67/548).
	Screening Information Data Set (SIDS)	Organization for Economic Cooperation and Development.
Korea	Korean Existing Chemicals Inventory (KECI)	Ministry of Environment.[a]
New Zealand	ERMA New Zealand's Register	Environmental Risk Management Authority.
Philippines	Philippines Inventory of Chemicals and Chemical Substances (PICCS)	Department of Environment & Natural Resources.
United States	Toxic Substances Control Act (TSCA) High Production Volume (HPV)	EPA.

[a] Since 1995, authority for conducting toxicity reviews has been delegated to the National Institute of Environmental Research (NIER).

all chemicals (single chemical substances, mixtures/preparation and treated articles containing releasable chemical substances, unless they are explicitly exempted). Exemptions from REACH include those chemicals explicitly being regulated under other legislations such as those for drugs, food, pesticides, etc.; low risk substances; naturally occurring substances such as minerals, ores, certain polymers; and those listed in Annex IV and V of REACH. REACH also stipulates specific regulatory processes, priority lists, cost sharing on testing, compensation of use of toxicity data, responsibilities of downstream users, etc.

REACH regulates dangerous substances, their mixtures (preparations), and articles treated with the substances through four major processes: registration, evaluation, authorization, and restriction.

Registration: Under REACH, each manufacturer, importer of a substance (chemical, its mixtures, or articles treated with the chemical), unless it is explicitly exempted, has to register the substance with ECHA before manufacturing or placing on the market. Under REACH, substances are categorized as *phase-in* or *non-phase-in* substance. Phase-in substances are those that have already been manufactured and/or placed in the market before REACH was in force, or those previously exempted polymers that have been reclassified as *no longer polymer*. Non-phase-in substances are those that do not meet the afore mentioned criteria. Upon on-time preregistration, phase-in substances have a longer transitional registration deadline than *non-phase-in* substances. Chemicals for research and development purposes are also exempted from registration for a period of 5 years.

Registration under REACH is based on the principle of one registration for one substance (chemical itself, mixtures containing the chemical, or article treated with the chemical). This means that all manufacturers and importers of an identical substance must submit registration application together sharing costs and information.

Under REACH, registrant(s) of a substance must submit to ECHA a registration dossier (technical dossier and chemical safety report) containing relevant information on the substance itself, hazard identification (and testing proposal if appropriate), risk assessment, as well as information on how the risk can been managed and communicated, including classification, labeling, and packaging (CLP). After the dossier has passed the administrative and technical checks for completeness for meeting all registration requirements, it will be assigned a registration number. All information submitted, except those that can be claimed as confidential, will be published for public involvement in the registration and decision making process.

Evaluation: ECHA and an component authority of a Member State will conduct evaluation of information submitted on some substances on the quality of the registration dossier, testing proposals, as well as whether the substance can pose an unreasonable risk to health and the environment. The outcome

of the evaluation could be the risk associated with the use of the chemical is sufficiently under control and adequate measures in place, thus require no further action; or information submitted is insufficient for the assessment of the risk, thus require submission of further information; or the risk is not sufficiently under control and thus require risk management measures such as harmonized CLP, inclusion on the authorization list (Annex XIV), banning the substance, or imposing/amending restrictions on the uses of the substance (Annex XVII).

Authorization: Once a substance is placed in the authorization list (Annex XIV), manufacturers/importers need to apply for special authorization for specific uses. Granting of authorization will be based on a weight of risk/benefit assessment through two committees, the ECHA's Risk Assessment Committee (RAC) and the Committee for Socio-Economic Analysis (SEAC), public consultation, as well as the availability of lower-risk alternatives.

Restriction: A member state, or EU Commission through ECHA, can initiate the process of imposing restrictions on the use of a substance, or even banning it, if they have a concern that the substance could pose an unreasonable risk to health and the environment. The process of restriction is based on the Committees' opinion and public consultation. After the Commission has made the decision, the restrictions will be imposed (amendment to Annex XVII).

REACH aims for a whole supply chain involvement in controlling the risk associated with the production and uses of chemicals. Downstream users, such as formulators, end users, treated articles producers, refillers, reimporters, etc., also have certain responsibilities under REACH, which include: provide use information to suppliers enabling them to conduct safety assessment, comply to the safety measures and use restrictions provided by suppliers, inform suppliers of any new information on hazard and/or risk on the chemical, as well as prepare and submit their own chemical safety report to ECHA, if needed.

Implementation of REACH started on June 1, 2008. REACH provisions are being phased-in over 11 years for all existing chemicals (phase-in chemicals) in accordance to a priority-based time schedule:

- November 30, 2010—Registration completed for chemicals of high concerns (≥1 metric ton/year of CMR, carcinogens, mutagens, and reproductive toxins; ≥100 metric tons/year of chemicals very toxic to the aquatic environment [PBT/vPvBs—very persistent and very bioaccumulative substances—classified with N: R50-53]; and ≥1000 metric tons/year of other chemicals)
- May 31, 2013—Registration completed for chemicals ≥100–1000 metric tons/year
- May 31, 2018—Registration completed for chemicals ≥1–100 metric tons/year

Data requirement under REACH is based on production or imported volume—the higher the volume, the larger the amount of data required. A technical dossier including a chemical safety report is required for the chemicals with an annual production volume of 10 metric tons or above. Information required includes chemical identity, physic–chemical properties, toxicity, ecotoxicity, environmental fate, exposure, and instructions for appropriate risk management. REACH lists certain higher tier tests required for chemicals with annual production volume of 100 metric tons or more.

Since entering into forces on June 1, 2007, the complexity of implementation of REACH has emerged and some controversial issues have surfaced. The EU Commission on Environment is planning to conduct a comprehensive review on REACH in 2012 with respects to its impact on risk caused by chemicals in 2012 compared to the 2007; its health and environmental benefits; its contribution to the development, commercialization, and uptake of products of emerging technologies; its impact on innovation and functioning of EU chemical industry; and issues related to operation, implementation and enforcement, CLP, technical assistance offered, and registration requirements for 1–10 metric tons substances and polymers; as well as REACH issues concerning nanotechnology.

Test Guidelines

The regulatory status of test methods used to evaluate the acute toxic and ocular irritation potential of test materials is in a state of flux. Most international regulatory agencies are attempting to reduce both the number of animals necessary to assess the acute toxicity potential and ocular hazard and to minimize pain and suffering. The primary acute toxicity and ocular irritation test guidelines that have been adopted by various regulatory agencies are summarized in Table 22.17.

The *International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use* (ICH) is an ongoing effort bringing together regulatory authorities of the United States (FDA), European Medicines Agency (EMA), and Japan and representatives from the pharmaceutical industry with the goal of harmonizing the technical aspects of test method design, data interpretation, and product registration [100]. While acute toxicity testing is a critical first step in the evaluation of a new pharmaceutical compound, the only recommendation pertaining to acute toxicity testing made by ICH is that the LD_{50} determination for pharmaceuticals should be abandoned [100].

Many countries have adopted the OECD toxicity test guidelines for regulatory purposes including notification, registration, and CLP of chemicals. A list of these globally accepted toxicity test guidelines for acute toxicity and irritation tests is given in Table 22.12.

After decades of advocates from animal welfare organizations, alternative toxicity tests have been gradually adopted for regulatory purposes. For example, under the REACH legislation, an increased use of animals in testing is expected, as the hazardous properties of chemicals cannot be sufficiently determined by using currently available in vitro nonanimal test methods, and regulatory action cannot solely rely on these tests. However, REACH encourages adaptation of alternative toxicity tests in favor of animal tests whenever it is possible, and a fast

TABLE 22.17
Summary Test Method Guidelines

	Acute Toxicity			
Regulatory Authority	**Oral**	**Dermal**	**Inhalation**	**Ocular Irritation**
EPA[a]	870.1100	870.1200	870.1300 870.1350	870.2400
CPSC[b]		16CFR1500.40		16CFR1500.42
OECD[c]	TG 420 TG 423 TG 425	TG 402	TG 403	TG 405
European Union[d]	B.1 bis B.1 tris	B.3	B.2	B.5
MAFF[e]	2-1-1	2-1-2	2-1-3	2-1-5

[a] EPA (U.S.)—Federal Insecticide Fungicide and Rodenticide Act. Series 870—Health Effects Testing Guidelines. Office of Prevention, Pesticides and Toxic Substances (Title 40 Code of Federal Regulations Part 799).

[b] CPSC (U.S.)—Federal Hazardous Substances Act. Title 16 Code of Federal Regulations Part 1500.

[c] Organization for Economic Cooperation and Development (European Union). Guidelines for the testing of chemicals. Section 4: Health Effects. www.OECD.org.

[d] European Union. Council Directive 67/548/EEC Annex V Part B: Methods for determination of toxicity. *Official Journal of the European Communities*, 196, 1–98.

[e] Ministry of Agriculture, Forestry and Fisheries (Japan). Appendix to Director General Notification No.12-Nousan-8147, November 24, 2000. Guidelines related to the study reports for the registration application of pesticides. Implementation methods.

tracking process (TSAR, Tracking System for Alternative test methods Review, Validation and Approval in the Context of EU Regulations on Chemicals) has been put in place to adopt promising new alternative test methods for regulatory purposes.

Classification Schemes

Two examples of how acute toxicity and ocular irritation data are used for the purposes of classifying and labeling chemicals and products are those detailed in European Council Directive 67/548/EEC Annex VI [53] and U.S. EPA Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) guidelines [59]. While the types of chemicals and products evaluated in the European and U.S. schemes are different, both schemes (as shown in Tables 22.10, 22.18, and 22.19) highlight the manner in which acute toxicity and ocular irritation data are used to define the hazard potential of individual chemicals and products. Chemicals and products evaluated in accordance with criteria defined in Council Directive 67/548/EEC Annex VI [53] are assigned risk or "R" phrases [54]. The choice of R phrases is made on the basis of the classification to ensure that the potential dangers identified in classification are expressed on the label.

However, all the classification and labeling systems will be ultimately replaced by the UN initiative, the GHS. For example, in European Union, from June 1, 2015, all mixture products must be classified and labeled in accordance with Regulation (EC) No. 1272/2008 (GHS); thereafter, Directives 67/548/EEC (on dangerous substances) and Directive 1999/45/EC (on dangerous preparations or mixtures) will no longer have any legal status.

Globally Harmonized System of Classification and Labeling of Chemicals

One practical use of acute toxicity data is for hazard classification and labeling of chemicals. The criteria for classification and labeling vary from country to country, and sometimes from regulatory agency to agency. The variation in classification and labeling has been an obstacle to international trade, and the need for harmonization is long overdue.

The GHS of Classification and Labeling of Chemicals was initiated at the *United Nations Conference on the Environment and Development* in Rio de Janeiro in 1992. It attempts to harmonize the hazard classification and the hazard communication of chemicals (labeling and MSDS), aiming to promote international trade and to set an internationally recognized safety standard of chemicals. The primary components of GHS include definition of hazard endpoints, classification criteria for hazard endpoints, mixture rules, safety data sheets (SDS) or MSDS, label elements, and implementation plans. GHS does not require testing or establishment of test methods; rather, GHS is a self-classification system using relevant data available from all sources. The GHS classification process is designed to be simple and transparent with a clear distinction between hazard classes and categories in order to allow for *self-classification* as far as possible. For many hazard classes, the criteria are semiquantitative or qualitative and expert judgment is required to interpret the data. Decision tree approach is provided in the GHS *Purple Book* [197] for many hazards (e.g., eye irritation) to assist in classification. GHS is a voluntary system to be adopted into local or

TABLE 22.18
EU Dangerous Substances Directive Acute Toxicity Classification Scheme

	Criteria[b]			
			Inhalation	
Classification (R Phrase)[a]	Oral	Dermal	Gas/Vapor	Aerosol/Particulate
Harmful (R20/R21/R22)	$200 < X \leq 2000$ mg/kg	$400 < X \leq 2000$ mg/kg	$2 < X \leq 20$ mg/L/4 h	$1 < X \leq 5$ mg/L/4 h
Toxic (R23/R24/R25)	$25 < X \leq 200$ mg/kg	$50 < X \leq 400$ mg/kg	$0.5 < X \leq 2$ mg/L/4 h	$0.25 < X \leq 1$ mg/L/4 h
Very toxic (R26/R27/R28)	$X \leq 25$ mg/kg	$X \leq 50$ mg/kg	$X \leq 0.5$ mg/L/4 h	$X \leq 0.25$ mg/L/4 h

Note: $X = LD_{50}$ (oral, dermal) or LC_{50} (inhalation).
[a] EEC.[54]
[b] EEC.[53]

TABLE 22.19
FIFRA Classification Scheme for Acute Toxicity and Eye Irritation

	Toxicity Categories			
Toxicological Endpoint	I	II	III	IV
Acute oral toxicity	$X \leq 50$ mg/kg	$50 < X \leq 500$ mg/kg	$500 < X \leq 5000$ mg/kg	$X > 5000$ mg/kg
Acute dermal toxicity	$X \leq 200$ mg/kg	$200 < X \leq 2000$ mg/kg	$2000 < X \leq 5000$ mg/kg	$X > 5000$ mg/kg
Acute inhalation toxicity	$X \leq 0.05$ mg/L/4 h	$0.05 < X \leq 0.5$ mg/L/4 h	$0.5 < X \leq 2$ mg/L/4 h	$X > 2$ mg/L/4 h
Primary eye irritation	Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days	Corneal involvement or other eye irritation clearing in 8–21 days	Corneal involvement or other eye irritation clearing in 7 days or less	Minimal effects clearing within 24 h

Source: Adapted from EPA, Precautionary statements, in *Label Review Manual*, 3rd edn., EPA document 735-B-03-001, U.S. EPA, Office of Prevention, Pesticides and Toxic Substances, chapter 7, 2003.
Note: $X = LD_{50}$ (oral, dermal) or LC_{50} (inhalation).

national regulations. Each country set its own implementation schedule. Some variations and flexibilities are provided, but ultimately, GHS will replace all CLP legislations and practices worldwide; for example, all current CLP legislations in the European Union will be replaced by GHS by 2015.

GHS harmonizes most classification criteria based on the substance's intrinsic health, physical, and environmental hazards. It uses pictograms, hazard statements, and signal words *danger* and *warning* to communicate hazard information on the labels and MSDS, and is based on the intrinsic hazard of the substance. The GHS allows individual countries or regions to implement building blocks (a set of GHS hazard classification and communication criteria) to meet the needs of their audience and sectors. The building blocks, however, may not be altered. In addition, there is room for Competent Authority Options and special limits for communication of components in mixtures. The GHS is a dynamic system being continuously updated based on implementation experience and the availability of relevant scientific information. The first edition of the GHS was approved by the Committee of Experts at its first session in December 2002 for initial global implementation. Since then, the GHS has been updated several times based on the experience of implementation. The latest update is the fourth edition published in 2011 [197]. The GHS has been implemented in almost in all the major economies, including United States, European Union, China, Japan, Korea, Southeast Asia, etc., for the transportation of dangerous substances. The implementation of GHS in other sectors, supply and use, workplace, etc., varies among countries. Eventually, the GHS is expected to replace all the current classification and labeling systems globally.

GHS is built on 16 physical, 10 health, and 3 environmental hazard classes, and comprises the following communication elements: 9 pictograms, 2 signal words *danger* or *warning*, 72 individual and 17 combined *hazard statements*, and 116 individual and 33 combined precautionary statements.

The 16 physical hazard classes are explosives, flammable gases, flammable aerosols, oxidizing gases, gases under pressure, flammable liquids, flammable solids, self-reactive substances, pyrophoric liquids, pyrophoric solids, self-heating substances, substances which in contact with water emit flammable gases, oxidizing liquids, oxidizing solids, organic peroxides, and corrosive to metals. The 10 health hazard classes are acute toxicity, skin corrosion/irritation, serious eye damage/eye irritation, respiratory or skin sensitization, germ cell mutagenicity, carcinogenicity, reproductive toxicology, target organ systemic toxicity (single exposure, target organ systemic toxicity, and repeated exposure), and

aspiration toxicity. The three classes of environmental hazards are hazardous to the aquatic environment (acute aquatic toxicity and chronic aquatic toxicity), bioaccumulation potential, and rapid degradability. The GHS classification categories for health hazards are summarized in Table 22.20.

The hazard statements are assigned a unique alphanumerical code that consists of one letter and three numbers as follows: the letter "H" (for *hazard statement*); a number designating the type of hazard as follows: "2" for physical hazards, "3" for health hazards, and "4" for environmental hazards; and two numbers corresponding to the sequential numbering of hazards arising from the intrinsic properties of the substance or mixture, such as explosive properties (codes from 200 to 210), flammability (codes from 220 to 230), etc.

The hazard classification criteria of toxicity and irritation of single substance under the GHS are listed in Tables 22.21 through 22.24, whereas the major pictograms used in GHS are listed in Table 22.25.

Classification and labeling of mixtures under GHS: While the classification and labeling of a single toxic substance are relatively straight forward under GHS, the process of classifying a mixture is tiered, and is dependent on the amount of information available for the mixture and its components, that is, (1) available data for the mixture itself, and/or (2) similar mixtures, and/or (3) data for the ingredients of the mixture. The process for the classification of mixtures is based on the following steps: (1) if test data are available for the mixture itself, the classification of the mixture will be based on that data (except carcinogens, mutagens, and reproductive toxicants); (2) if test data are not available for the mixture itself, then the appropriate bridging principles should be used; and (3) if (a) test data are not available for the mixture itself and (b) the bridging principles cannot be applied, then the calculation or cutoff values should be used to classify the mixture. When a mixture has not been tested, but there are sufficient data on the components and/or similar tested mixtures, then these data can be used in accordance with the following six bridging principles: (1) Dilution: If a mixture is diluted with a diluent that has an equivalent or lower toxicity, then the hazards of the new mixture are assumed to be equivalent to the original; (2) Batching: If a batch of a complex substance is produced under a controlled process, then the hazards of the new batch are assumed to be equivalent to the previous batches; (3) Concentration of highly toxic mixtures: If a mixture is severely hazardous, then a concentrated mixture is also assumed to be severely hazardous; (4) Interpolation within one toxic category: Mixtures having component concentrations within a range in which the hazards are known or assumed to have those known hazards; (5) Substantially similar mixtures: Slight changes in

TABLE 22.20
Summary of the GHS Health Hazard Classifications Categories

Hazard Class	Hazard Classification Category				
Acute toxicity	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5
Skin corrosion/irritation	Cat 1A	Cat 1B	Cat 1C	Cat 2	Cat 3
Serious eye damage/eye irritation	Cat 1	Cat 2A	Cat 2B	—	—
Respiratory or skin sensitization	Respiratory Cat 1	Skin Cat 1	—	—	—
Germ cell mutagenicity	Cat 1A	Cat 1B	Cat 2	—	—
Carcinogenicity	Cat 1A	Cat 1B	Cat 2	—	—
Reproductive toxicity	Cat 1A	Cat 1B	Cat 2	Lactation	—
Specific target organ toxicity—single exposure	Cat 1	Cat 2	Cat 3	—	—
Specific target organ toxicity—repeated exposure	Cat 1	Cat 2	—	—	—
Aspiration hazard	Cat 1	Cat 2	—	—	—

TABLE 22.21
GHS Classification Criteria for Acute Toxicity

Acute Toxicity	Category 1	Category 2	Category 3	Category 4	Category 5
Oral (mg/kg)	≤5	>5, ≤50	>50, ≤300	>300, ≤2000	*Criteria*:
Dermal (mg/kg)	≤50	>50, ≤200	>200, ≤1000	>1000, ≤2000	Anticipated oral LD_{50} between 2000 and 5000 mg/kg
Gases (ppm)	≤100	>100, ≤500	>500, ≤2500	>2500, ≤5000	Indication of significant effect in humans[a]
Vapors (mg/L)	≤0.5	>0.5, ≤2.0	>2.0, ≤10.0	>10, ≤20	Any mortality at class 4[a]
Dust or mist (mg/L)	≤0.05	>0.05, ≤0.5	>0.5, ≤1.0	>1.0, ≤5	Significant clinical signs at class 4[a]
					Indications from other studies[a]

[a] If assignment to more hazardous class is not warranted.

TABLE 22.22
GHS Classification Criteria for Skin Corrosion/Irritation Toxicity

Skin Corrosion Category 1			Skin Irritation	Mild Skin Irritation
			Category 2	Category 3
Destruction of dermal tissue: visible necrosis in at least one animal			Reversible adverse effects in dermal tissue	Reversible adverse effects in dermal tissue
Subcategory 1A	Subcategory 1B	Subcategory 1C		
Exposure <3 min	Exposure <1 h	Exposure <4 h	Draize score: ≥2.3 <4.0 or persistent inflammation	Draize score: ≥1.5 <2.3
Observation <1 h	Observation <14 days	Observation <14 days		

TABLE 22.23
GHS Classification Criteria for Eye Corrosion/Irritation Toxicity

Category 1	Category 2	
Serious Eye Damage	**Eye Irritation**	
Irreversible damage 21 days after exposure	Reversible adverse effects on cornea, iris, conjunctiva	
	Draize score:	
Draize score:	Corneal opacity ≥1	
Corneal opacity ≥3	Iritis >1	
Iritis >1.5	Redness ≥2	
	Chemosis ≥2	
	Irritant	Mild irritant
	Subcategory 2A	Subcategory 2B
	Reversible in 21 days	Reversible in 7 days

TABLE 22.24
GHS Classification Criteria for Target Organ Toxicity from Single Exposure

Category 1	Category 2	Category 3
Significant toxicity or presumed to have significant toxicity in humans; classified based on:	*Presumed to be harmful to human health; classified base on*:	*Transient target organ effects; classified based on*:
Reliable, good quality human case studies or epidemiological studies	Animal studies with significant toxic effects relevant to humans at generally moderate exposure (guidance)	Narcotic effects
Animal studies with significant and/or severe toxic effects relevant to humans at generally low exposure (guidance)	Human evidence in exceptional cases	Respiratory tract irritation

the concentrations of components are not expected to change the hazards of a mixture and substitutions involving toxicologically similar components are not expected to change the hazards of a mixture; (6) Aerosols: An aerosol form of a mixture is assumed to have the same hazards as the tested, nonaerosolized form of the mixture unless the propellant affects the hazards upon spraying. The basic GHS approach to the classification and labeling of health hazards is summarized in Table 22.25.

For acute toxicity, GHS allows the use of two formulas to calculate the acute toxicity estimate (ATE) for mixtures for classification and labeling purposes [197] as follows:

1. When data available for all ingredients (ignoring acutely nontoxic components, such as water, sugar, etc., and ingredients that fall at the upper threshold of Category 4 from a limit dose test), or where acute toxicity data are unavailable on total concentration of ingredients at <10%, ATEmix is determined using the following formula:

$$\frac{100}{\mathrm{ATE(mix)}} = \sum_{n} \frac{C_i}{\mathrm{ATE}_i}$$

where
C_i is the concentration of ingredient i
n is ingredients
i is running from 1 to n
ATE_i is the acute toxicity estimate of ingredient
ATE(mix) is the acute toxicity estimate of the mixture

TABLE 22.25
Major Hazard Pictogram Used in GHS

Description	Pictogram	Hazard Class and Hazard Category
1. Exploding bomb		Unstable explosives Explosives of Divisions 1.1, 1.2, 1.3, and 1.4 Self-reactive substances and mixtures, Types A and B Organic peroxides, Types A and B
2. Flame		Flammable gases, Category 1 Flammable aerosols, Categories 1 and 2 Flammable liquids, Categories 1, 2, and 3 Flammable solids, Categories 1 and 2 Self-reactive substances and mixtures, Types B, C, D, E, and F Pyrophoric liquids, Category 1 Pyrophoric solids, Category 1 Self-heating substances and mixtures, Categories 1 and 2 Substances and mixtures, which in contact with water, emit flammable gases, Categories 1, 2, and 3 Organic peroxides, Types B, C, D, E, and F
3. Flame over circle		Oxidizing gases, Category 1 Oxidizing liquids, Categories 1, 2, and 3
4. Gas cylinder		Gases under pressure: Compressed gases Liquefied gases Refrigerated liquefied gases Dissolved gases
5. Corrosion		Corrosive to metals, Category 1 Skin corrosion, Categories 1A, 1B, and 1C Serious eye damage, Category 1
6. Skull and crossbones		Acute toxicity (oral, dermal, inhalation), Categories 1, 2, and 3
7. Exclamation mark		Acute toxicity (oral, dermal, inhalation), Category 4 Skin irritation, Category 2 Eye irritation, Category 2 Skin sensitization, Category 1 Specific target organ toxicity—single exposure, Category 3
8. Health hazard		Respiratory sensitization, Category 1 Germ cell mutagenicity, Categories 1A, 1B, and 2 Carcinogenicity, Categories 1A, 1B, and 2 Reproductive toxicity, Categories 1A, 1B, and 2 Specific target organ toxicity—single exposure, Categories 1 and 2 Specific target organ toxicity—Repeated exposure, Categories 1 and 2 Aspiration hazard, Category 1
9. Environment		Hazardous to the aquatic environment Acute hazard, Category1 Chronic hazard, Categories 1 and 2

2. When acute toxicity data are unavailable on total concentration of ingredients at >10%, ATEmix is determined using the following formula:

$$\frac{100-\left(\sum C(\text{unknown})\right)}{\text{ATE(mix)}}=\sum_{n}\frac{C_i}{\text{ATE}_i}$$

where
C_i is the concentration of ingredient i
C (unknown) is the total concentration of ingredients with unknown acute toxicity data
n is ingredients
i is running from 1 to n
ATE_i is the acute toxicity estimate of ingredient
ATE (mix) is the acute toxicity estimate of the mixture

The classification of skin and eye corrosion/irritation of mixtures under GHS generally follows the bridging and additivity principles. When no test data are available for the mixture and bridging principles do not apply, the classification is based on additivity which assumes that each corrosive or irritant component contributes to the overall corrosivity/irritancy of the mixture in proportion to its potency and concentration. Whether the mixture is considered to be an irritant or seriously damaging to the skin or eye is based on cutoff values (Tables 22.26 and 22.28). If additivity approach does not apply as many substances are corrosive or irritant at concentration <1% (e.g., acids and bases, inorganic salts, aldehydes, phenols, and surfactants), then different criteria are used [Tables 22.27 and 22.29]).

TABLE 22.26
GHS Skin Corrosive/Irritant Hazard Classification of Mixtures Triggering Criteria

	Concentration (%) Triggering Classification of a Mixture As		
	Skin Corrosive	Skin Irritant	Skin Irritant
Sum of Ingredients Classified As	Category 1	Category 2	Category 3
Skin Category 1	≥5	≥1 but <5	
Skin Category 2		≥10	≥1 but <10
Skin Category 3			≥10
(10 × Skin Category 1) + Skin Category 2		≥10	≥1 but <10
(10 × Skin Category 1) + Skin Category 2 + Skin Category 3			≥10

TABLE 22.27
GHS Skin Corrosive/Irritant Hazard Classification of Mixtures Triggering Criteria (When Additivity Approach Does Not Apply)

Ingredient	Concentration (%)	Mixture Classified as Skin
Acid with pH ≤ 2	≥1	Category 1
Base with pH ≥ 11.5	≥1	Category 1
Other corrosive (Category 1) ingredients for which additivity does not apply	≥1	Category 1
Other irritant (Category 2 or 3) ingredients for which additivity does not apply, including acids and bases	≥3	Category 2

TABLE 22.28
GHS Eye Corrosive/Irritant Hazard Classification of Mixtures Triggering Criteria

	Concentration (%) Triggering Classification of a Mixture As	
	Irreversible Eye Effects	Reversible Eye Effects
Sum of Ingredients Classified As	Category 1	Category 2
Skin Category 1	≥3	≥1 but <3
Skin Category 2/2A	—	≥10
(10 × Eye Category 1) + Eye Category 2/2A	—	≥10
Skin Category 1 + Eye Category 1	≥3	≥1 but <3
10 × (Skin Category 1) + Eye Category 1 + Eye Category 2/2A	—	≥10

TABLE 22.29
GHS Eye Corrosive/Irritant Hazard Classification of Mixtures Triggering Criteria (When Additivity Approach Does Not Apply)

Ingredient	Concentration (%)	Mixture Classified as Eye
Acid with pH ≤ 2	≥1	Category 1
Base with pH ≥ 11.5	≥1	Category 1
Other corrosive (Category 1) ingredients for which additivity does not apply	≥1	Category 1
Other irritant (Category 2) ingredients for which additivity does not apply, including acids and bases	≥3	Category 2

QUESTIONS

Q. What is the importance of acute toxicity testing and is a precise LD_{50} value necessary to adequately define acute toxicity?

A. Acute toxicity testing is the way in which we define the intrinsic toxicity of a chemical, identify target organs, provide information for risk assessment of acute exposure, provide information for the design and selection of dose levels for more prolonged studies (i.e., subchronic, chronic), and most importantly, provide information to clinicians for use in treatment of acute chemical poisoning. Information from acute toxicity testing is also used to provide insight into the mechanism of action of a chemical, to formulate safety measures during the early stages in the development of a new chemical, and for categorization and labeling purposes for handling and shipping chemicals. One should not confuse the concept of acute toxicity with the term LD_{50}. The LD_{50} is a statistically defined measure of acute toxicity but is only one of many ways to define acute toxicity. Indeed, a precise LD_{50} is seldom required in acute toxicity testing, and its use is being de-emphasized to reduce the total number of animals and pain and suffering involved in their use. The LD_{50} is being replaced by more modern methods. The up-and-down procedure and the acute toxic class method are alternatives to the LD_{50} test that can be used to estimate the medial lethal dose and to provide hazard classification for labeling, respectively.

Q. Name some of the factors that can influence the results of an acute toxicity study.

A. Physiochemical properties of the test article (lipophilicity, molecular weight, and solubility), species used, age of the animals, route of exposure, and rate of test article metabolism.

Q. The following mortality data were obtained from an acute oral toxicity study.

Dose (mg/kg)	1	2	4	8	16	32
Mortality	0/10	1/10	3/10	4/10	7/10	10/10

Calculate the LD_{50}, the SE of the LD_{50}, the fiducial limits, and the slope of the dose–response curve.

		Probits		Probabilities	Responses		
Log Dose (x)	n	Observed	Expected	Expected (P)	Observed	Expected	χ^2
0.30	10	3.72	3.82	11.9	1	1.19	0.0344
0.60	10	4.48	4.36	26.1	3	2.61	0.0344
0.90	10	4.75	4.75	46.4	4	4.64	0.0789
1.20	10	5.52	4.45	67.4	7	6.74	0.1646
1.50	10	—	—	—	10	—	0.0307
						$\Sigma\chi^2 = 0.386$	
						$df = 2$	

A. Procedure

1. Determine the log dose and probits:

Log dose	0.0	0.3	0.6	0.9	1.2	1.5
Probits	—	3.72	4.48	4.75	5.52	—

2. Plot log dose versus probits (Figure 22.7) and fit the best point(s) to a straight line (see Figure 22.7).
3. From the log dose probits line, extrapolate the log $LD_{50} = 0.95$; then LD_{50} = antilog 0.95 = 8.91 mg/kg body weight.
4. From the same line, calculate the slope as: (numbers of probit units)/unit log dose = 2/11 = 1.818.

Thus, $\sigma = 1/\text{slope} = 0.55$ (Figure 22.7).

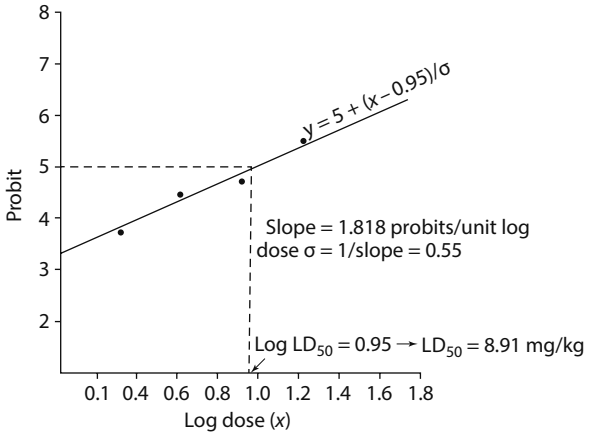

FIGURE 22.7 Example of probit versus log dose plot.

5. χ^2 *test of goodness of fit.* Expected probability is converted from the expected probits. The test is conducted by converting each expected probit (y) back to the expected probability (P) and then to the number of expected responses (E) (i.e., multiply the expected probability P by n). The difference between expected and observed number of responses will be used to calculate the χ^2 statistic, but instead of using $\Sigma[(E-0)^2/E]$, the weighted value will be used, that is, $\Sigma[(E-0)^2/E(1-P)]$. The degree of freedom (df) is $N-2$, where N is the number of dose levels used in the calculation of χ^2. The critical χ^2 for $(4-2)=2$ degrees of freedom is 6.0 at a $P=0.05$, and the calculated $\chi^2=0.386$, which is less than the critical value, indicating that the fitted line is adequate.
6. *Determination of precision of LD_{50} by weighting.* The SE of $\log \mathrm{LD}_{50} = S_m = \sigma / \sqrt{\Sigma mW} = 0.55 / \sqrt{18.5} = 0.129$.

   The approximation of SE (LD_{50}) = $(10^m) \cdot (S_m)$ = 8.91 × 2.302 × 0129 = 2.646. The precision of LD_{50} = 8.91 ± 2.646 mg/kg.

Dose (mg/kg)	2	4	6	16
W	0.277	0.423	0.541	0.564
nW	2.77	4.23	5.41	5.64
ΣnW		18.05		

7. *Fiducial limits.* Using the approximation formula, the fiducial limit calculated at the F = 95% level is given by log LD_{50} ± 1.96 (S_m). Thus the lower log LD_{50} limit = 0.5 − 1.96 × 0.129 = 0.697, and the antilog 0.697 = 4.977. The upper log LD_{50} limit = 0.95 + 1.96 × 0.129 = 1.20 = 15.849. Antilogs of 0.697 and 1.2 give the fiducial LD_{50} limit 4.98–15.85 mg/kg.

Q. What type of distribution provides a precise description of a lethality response to a toxic test article?

A. Lognormal distribution.

Q. Compare and contrast eye irritation and eye corrosion including in the discussion a description of the observation endpoints usually associated with irritancy in the three major tissues of the eye.

A. Eye irritation can be defined as reversible inflammatory changes in the eye and its surrounding mucous membranes following exposure to a material on the surface of the anterior portion of the eye. By contrast, corrosion represents irreversible tissue damage to the eye following exposure to a material. The amount of damage to each of the three major eye tissues, the cornea, the conjunctiva, and the iris, is what differentiates irritancy from corrosion. Gross tissue destruction that follows rapidly after exposure and persists for an extended period in any or all of these tissues is usually an indication of eye corrosion. Irritancy, however, can occur to various degrees. Assessment of injury is based on the presence and severity of cloudiness (opacity) and swelling of the cornea; redness, edema (chemosis), and discharge in the conjunctiva; and increased vascularity, edema, absence of reaction to light, and cloudiness (aqueous flair) in the iris.

Q. A consumer aerosol product contains ingredient A (20%), ingredient B (30%), and water (50%); there are no inhalation data on the product itself, but the acute inhalation LC_{50} (rat, 4 h) is 0.5 mg/L for ingredient A, and 5 mg/L for ingredient B. What is the GHS acute toxicity classification category and pictogram for this product?

A. Use the ATE (mixture) formula to calculate the LC_{50} for the product, ignoring the water component. The calculated LC_{50} (product) = 2.17 mg/L; thus the product is in Category 4 with an exclamation pictogram.

KEYWORDS

Acute toxicity; Eye irritancy; Clinical signs and observation; Oral acute toxicity; Dermal acute toxicity; Inhalation acute toxicity; Eye irritation; Eye corrosion; Lethal medium dose (LD_{50}); Lethal medium concentration (LC_{50}); Probit analysis; Fluorescein staining for corneal damage; Slit lamp microscopy; Corneal pachymetry; Confocal microscopy; Alternative toxicity test methods; Bovine corneal opacity permeability (BCOP) assay; Isolated chicken eye test; EpiOcular assay; HCE-T TEP assay; MDCK PL assay; Tier testing strategy; Driaze's test for eye irritation; EU registration, evaluation, authorization, and restriction of chemicals (REACH); Classification, labeling, and packaging of chemicals (CLP); Globally harmonized system (GHS) of classification and labeling of chemicals

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23 Genetic Toxicology

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CONTENTS

INTRODUCTION

Genetic toxicology addresses the identification, analysis, and management of agents with toxicity directed toward the hereditary components of living organisms. A large proportion of human disease is either directly or indirectly associated with altered genetic information. Although many agents are capable of indirectly altering DNA functionality at excessively high exposure concentrations, the primary objective of genetic toxicology is to identify and assess genetic

hazard from agents that specifically interact with nucleic acids or are capable of altering the expression of genomic information. Such agents are classified as *genotoxic*.

The term genotoxic is a general descriptor used to distinguish chemicals that directly change normal genomic function from those that do not; however, categorization of a chemical as genotoxic is not a priori an indication of a health hazard.[1]

Genotoxicants are characterized by several properties including those which alter (1) the nucleotide sequence of genes, (2) chromosome structure, (3) chromosome number, or (4) expression of genes. Change in nucleotide sequence is described as *mutation*, structural chromosomal damage is referred to as *clastogenicity*, change in chromosome number is called *aneuploidy*, and change in gene expression in the absence of any alteration in DNA sequence is identified as an *epigenetic* effect. These major classes of genotoxic damage are responsible for an array of human genetic diseases, congenital malformations, and key steps in cancer initiation (Table 23.1).

Genetic toxicology began in the late 1960s with concerns raised regarding potential genetic and reproductive effects arising from man-made chemicals entering the environment. Early objectives of genetic toxicology were focused on the integrity of the human reproductive process and the induction of new transmissible DNA damage. Test methods employed initially were largely in vivo and oriented toward the detection of alterations to germ cells. At the end of the 1960s, reports from Bruce Ames and other investigators were available showing a strong relationship between results in tests for genotoxicity and bioassays for rodent carcinogenicity.[2–4] More recently, attention has been drawn to a class of agents that do not change the primary structure of DNA, but alter phenotypes through environmentally induced alterations in gene expression patterns.[5]

Over the past 40 years, genetic toxicology testing has evolved to a point where it now plays a dual role in safety evaluation programs. One role is the identification and risk assessment of genotoxic agents capable of altering the integrity of the human genome. The second role is the application of genetic toxicology data to achieve a better mechanistic understanding of chemicals that produce somatic cell diseases such as cancer.

BASIC GENETIC CONCEPTS

Gene Structure

DNA is responsible for the hereditary characteristics of all living systems, with the exception of some viruses that use RNA. Even those organisms that store their hereditary information in RNA go through a DNA intermediate during replication. The structure and biochemical characteristics of human DNA has been summarized by Baltimore.[6]

The basic functional unit in a DNA molecule is termed a *gene*. Most of the early knowledge concerning structure and operation of genes was acquired from studies with bacteria or bacteriophages. Advances in understanding the molecular biology of mammalian cells have resulted in equivalent information in eukaryotic cells. The nucleotide composition and the mechanisms by which information encoded in a gene is transformed into gene products are universal. Universality was established through recombinant DNA engineering studies, which demonstrated that genes continue to function properly after having been transplanted from human cells to bacterial cells or from bacterial cells to plant cells.[7,8]

DNA found in prokaryotic (bacteria) and eukaryotic cells (plant and animal cells) differs in a number of ways. In prokaryotic cells, DNA forms a single *chromosome* with little or no substructure along the molecule. DNA in eukaryotic cells, on the other hand, is organized in combination with structural and regulatory proteins (*histones*) into highly differentiated chromosomes. The basic structure of chromosomes is similar across species with most having their genomic DNA distributed across multiple chromosomes. Chromosomes contain genes with functional coding sequences called *exons* separated by nonfunctional, repeat DNA sequences and regions of noncoding DNA called *introns*. The exact role of intron regions is not known, but in the human genome, the numbers of introns and exons are roughly equal.

In eukaryotic cells, the process of gene transcription is controlled by regulatory genes.[9] There are two major types of regulatory regions: promoters and enhancers. Promoters are found immediately adjacent to genes and contain specific DNA sequences (e.g., TATA) that serve as recognition sites for polymerases and other proteins needed to initiate transcription. During *transcription* (reading of the DNA code into mRNA), RNA polymerase transcribes both exons

TABLE 23.1
Examples of Effects from Genotoxic Agents

Mutation Type	Examples of Inherited Effects	Examples of Somatic Effects
Single base changes	Sickle cell disease, Phenylketonuria Hemophelia	Epithelial cancers, activation of ras oncogenes
Small deletions and/or Translocations	Duchenne muscular dystrophy	Lymphomas, leukemias, enhanced activation of Myc, abl oncogenes
Whole chromosome losses or gains	Down's syndrome (trisomy 21) Turners syndrome (monosomy X)	Loss of tumor suppressor genes, retinoblastoma Wilm's tumor, breast cancer

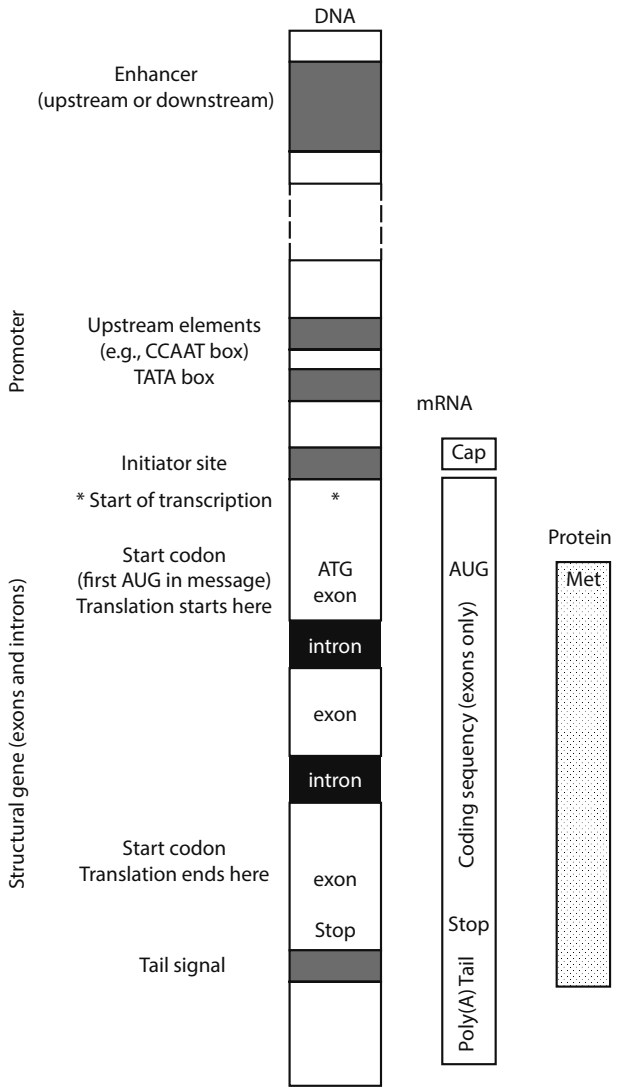

FIGURE 23.1 Gene structure, function, and regulation.

and introns into pre-mRNA. Proteins called spliceosomes located in the nucleus of the cell excise intron regions and splice the coding sequence together. The resulting mature mRNA is then transported to *ribosomes* outside the nucleus for *translation* (reading of the mRNA into polypeptides) (Figure 23.1). In some instances, the resultant polypeptides undergo posttranslational modification (glycosylation, hydroxylation, proteolytic cleavage) depending on their cellular functions. Enhancers are specific DNA sequences that function to enhance transcription but are not necessarily adjacent to the genes they affect. Enhancers may occur on either strand of the DNA and affect genes located hundreds or thousands of bases away.

Somatic and Germ Cell Characteristics

From a genetic perspective, multicellular eukaryotic organisms are composed of two cell types: somatic cells and germ cells. Somatic cells constitute the major portion of the mammalian organism. The adult human body is estimated to be made up of 10^{14} somatic cells. The number of germ cells (ova) is fixed by sexual maturity in females, while males continuously produce male germ cells (sperm) from spermatogonial stem cell populations. The total DNA composition (*the genome*) of most somatic cells is found in the *diploid* (two complete sets of chromosomes) configuration. Virtually all in vitro mammalian cell assays used in genetic toxicology employ somatic cell types. Germ cells, or gametes, constitute a special cell population in multicellular organisms. Germ cells are derived from diploid stem cell, and following meiosis, they are left with a single (*haploid*) set of chromosomes. Their function is to pass genomic information from one generation to the next. Mutations found in germ cells produce a broad array of heritable genetic diseases, congenital malformations, and predisposition to other disorders. Mutations found in somatic cells are generally less damaging unless they result in the initiation of malignant cell transformation.

Mutation and Human Disease

The biological consequences of DNA damage are dependent on which type of cell (somatic or germ) carry the alteration. The mutational basis for many human disorders and anomalies is well documented.[10] The human genome contains preexisting mutations forming what is called the *genetic disease burden*. The extent of the preexisting genetic disease burden is estimated to be as high as 5% (Table 23.2). Diseases associated with this genetic burden contribute significantly to the healthcare costs of most developed countries and the high mortality rates in less well-developed regions. The precise origin of preexisting DNA alterations found in the human gene pool is unknown, but Table 23.3 provides some of the accepted sources for the genetic disease burden. Although human mutagenicity has not been demonstrated in epidemiology investigations, experimentation in animal models amenable to the study of transmissible mutations provides convincing evidence that environmental genotoxicants would be capable of inducing transmissible mutations in human germ cells.[11,12] Genetic changes in somatic cells are associated with a range of dysfunctions, including inherited and induced tumors,[13] teratogenesis,[14] reproductive failure,[15] and atherosclerosis,[16] and appear to be involved in aging.[17]

It is estimated that the human genome consists of approximately 25,000 genes controlling all aspects of an organism's biology and behavior. Our knowledge of the function of these genes remains limited, but research continues to identify functionality at an increasing pace. As of 2008, the *Online Mendelian Inheritance in Man* database established in 1997 by Dr. V.A. McKusick reported 387 genes of known sequence with a known phenotype and 2310 human phenotypes with a known molecular basis.

The deleterious consequences of germ cell alterations vary according to the nature of the alteration. Other than certain DNA alterations (e.g., balanced chromosomal translocations or dicentric chromosomes) that are compatible with cell replication, the vast majority of damage to chromosomes

TABLE 23.2
Composition of the Existing Human Genetic Burden

Type of Genetic Alteration	Incidence per Thousand Newborns (Approximate)	Examples of Diseases Associated with Alteration
Chromosomal abnormalities (e.g., translocations, aneuploidy)	6.9	Down's syndrome (trisomy 21) Klinefelter's syndrome (XXY) Cri du chat (chromosome deletion)
Autosomal dominant mutation	2.5	Familial polyposis (colon cancer) Huntington's disease Retinoblastoma
Autosomal recessive mutation	2.5	Sickle cell disease Phenylketonuria
X-linked recessive mutation	1.7	Duchenne muscular dystrophy Hemophilia
Polygenic or congenital effects	30	Cleft lip Spina bifida
Total	43.6	

TABLE 23.3
Probable Sources of Preexisting Mutations Found in the Human Genome

Spontaneous errors occurring during normal DNA replication and repair
Unavoidable environmental exposures (e.g., food, radiation, products of combustion, mycotoxin, pesticides, manufacturing emissions)
Therapeutic treatments that are directly mutagenic (e.g., radiation or chemotherapy)
Effective treatment of formerly lethal genetic diseases thereby elevating the probability of their contribution to the gene pool
Production of genotoxic by-products of normal oxidative metabolism processes (i.e., reactive oxygen species, lipid peroxidation)

(i.e., breaks or deletions) results in lethality to the respective germ cell or to the embryo derived from that germ cell. Consequently, most genetic damage produced in human germ cells is observed as reproductive failure (e.g., spontaneous abortion). A much smaller proportion of the damage is of a type that is transmissible. Newly generated dominant gene mutations will be expressed in the first generation (F_1). The impact of dominant mutations on the gene pool (*all genes contained in a given population*) is generally limited because the *affected* individuals are aware that they are carrying the mutant form of the gene and are aware that they can transmit the mutant gene to their children. Thus, depending on the severity of the disease or effect, parents can decide, prior to reproducing, if the health risk associated with possible transmission to a child is acceptable.

Unlike dominant mutations, recessive disease mutations will not be expressed unless both alleles of the gene pair are mutant. Two phenotypically normal heterozygous carriers for a recessive mutant allele (on autosomal chromosomes) will theoretically produce offspring that have a 25% incidence of exhibiting a recessive disease. Additionally, 50% of their offspring will be the same phenotypically normal heterozygous carriers as the parents. The frequencies of recessive mutations in the gene pool are maintained by the phenotypically normal heterozygous carriers. An increase in the incidence of new recessive mutations poses the most serious threat to human populations as these mutations are usually not expressed immediately after induction. New recessive mutations tend to accumulate in the gene pool in the heterozygous configuration. Due to the resultant generational latent period, the ultimate expression of a *new* recessive mutation in the population may have no apparent association with the environmental exposure that induced it until several generations later. This situation severely limits the opportunity to use human epidemiological studies to detect human genetic risk.[18,19] Finally, sex-linked recessive mutations are alterations on the X chromosome and appear as dominant mutations when transmitted to male offspring as the Y chromosome has no corresponding alleles to offset the effects of the X-linked mutation.

The discipline of genetic toxicology initially relied upon a set of in vivo tests focused on the detection of DNA damage to germ and somatic cells of rodent species with an intent to extrapolate the results to human hazard and risk assessments.[15,19,20] Early testing approaches employed rodent assays, which detect dominant lethality (implantation failure or early embryonic lethality), heritable translocation induction (semisterility), and specific locus mutation induction in somatic and germ cells.[21] Genetic risk assessment using an extrapolation approach will be discussed later in this chapter; however, the lack of a set of known human mutagens makes the validation of this approach difficult. As a consequence, alternative approaches have been developed for assignment of heritable mutation risks to humans.[22]

In summary, deleterious genes (diseases) are present in the human gene pool with predictable rates. The origin of this genetic disease burden in humans is not completely known, but it is imperative that we, as current caretakers of the human

gene pool, use all precautions to transmit the gene pool to the next generation in no worse shape than it was received.

Carcinogen–Mutagen Relationship

During the late 1960s, a substantial amount of scientific evidence demonstrated that most chemical carcinogens, or their metabolites, had electrophilic properties and would directly bind to DNA.[23] Early studies of carcinogen binding used purified DNA and did not directly demonstrate a relationship between the binding, mutation formation, and cancer initiation. The introduction of a *Salmonella* assay for detecting mutation (Ames test), combined with an in vitro metabolic activation system, appeared to offer a rapid, inexpensive method to identify DNA-reactive/DNA-binding chemicals capable of inducing mutation and cancer.[2,24] The Ames test was the forerunner of a broad array of in vitro submammalian and mammalian cell assays that were later proposed as rapid screens for carcinogens.[3,25]

The relevance of genotoxicity assays as predictors of carcinogenicity was later supported by studies demonstrating that normal, nonmalignant mammalian cells could be transformed into cancer cells by transfecting them with DNA isolated from malignant cells.[26] Ultimately, specific genes were identified in the transfected DNA that, when mutated, were responsible for the transformation.[27] These *cancer* genes (*oncogenes*) are highly conserved genes in most eukaryotic organisms. Oncogenes (e.g., *ras*, *myc*) are activated by nucleotide substitution mutations or chromosome breaks[13,28] in specific exons. The spectra of nucleotide substitutions induced may be characteristic of a particular carcinogen. In vivo experiments with mice documented that nucleotide changes in K-*ras* oncogenes isolated from tumors induced by some chemical carcinogens are agent specific and serve as signatures specific to that chemical.[29] Identification of the signature nucleotide changes in *ras* gene sequences isolated from tumors of humans exposed to the chemical in question are then able to link the human tumor to prior exposures.

Other cancer genes called *tumor suppressor* genes (e.g., p53) have been identified. These genes in their normal configuration produce proteins responsible for suppressing tumor cell development. DNA mutations that result in the inactivation of the suppressor proteins will result in increased tumor development. One specific tumor suppressor gene, the p53 gene, is involved in the process of apoptosis and has been used as the basis of a short-term model of tumorigenesis in transgenic mice made heterozygous for the p53 gene.[30] This model primarily detects mutagenic carcinogens through mutation-induced inactivation of the single normal p53 allele.

Initial optimism that genotoxicity tests might be a shortcut to animal and/or human carcinogen prediction was generated by studies correlating the results of specific genotoxicity tests (or batteries of tests) with rodent bioassay responses for the same chemicals. By 1974, concordance between rodent carcinogenicity and results from the Ames test, alone, appeared to be as high as 90%–95%, and a paper published by Ames et al.[24] was confidently titled *Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection*. However, as more chemicals were added to the database of Ames test results over the next decade, the predictivity declined, and by 1984, the concordance between the Ames test and rodent bioassay results dropped to just over 60%.[31] There are several reasons for the reduction in concordance[32]; however, the factor that had the most influence was an expansion in the sets of chemical classes used in the comparisons. Reports in the early to mid-1970s showing high concordance were based on sets of chemicals highly biased toward inclusion of electrophilic carcinogens. Later studies included a heterogeneous range of animal carcinogens, including those that were not electrophilic. These *epigenetic carcinogens* failed to give the appropriate responses.[33] The initial response to the declining concordance was an expansion in the range of different tests used to evaluate chemicals. This leads to the development of test batteries, which included the Ames test plus other microbial- or cell-based assays.[3] The result of this action was an increase in overall sensitivity (more positive responses for carcinogens) but an even greater decline in concordance specificity (fewer noncarcinogens without a positive response). The analysis of the declining concordance between short-term tests and animal cancer bioassays leads to the conclusion that genotoxicity tests, particularly in vitro ones, were good in identifying carcinogens but were less reliable in predicting noncarcinogenicity. This issue is clearly evident in the result provided in Table 23.4, which shows that test batteries generally improve correlation sensitivity but have too many false positives to be of value in identification of noncarcinogenic chemical. In fact, most chemicals classified as noncarcinogens by agencies such as the National Toxicology Program (NTP) and the International Agency for Research on Cancer (IARC) produced positive responses in one or more tests in a battery of in vitro genetic assays, suggesting that the specificity of

TABLE 23.4
Predictivity Performance of a Battery of Three Common In Vitro Assays

Characteristic	Ames (%)	Mouse Lymphoma (%)	Chromosome Aberrations (%)	All Tests Combined (%)
Sensitivity	58.8	73.1	65.6	84.7
Specificity	73.9	39.0	30.8	22.9
Concordance	62.5	62.9	67.8	—

in vitro tests is too poor to rely upon individual tests or test batteries to predict carcinogenic profiles for new molecules.[31] Addition of in vivo results to the process of predicting carcinogenicity with tests detecting DNA damage (e.g., mouse micronucleus) also failed to improve the overall predictive performance.[33] The Ames test has the best overall combination of sensitivity and specificity and may be the most effective screen for carcinogenic activity. The claim of accurately predicting animal carcinogenicity, and noncarcinogenicity, using in vitro genotoxicity tests has not yet been fulfilled.

A valuable application of genetic toxicology results to cancer risk estimation has been the use of genotoxicity data by regulatory agencies as a key component in determining the mode of action (MOA) for carcinogens.[34,35] Carcinogens with positive effects in genetic tests are considered to have an MOA through direct effects on DNA. Carcinogens that test negative for genotoxicity are presumed to produce tumors through one of several epigenetic MOAs. The U.S. Environmental Protection Agency (EPA) has developed a decision tree approach, which uses genetic test results to aid in the selection of the most appropriate data extrapolation model for cancer risk assessment.[36] A genotoxic mechanism implies a no-threshold mechanism whereas a threshold may exist for epigenetic MOAs.

An important factor affecting an appreciation for the complexity of DNA alterations and accurate carcinogen predictivity is the need to define epigenetic mechanisms and develop tests to detect agents that induce them. Several diseases, including cancer, are associated with aberrant DNA methylation.[37,38] Inclusion of tests for epigenetic effects would increase the predictivity of tests assessing genotoxicity and move short-term testing closer to the goal of carcinogen identification.

MECHANISMS OF GENOTOXICITY

Background and Spontaneous DNA Alterations

DNA synthesis and replication are not flawless processes, and in rare instances, genetic alterations occur spontaneously during regular cell division. In addition, aerobic metabolism produces reactive by-products (e.g., hydroxyl radicals, formaldehyde) capable of damaging DNA. Peroxisomes compartmentalize oxidative metabolism leading to reactive products that would otherwise be detrimental to the cell, although under certain conditions, these products may be released. The vast majority of oxidized DNA base lesions are repaired by DNA repair systems, but in certain situations of excessive toxicity, unrepaired endogenous DNA damage is a cause of background or spontaneous mutation.

Background mutation can also arise from exposure to environmental agents such as radiation (e.g., radon gas, cosmic, solar), chemical pollution (e.g., combustion hydrocarbons), and diet. Perturbation of endogenous DNA damage or disease-related inflammation (e.g., osteoarthritis[39]) will result in increased levels of mutation and chromosome damage. Although DNA damage due to endogenous processes and other natural sources can be minimized, it cannot be totally eliminated.

Classification Scheme for Genotoxic Effects

DNA damage may be classified into several broad categories based on the nature (presumed mechanism) of the DNA effects (Table 23.5).

DNA binding and nucleotide changes are alterations occurring at the nucleotide level. Nucleotide damage generally produces point mutations through base-pair substitution or insertion/deletion. Point or gene mutations are generally induced by agents that specifically target nucleophilic sites on individual bases in nucleic acids. The normal DNA base pairings are shown in Figure 23.2. Adenine and thymine form two hydrogen bonds, and guanine and cytosine form three. Hydrogen bonds are weak electrostatic forces involving oxygen and nitrogen atoms at specific sites on the purine and pyrimidine molecules. When electrophilic chemical species covalently bind (*adduct*) to portions of the DNA bases involved in the formation of hydrogen bonds, the resultant structures (Figure 23.3) can produce electron shifts from the H-bonding sites to areas within the molecules giving rise to opportunities

TABLE 23.5
Major Categories of DNA Damage

Mechanism	Description
Covalent binding	A chemical bond formed when electrons are shared between two atoms. Electrophilic agents form covalent bonds with DNA. Chemicals that form covalent bonds alter the normal electron sharing between DNA base pairs (i.e., G:C) leading to mispairing and gene mutation.
Intercalation	Insertion of an agent between base pairs in double-stranded DNA. This deforms the DNA configuration and adversely affects processes of DNA replication and repair. The typical alteration generated is a frameshift mutation. Intercalation does not involve covalent bonds.
Cross-linking	Agents that can form covalent bonds with multiple sites on the DNA may form interstrand or intrastrand cross-links that prevent normal DNA replication that leads to breaks in the DNA backbone.
DNA strand breakage	SSB or DSB are produced by cross-linking agents as well as reactive oxygen species. DNA breaks can produce chromosome aberrations.
Epigenetic	Agents that alter gene expression without changing the base sequence of DNA are classified as epigenetic genotoxicants. Examples include DNA methylation and histone protein acetylation and deacetylation.

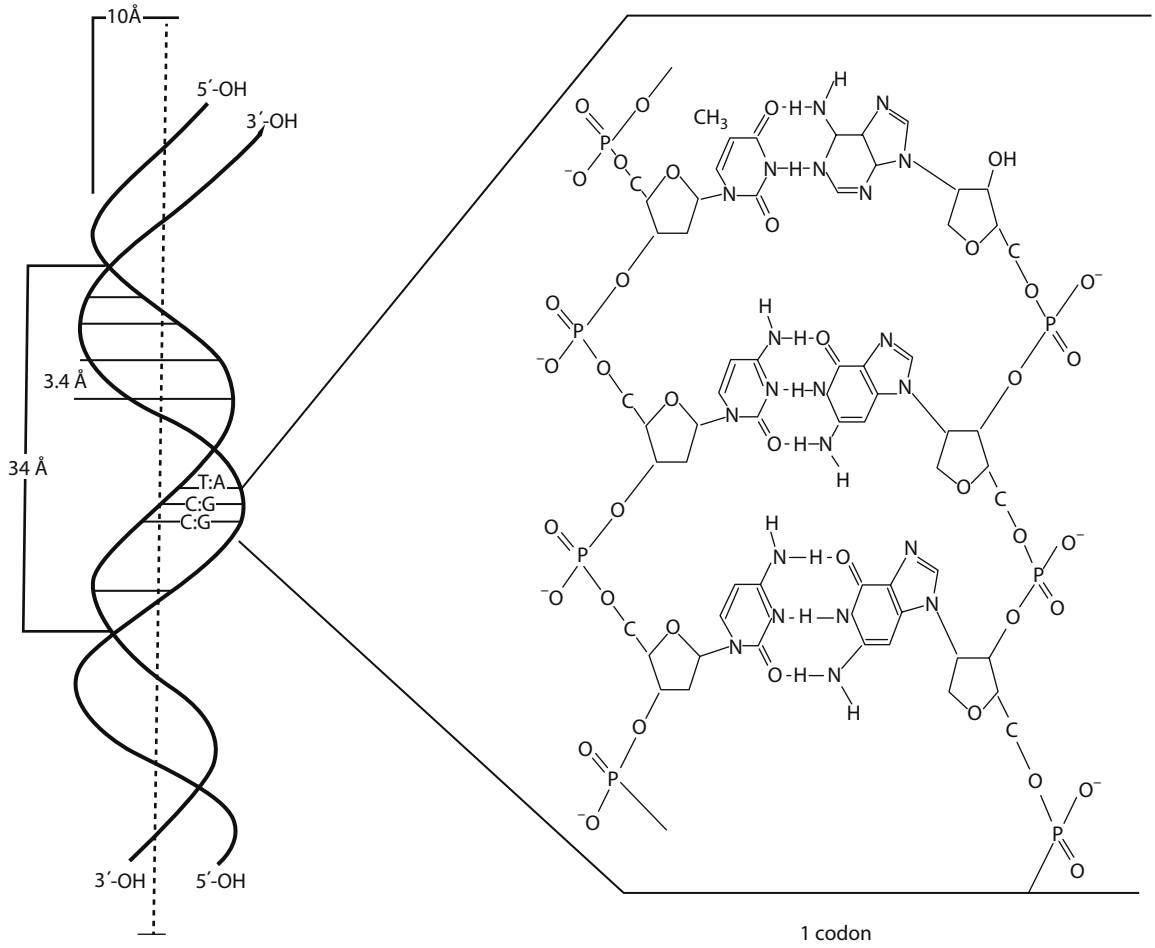

FIGURE 23.2 Structure, hydrogen bonding, and helical configuration of DNA.

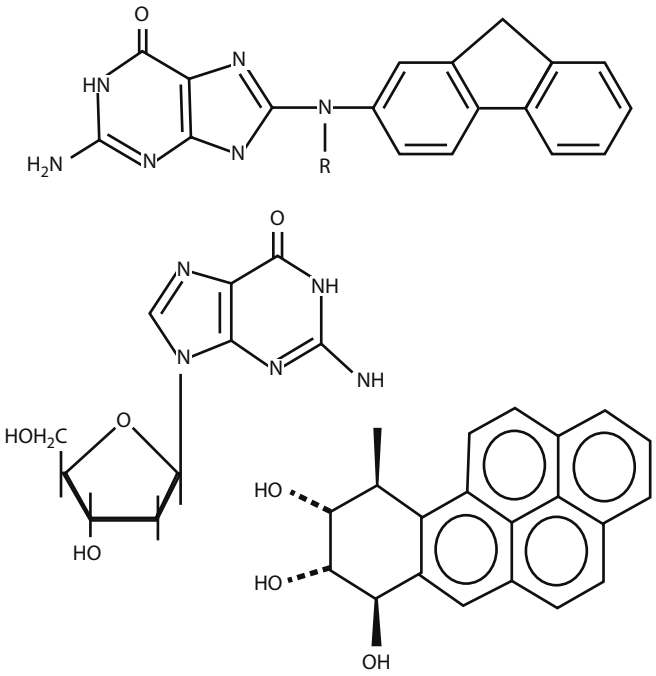

FIGURE 23.3 DNA adducts. Top: aromatic amine adduct of guanine. Bottom: benzo(*a*)pyrene adduct of guanine.

for short-lived mispaired bases (e.g., A:C or G:T). If this aberrant pairing occurs before or during a DNA replication cycle, the result may be the substitution of a new base pair in place of the original one. One cycle of DNA replication is needed to fix the error in daughter DNA strands and to create base-pair substitution mutations. This process is referred to as the *expression period* in mutation assay protocols. Base-pair addition/deletion mutations, also called *frameshift mutations*, result from the addition or deletion of one or a few nucleotide pairs from the nucleotide sequence in an exon or gene. Because the codon sequence reads in one direction and is not punctuated, the loss or gain of a single base pair changes the reading frame of the gene—hence, frameshift mutation. The deletion of base pair shifts the reading frame to the right, and the addition of base pair shifts it to the left. This type of mutagenic mechanism is illustrated in Figure 23.4. Both frameshift and base-pair substitution gene mutations result in alterations in translation of mRNA into the proper amino acid sequence in the gene products producing a mutant cell or organism. Base-pair substitution and frameshift mutations are responsible for both human genetic diseases and cancer initiation in proto-oncogenes and tumor suppressor genes.

Detection of DNA binding or even specific adducts is not equivalent to the detection of mutation.[40,41] When electrophilic

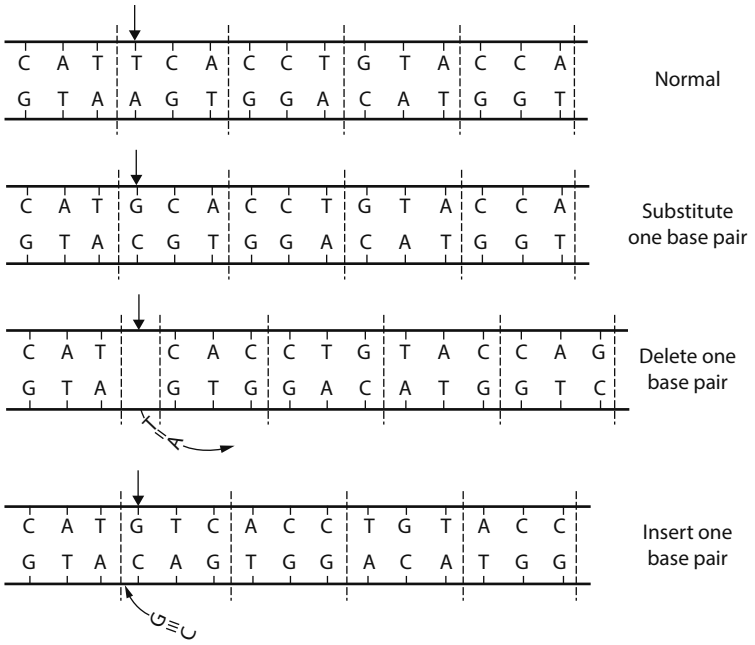

FIGURE 23.4 Base substitution and frameshift mutation mechanisms.

chemicals react with DNA, they form both unstable and stable adducts. Unstable DNA adducts tend to be removed forming abasic sites in the DNA that are generally repaired. Stable adducts can result in mispairing and mutation. The ratio of stable to unstable adducts differs by chemical even among closely related structures. Consequently, adduct levels, per se, are not an accurate indicator of mutagenicity. However, adduct detection in vivo is a sensitive method to demonstrate exposure to electrophilic agents. Unfortunately, not enough is known about adduct processing and repair for this technique to accurately define genetic or cancer risk.[42] At the present time, the qualitative and quantitative relationships between DNA adduct formation and mutation are known for only a small number of agents. It is known that organs and tissues in mammals have different capacities for repairing adducts, and that some adducts, such as the O-alkyl adducts, are repaired less efficiently than N-7 adducts, for example, and are believed to produce most of the mutagenic damage responsible for tumor initiation.[40] In summary, when comparing different carcinogens, no quantitative correlation exists between the level of DNA adduct formation and mutation.

DNA breakage, *chromosome aberrations*, and *abnormal chromosome segregation* involve the structural integrity of chromosomes as well as chromosome number. Clastogenic effects are visible through cytologic analysis of condensed chromosomes. During most of a cell's existence, DNA is packaged in the nucleus of somatic cells as uncondensed chromatin and is not visible except during mitosis when chromatin is condensed. Figure 23.5 illustrates the generalized anatomy of a chromosome at metaphase. The condensed chromosome shows distinctive banding with Giemsa staining. It contains a constricted region known as the centromere. To either side of the centromere are the chromosome arms that terminate in unique sequences called *telomeres*. The darker bands (G+ bands) in the stained chromosome represent condensed DNA (heterochromatic) believed to represent areas of little or no gene expression (transcription). The lighter bands (G− bands) represent relaxed DNA sequences believed to be active genomic areas where gene expression (transcription) is occurring. The telomeric regions at the ends of the chromosome structures are important for chromosome stability and cell longevity.[43] Telomeres consist of repetitive sequence of a small number of nucleotides (TTAGGG) that protect the ends of chromosomes from fusing. During somatic cell replication, telomeres are not completely replicated. They are gradually shortened, and when the telomeres reach a critical threshold, cell division stops in what is called *replicative senescence*.[43,44] The enzyme telomerase (a reverse transcriptase) can replace lost telomeric DNA. Telomerase is almost nonexistent in somatic cells but is found in embryonic stem cells and tumor cells.

Chromosomal effects scored microscopically are the result of damage to the integrity of the DNA molecule producing strand breaks, either single-strand breaks (SSBs) or double-strand breaks (DSBs). DNA strand breakage can be the result of exposure to reactive oxygen species or cross-linking DNA adducts. Chemicals that interfere with DNA synthesis and DNA repair processes may also produce increased levels of DNA strand breaks. In addition to DNA-reactive agents, chromosome alterations are also produced by secondary mechanisms, especially in vitro, by extreme shifts in cellular homeostasis such as high temperature, low pH, or osmotic changes.[45–47]

Damage to chromosomal DNA can be subdivided into changes in chromosome number resulting in aneuploidy (gain or loss of less than a complete set of chromosomes) or

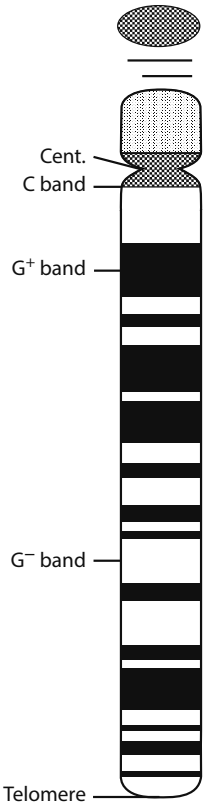

FIGURE 23.5 Diagram of chromosome G⁻ bands are areas of light staining indicating active transcription. G⁺ bands are highly stained areas with minimal transcription.

polyploidy (multiple full sets of chromosomes)[48] or changes in chromosome structure (breaks, deletions, rearrangements). A wide range of in vitro and in vivo genetic test methods are available to assess numerical changes and structural breaks. Since most chromosome breakage of the types scored in aberration analyses results in cell death, the breaks and gaps that are found serve as signals indicating the probable induction of other more stable and, therefore, more relevant chromosome damage such as balanced translocations, dicentric chromosomes, stable deletions, and numerical changes such as aneuploidy. It is these stable aberrations that are associated with human genetic diseases and cancer.

Sister chromatid exchange (SCE) is another type of chromosomal event, which can be visualized in cells by using a specific bromodeoxyuridine staining process; however, SCEs are exchanges between identical DNA chromatid sequences and are not, per se, a DNA alteration resulting in any known adverse outcome. SCEs occur normally but are increased by genotoxic agents and can, in a manner similar to DNA adducts, be used as a dosimeter of exposure, but not as a predictor of mutation or cancer.

DNA changes not altering the primary sequence affect gene expression and, in some cases, such as genetic imprinting, the effects are transmissible. DNA methylation and histone protein acetylation are two epigenetic changes that result in gene expression and contribute to genetic-based diseases.[49–51] Epigenetic phenomena play a key role in regulating gene expression.[49] One of the most widely studied epigenetic modifications is DNA methylation at cytosine residues in gene promoters. Genomic imprinting refers to methylation of genes that results in monoallelic expression depending on the parental origin. This is typically accomplished by methylation of specific genes. An individual normally has one active copy of an imprinted gene. Improper imprinting or aberrant methylation can result in an individual having two active copies or two inactive copies. Although only a minority of human genes are imprinted, unscheduled methylation can lead to altered expression producing severe developmental abnormalities, cancer, and other problems.[39] Some environmental agents (e.g., cadmium, folate, zinc, and nickel) have been shown to alter DNA methylation patterns.[37]

In eukaryotes, genomic DNA is packaged with histones to form chromatin, which in turn condenses to form more compact structures. The condensation of chromatin affects processes requiring access to the DNA, such as transcription, replication, DNA repair, or recombination. A second epigenetic process is associated with acetylation of histone proteins found in chromosomes. Acetylation of chromosomal proteins is produced by two types of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs acetylate histones, while HDACs deacetylate histones, and together they regulate transcription profiles for specific functions. Acetylation of the lysine residues at the N terminus of histone proteins removes positive charges, thereby reducing the affinity between histones and DNA.[51] This makes RNA polymerase and transcription factors easier to access the promoter region. Therefore, in most cases, histone acetylation enhances transcription, while histone deacetylation represses transcription. Research has demonstrated that histone deacetylation can repress tumor suppressor gene expression increasing cancer risks.[52] Inhibitors of HDAC (HDACI) are highly effective in upregulating tumor suppressor gene expression, reducing tumor growth and inducing programmed cell death in vitro and in cancer patients in phase I and II clinical trials.[53] Some short-chained fatty acids (e.g., valproic acid), benzamides, and ketones are HDACIs and under investigation as possible anticarcinogenic treatments. Studies with one histone deacetylase (HDAC2) have shown that it can be elevated in mice by amyloid beta, which may then result in downregulation of genes responsible for cognition.[54]

Factors Influencing Genotoxic Hazard/Risk

Repair of DNA Damage

The fidelity and integrity of genetic information in organisms are maintained by several types of enzymatic DNA repair. The characteristic of self-repair is unique to DNA and emphasizes importance of the integrity of this molecule to the survival of an organism. Most components of DNA repair operate constitutively in organisms, although a few repair processes are adaptive (inducible) following cellular exposure to genotoxicants.[55,56]

There are more than 20 different oxidative DNA base lesions involved in the estimated 10,000 oxidative hits, which occur per cell per day in the mammalian genome.[57] Constitutive cellular repair capacities are generally adequate to compensate for the typical endogenous background damage; however, recurrent exposures to high concentrations of exogenous genotoxic agents may saturate the DNA repair capacity, leading to mutation and associated chronic diseases such as cancer and aging.

The common feature of repair processes is their ability to detect, remove, and replace damaged segments of DNA.[58] If a DNA lesion (i.e., DNA strand break or adduct) can be repaired prior to mutation fixation, the net effect of the DNA damage to an organism may be nil. This is especially true following intermittent, low-level exposures to genotoxicants where repair enzymes are not fully saturated. DNA adducts are not all recognized or repaired equally by excision repair.[59]

Test systems measuring some parameter of the DNA repair process have been used as screens for detection of primary DNA damage. Normal organisms are capable of some type of DNA repair activity following chemical insult; thus, stimulation or induction of repair activity following chemical treatment at sublethal concentrations is a good indicator that the target organism has experienced DNA-directed toxicity. DNA repair induction (i.e., induction of unscheduled DNA synthesis [UDS]) can be used as a screen for genotoxicity.[60]

Studies of DNA repair kinetics indicate that once premutational lesions have been induced in the DNA, both error-prone and error-free repair processes are activated. Error-prone systems attempt to maintain DNA continuity and, in the process, may actually generate nucleotide mismatches (e.g., A:C or G:T) that result in mutations. Error-free repair replaces the damaged DNA site with a correct nucleotide sequence. The fidelity of repair depends on the degree to which the two different processes are involved. Factors that determine whether error-prone or error-free pathways predominate include (1) the target organism species, (2) the cell type involved, (3) the chemical mutagen, and (4) the specific DNA lesion induced. Some data suggest that the error-free repair pathways predominate at low exposure levels, and error-prone pathways come into play only following saturation of the error-free enzymes.

Some repair processes directly reverse the damage in a single step. Thymidine dimers (covalently linked adjacent thymidine bases) induced by ultraviolet light exposure are reversed by a photolyase enzyme. Alkylating agents form adducts at the guanine-O^6 position and can be reversed by alkylguanine transferase. This enzyme removes the alkyl adduct from DNA transferring it to a cysteine residue in a single step.[61]

The remaining DNA repair processes involve multiple steps with multiple enzymes recruited to the repair sites as a repair complex. Various proteins (i.e., p53, CHEK1, CHEK2) are also involved in preventing damaged cells from entering S-phase to give repair processes time to remove the damage.[62]

Base excision repair (BER) and nucleotide excision repair (NER) are the primary repair mechanisms for chemical damage. The general processes involved in BER and NER are shown in Figure 23.6. BER corrects damaged bases or

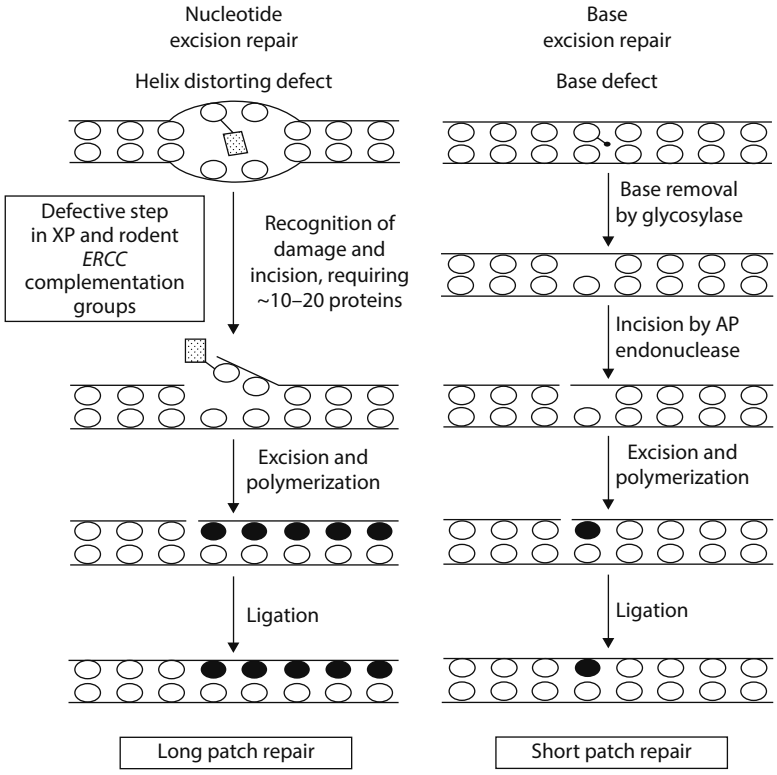

FIGURE 23.6 Diagrams illustrating NER and BER processes.

abasic sites in DNA. In BER, glycosylases also involved in mismatch repair (MMR) catalyze release of the inappropriate or damaged base followed by replacement and ligation.[63] The enzyme complex responsible for NER of bulky adducts requires more than 20 proteins and consists of several steps.[64] An endonuclease cleaves the DNA at the site of the damage, an exonuclease cuts out the damaged region including nucleotides to either side (i.e., 25–30 nucleotides), and new bases are replaced by a DNA polymerase using an editing function to ensure that the correct bases are incorporated into the repair patch. Finally, DNA ligase seals the repair patch. NER serves as the genome on a global basis (scanning the entire length of the DNA for damage) in addition to repair localized around DNA sites actively engaged in transcription where transcribed genes are scanned by translocating RNA polymerases, which sensitively detect DNA damage and initiate transcription-coupled repair (TCR), a subpathway of NER.[65] Occasionally, even in error-free repair, DNA polymerase can incorporate incorrect bases leading to mismatched pairings that do not properly hydrogen bond and escape proofreading. Failure to detect and repair these mismatches leads to general genome-wide increases in gene mutation. A specific repair process identified as mismatch repair (MMR) uses proteins that recognize aberrant DNA pairing caused by replication errors and targets the newly synthesized DNA strand. A short segment of the DNA duplex is excised and filled by the repair polymerase.[66] This is a second-chance repair process that occurs after BER and NER and improves the accuracy of those processes. Deficiencies in MMR proteins are associated with increased cancer risk.

Several less well-studied repair systems have been identified, one of which is specific for sealing (ligase) SSBs after remodeling of the opened ends by poly(adenosine diphosphate [ADP]-ribose)polymerase and another that repair interstrand DNA cross-links that block DNA synthesis at the replication fork. The repair enzymes break the DNA at the site of the cross-link followed by restoration of the replication fork, which removes lesions from the template DNA strands of actively transcribed genes.[67] Table 23.6 summarizes the major DNA repair processes that exist in organisms.

Theoretical assumptions and data from studies of repair in many species support the belief that at background or low exposure levels, an error-free removal of adducts from DNA can be virtually 100% effective, which supports the arguments for thresholds in cancer initiation.[68] Thus, one observes survival shoulders and nonlinear kinetics for mutation induction in repair-proficient cells and the loss of apparent no-effect regions in repair-deficient cells.

Genetic Susceptibility to Genotoxic Damage

Gene polymorphisms affecting traits such as xenobiotic metabolism and DNA repair capacity are likely to influence human susceptibility to genetic damage. Identification of gene polymorphisms that influence the susceptibility of an individual to the pharmacologic and toxic effects of therapeutic agents has developed into a major field of medical genetics. Institutions have assembled alleles from hundreds of polymorphic genes, and large human populations can be screened for single-gene polymorphism with automated high-throughput screening systems employing techniques such as DNA binding and transcription activation. Studies in humans exposed to polycyclic aromatic hydrocarbons (PAHs) documented a genotypic influence on the level of adducts. Polymorphism in GSTMI 2 (glutathione transferase), CYP1A1, and CYP2D6 alleles all affect the formation of DNA adducts found in white blood cells of exposed individuals.[69] Individuals expressing different polymorphic forms of the same genes can vary significantly in their response to a toxic agent. Unlike the genetic uniformity desired in experimental models in toxicology testing, the diversity associated with metabolic and DNA repair kinetics in human populations is sufficient to influence the degree of risk from exposure to genotoxicants.[64,70,71] Studies comparing the DNA repair capacity of humans with that of experimental animals suggest some significant differences, complicating the extrapolation of genotoxic results from mice to humans.[72]

Additional information about repair genes comes from studies of human genetic diseases such as xeroderma pigmentosum (XP). Individuals with XP lack one of the enzymes in the NER repair process, and affected individuals, with as

TABLE 23.6
Major Mechanisms of DNA Repair

Mechanism	Target DNA Lesion	Process
Direct reversal	Thymidine dimers	Photolyase enzyme converts pyrimidine dimers into two adjacent pyrimidines.
	Methylated bases	DNA methyl transferase removes methyl adducts directly from DNA.
BER	Abasic sites and damaged single bases	Glycosylase enzymes remove altered base and fills the gap prior to ligase sealing.
Excision repair	Large and/or bulky DNA adducts	Multistep process involving damaged region containing multiple bases that are removed and replaced using the other DNA strand as template. The most common repair process and is associated with both global and transcription-coupled DNA repair.
MMR	Erroneously matched base pairs (i.e., A:C or G:T)	Mismatched sites are excised and replaced using existing strand as template and polymerase III. Multistep process. Repaired by MutS, MutL, and MutH proteins.
Recombinational or cross-link repair	DNA cross-links	Not well studied but the repair enzymes break the DNA at the site of the cross-link followed by restoration of the damaged region of the DNA replication fork.

little as 1%–2% of the normal repair capacity, usually experience high levels of intrinsic DNA damage.[73] Cancer susceptibility among XP individuals is believed to be a consequence of the genetic damage and can be as much as 1000 times greater than for non-XP individuals. Other human syndromes associated with reduced repair capacity (e.g., ataxia telangiectasia, Bloom's syndrome, Fanconi's anemia) are inherited traits that also exhibit increased cancer risk.[73] Because DNA repair capacities exhibit such a broad range, genetic effects induced in genetically homogenous animal models with uniform repair can only be extrapolated to human risk estimates with large margins of error. The integration of metabolomics information into a genetic profile may eventually be used to direct an individual's lifestyle optimization.

Lifestyle

It is well documented that occupation and lifestyle can influence genetic hazard.[74–76] Tobacco use and alcohol consumption are both associated with genotoxic risk.[77,78] Diet is also a direct and indirect source of mutagens. The average human consumes about 10 tons (dry weight) of food by the age of 50 years,[79] and genetic toxicology studies have demonstrated that a number of common foods contain substances that are mutagenic.[74,80] Genotoxic agents such as aflatoxin B1, acrylamide, furfural, caffeic acid, and formaldehyde can be present in foods at low levels.

Other lifestyle exposures that may represent important risks are less subject to individual control. Exposure to ultraviolet light and ionizing radiation are common. Ambient air in many industrial and urban areas contains silica dust and carbonaceous particles coated with agents (e.g., aromatic amines and hydrocarbons) producing mutagenic responses in a range of assays.[81]

A possible genotoxicity defense process is linked to agents known as antimutagens. Agents that eliminate or reduce the mutagenic activity of a known mutagen when administered prior to mutagen exposure have been labeled antimutagens. Some investigators suggest that consuming certain foods or vitamins can protect an individual from DNA-damaging agents in the environment. For example, antioxidant materials found in plants (e.g., flavonoids) can reduce the mutagenic activity of chemicals such as ethylmethanesulfonate, benzo(*a*)pyrene, and other common mutagens when studied in vitro.[82] Unfortunately, unequivocal evidence of significant antimutagenic activity for these agents at typical use exposures in vivo has been difficult to demonstrate.

GENETIC TOXICOLOGY TESTING

Since 1970, more than 200 tests have been used to identify agents with genotoxic properties. A perspective on the number of tests used and a method for organizing the data produced by the various tests can be obtained from a review of the genetic activity profile (GAP) program of the EPA.[83] This program collected and analyzed genetic testing results from a wide range of in vitro and in vivo tests.

A relatively small number of those tests have continued to be recommended for routine screening and/or research in genetic toxicology. Strategies developed for the detection and assessment of genotoxic hazard include both in vitro and in vivo tests. Test selection and application is generally determined by characteristics of the test agent. Some tests such as microbial mutation tests (e.g., Ames II, green screen) or in vitro cell assays measuring micronucleated cells with flow cytometry can be conducted using semiautomated methods and are useful to evaluate large numbers of agents for basic genotoxicity classification.[84,85] Other tests such as in vivo evaluations for chromosome damage, induction of DNA repair synthesis, or DNA strand breakage provide more detailed information regarding the impact of route of exposure and in vivo metabolism and detoxification on the genotoxic activity of the test agents. The international scientific and regulatory communities have developed guidance documents outlining strategies for the evaluation of new chemicals, pesticides, food additives, and pharmaceutical products.[86]

Testing Strategies and Data Evaluation

A genotoxic compound may be defined as *an agent that produces a positive response in a bioassay measuring any genetic end point* (e.g., mutation, DNA breaks, clastogenicity). Although this definition considers virtually all forms of damage to DNA to classify an agent as genotoxic, that classification should not be interpreted a priori as an indication of hazard or risk. Additional experimental information beyond this initial classification is necessary to resolve concerns of genetic hazard/risk to somatic or germ cells.

Genetic toxicology assessments should not consist of a single test. The multiplicity of mechanisms that could potentially be involved in genetic toxicity necessitates use of battery of tests that will detect, at least, the primary genotoxic mechanisms. Virtually all regulatory strategies include tests for gene mutation and clastogenicity and require at least one in vivo assay. It is important at the outset of testing to carefully define the objectives desired in a testing program. Screening to prioritize agents for further testing may employ different types of tests than would be used in hazard identification. The general guidance developed by most international bodies considers the overall strengths and limitations of the tests recommended in an attempt to minimize missing genotoxicants with high intrinsic risk.

Compared to conducting the tests, data assessment and interpretation can often be a more complex task. There is minimal guidance regarding interpretation of heterogeneous outcomes from a test battery. The general recommendation for the interpretation of mixed results (either all positive or all negative) is to conduct additional testing until a weight-of-evidence (WOE) assessment of the intrinsic properties of the agent is clearly defined.

Most decisions made for regulatory purposes are based on the response profile from a battery of tests specified by regulations or guidelines.[86] A few computer-based approaches have been developed for evaluating the results of multitest data sets. The EPA GAP approach of Waters et al.[83] and a method published by the International Commission for

TABLE 23.7
Possible In Vivo Follow-Up Tests to Support Isolated In Vitro Positive Responses

In Vitro Test Giving Positive Response	UDS in Rat Liver	Transgenic Mutation Tests	DNA Adducts	Comet Assay	Micronucleus Assay	Chromosome Aberration Assay
Gene mutation	+++	+++	+++	++*	–	–
Structural chromosome Damage (breaks, deletions, rearrangements)	–	+	+	++*	+++	+++
Numerical chromosome damage (aneuploidy or polyploidy)	–	–	–	–	+++ (aneu) ++ (poly)	++ (aneu) +++ (poly)

Source: Modified from Dearfield, K.I., et al., *Environ. Mol. Mutagen.* 52, 177, 2011.
+++ Efficiently detected, ++ detected depending on test design or * not enough data available, + not always detected, – not detected.

Protection against Environmental Mutagens and Carcinogens (ICPEM)[87] were designed to produce WOE classifications for chemicals characterized by large data sets including multiple trials of the same test. In 2006, the U.S. Food and Drug Administration (FDA) provided a guide for the integration of genetic toxicology results into the safety assessment of pharmaceutical products.[88]

The most problematic data evaluation situation is the unique, unexpected positive response found in a battery of otherwise negative results. The positive response may be an important piece of information defining potential hazard or it may be a false-positive result. Distinguishing between the two possibilities is not always a simple task. When the positive response is produced by an in vitro test that can be followed up by an in vivo analog, a decision can be derived from the results of the in vivo test. A well-conducted negative in vivo study often trumps the in vitro positive. This is especially true if the in vitro positive is associated with certain testing features linked to generation of false responses (e.g., excess toxicity, extreme osmolality). Important lessons have been learned that can be applied when testing in vitro that may avoid spurious results and unnecessary additional testing.[89]

Regulatory Guidance for Environmental Agents

Recommendations outlining methods for routine genetic toxicology assays have been published by the EPA,[90] Organisation for Economic Co-operation and Development (OECD),[91] Canadian Health and Welfare,[92] and the European Economic Community.[93] Over the past decade, substantial progress has been made in harmonizing genetic testing requirements as well as the protocols used to conduct the tests. Today, most regulatory test batteries include, at a minimum, (1) the Ames test, (2) a test for in vitro and/or in vivo cytogenetic analysis, and, in some batteries, (3) an in vitro test for gene mutation in mammalian cells. Other tiers and tests may be included to expand the profile on the agent if positive results were obtained in these tests (Table 23.7).[94] It is also possible to add special tests that may be particularly informative for special chemical classes. Table 23.8 summarizes a uniform strategy for genetic testing for environmental agents.

TABLE 23.8
Proposed Tier-Based Testing Strategy for Environmental Chemicals

Level 1	Three in vitro tests: Ames test In vitro gene mutation test in mammalian cells In vitro chromosome aberration test in mammalian cells
Level 2	One or more in vivo tests in mammalian somatic cells: Chromosome aberrations in somatic tissue Micronucleus Comet assay or other in vivo alternative tests (i.e., UDS, DNA adducts, transgenic mutation)
Level 3	In vivo tests in mammalian gonadal tissue (germ cell targets): Chromosome damage (aberrations, micronuclei, SCE) Dominant lethal Transgenic mutation models
Level 4	In vivo intergenerational tests in mammals: Visible or biochemical-specific locus mutation Heritable translocations

Regulatory Guidance for Pharmaceutical Products

A standard core battery of tests for evaluating pharmaceuticals was developed under the International Conference on Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). The ICH process resulted in two guidelines: (1) ICH S2A that provides guidance and recommendations for the conduct of genetic tests and (2) ICH S2B that establishes a standard genotoxicity test battery. In 2008, the ICH revised the recommendations[95] to include two options for a standard test battery:

Option 1

- A test for gene mutation in bacteria
- A cytogenetic test for chromosomal damage (the in vitro metaphase chromosome aberration test or in vitro micronucleus test) or an in vitro mouse lymphoma *tk* gene mutation assay
- An in vivo test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells

Option 2

- A test for gene mutation in bacteria
- An in vivo assessment of genotoxicity with two tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second in vivo assay

This new approach has been accepted for pharmaceutical registration in the United States, Europe, and Japan, and either option is considered appropriate.

In most situations, Option 1 tests include the Ames tests, in vitro cytogenetics using a cell line or cultured human lymphocytes (in vitro micronucleus is also accepted), and a test for micronuclei induction in mouse or rat bone marrow cells. The thymidine kinase heterozygous (TK+/–) mouse lymphoma assay was initially preferred because it can serve as an in vitro measure of both gene mutation and chromosome aberrations since data have demonstrated that induction of mutation at the target gene (TK+) can be produced by either base-pair substitution mutation in the normal allele or by deletions of the allele through chromosome breakage. The result is a tk–/– cell, which can grow in the selective media. Base substitution mutants result in large colony tk–/– mutants, and the deletion mechanism produces small mutant colonies; consequently, use of the mouse lymphoma assay for ICH purposes requires colony sizing.[96] Option 2 is a relatively new approach that includes the Ames test and either a combined micronucleus and comet assay or a comet assay evaluating two tissues (e.g., a presumed target tissue and one other). Other in vivo tests such as metaphase chromosome analysis in rodents, transgenic animals, or UDS may be used, especially for agents with unique testing issues. OECD protocols (as well as draft protocols) are readily available for tests commonly used in either option. The primary reasons for developing a second option was the knowledge that most of the mammalian in vitro tests were too sensitive to secondary effects from toxicity as well as the need to address the goal of reducing unnecessary animal use.

New guidance has also been proposed to detect and evaluate the presence of genotoxic impurities in drug substances (i.e., active ingredients).[97] The guidance recommends reliance upon a combination of structure–activity relationship (SAR) models and results from the Ames test for identification of genotoxic impurities. Limits of impurities permitted in the drug substances are based on the clinical use (duration of exposure) of the drug substance and are set as an allowable daily intake of the impurity.

Limitations of Current Testing Strategies

By 1980, a large number of test methods had been proposed for the identification of genotoxicity. Some involved unique methods or target organisms, but many were duplicative of other tests. In the following decade, extensive efforts were undertaken to validate and evaluate the best test or set of tests for the purposes of detecting genotoxicants. In the process, several valuable lessons were learned:

- Current testing strategies do not cover all genotoxic mechanisms associated with human disease or cancer. Prime examples would be the absence of test to specifically detect aneugens (agents producing aneuploidy) or tests for agents inducing epigenetic responses (acetylation of lysine or methylation of cytosine). It is possible to detect some aneugens with a properly designed micronucleus study.[98]
- Some test methods appear to be hypersensitive to chemicals, which are not directly genotoxic and pose no significant risk. SCE detection is an example of a method that has an extremely high level of sensitivity and can be produced by lifestyle activities such as exercise or stress. The in vivo comet assay that detects SSB and DSB is also susceptible to false-positive responses from excessive toxicity that generates apoptosis at the target tissue site.[99] When conducting in vivo comet tests, it is prudent to include supplemental evaluations for apoptosis and target tissue histopathology to preclude false-positive responses due to secondary toxicity.
- Test methods that worked extremely well in the laboratory of the developer have not always transferred to other testing laboratories. The Syrian hamster embryo (SHE) assay was an example of such a test. The problems surrounding successful transfer of the technology involved acquiring the skill to detect minor variations in scoring transformed colonies and coping with unique specifications for primary embryo cell culture conditions.
- Most mammalian cell in vitro tests are susceptible to false-positive responses generated by nonphysiological treatment conditions, excessive cytotoxicity, or the production of reactive oxidation species generated by S9 mix chemistry.[89]

Several actions were taken in an attempt to resolve these issues. Genetic toxicology testing schemes have been simplified, and most agencies now expect to see the results from a limited test battery consisting of tests for gene mutation and chromosome aberrations before asking for further tests. Further testing is then based on the profile from the limited battery. Treatment conditions and dose level limits for in vitro tests have been spelled out in more detail and modified to minimize false-positive responses.[95] Although data analysis and interpretation methods are not uniformly applied, more guidance is becoming available.

Supplemental Test Methods

Several supplemental technologies may be applicable to filling gaps identified in existing strategies or to provide confirmatory follow-up to equivocal or suspicious positive

responses in basic batteries. Generally, these methods are performed in vivo, which adds a dimension of relevance that is able to put in vitro findings into better perspective:

- The single-cell gel electrophoresis (comet) assay has eclipsed the in vivo UDS assay as the most common follow-up test for positive findings in the ICH core battery (Option 1) and as a primary in vivo test in Option 2. The comet assay measures both SSB and DSB in specific tissues and or cell types.[100,101] The method is extremely versatile and can be used in human monitoring.[102] The protocol is standardized, and the method permits analysis of tissue- or organ-specific genotoxicity not available in most other in vivo assays. By including supplemental analyses for cytotoxicity in the study design, the comet assay can be an extremely valuable test. The relevance of SSB and DSB to the prediction of genetic or cancer risk has not been determined making extrapolation of comet responses to risk uncertain.
- Transgenic animals for mutation detection were developed in 1990 using shuttle vector technology. A few models commercially available in the 1990s, such as the MutaMouse and BigBlue Mouse, measured *lac I* and *lac Z* gene activation.[103] These models include mice and rats, which contain multiple copies of chromosomally integrated shuttle vectors with reporter genes that can be recovered and evaluated for mutation and/or chromosomal damage in vitro. These tests have been proposed as in vivo confirmatory assays *for* in vitro gene mutation (e.g., Ames) responses. The OECD is presently developing standard protocols for transgenic gene mutation models.
- The SHE cell transformation assay has been used in special situations because of its high predictivity of rodent carcinogenesis.[104] The most common application of this assay has been following the discovery of a positive genetic toxicology response for a product, already in human use, that does not have an animal cancer bioassay completed. A negative result in the SHE assay provides some degree of comfort that the positive test result may not be relevant. Difficulties associated with establishing this assay in the laboratory have been discussed earlier.
- A new in vivo gene mutation assay, phosphatidylinositol glycan Class A (PIG-A), is currently under development as a possible confirmatory test for in vitro gene mutation positive responses.[105] The PIG-A gene is located on the X chromosome and codes for a catalytic subunit of the *N*-acetylglucosamine transferase complex involved in an early step of the glycosylphosphatidyl inositol (GPI) cell surface anchor that binds specific protein markers to the exterior cell membrane. Mutation in the gene renders cells deficient in GPI anchors and, as a consequence, deficient in GPI-anchored cell surface markers. Mutant cells (PIG-A) deficient in a GPI-anchor protein called CD59 can be detected using labeled anti-CD59 with flow cytometry.

PIG-A is the only gene involved in GPI-anchor synthesis located on the X chromosome. The preferred method for detecting *PIG-A* mutant cells is the staining of peripheral red blood cells (RBCs) with fluorescently labeled anti-C59 antibodies followed by flow analysis. Both white and red blood cells can be used for the assay; however, larger sampling volumes with RBCs favor the use of this cell type. Early studies indicate that base-pair substitutions, deletions, and possibly other end points can be detected. The assay has several advantages including (1) a low spontaneous background ($<5 \times 10^{-6}$), (2) a cycle of time of several hours using flow analysis methods, (3) minimal blood volumes required when performed on RBCs, and (4) the ability to detect gene mutations in humans as well as rats. The method appears promising; however, the location of the gene on the X chromosome may suggest gene regulation processes unlike those associated with autosomal genes. A substantial amount of research on the susceptibility of this gene to multiple mutation pathways is ongoing, and several reference chemical mutagens have been shown to produce *PIG-A* mutations of both base-pair substitutions and frameshift types.

ASSESSING GENETIC HAZARD AND RISK

Germ Cell Risk in Human Populations

Observations of new dominant mutations arising in human populations and extensive experimental data demonstrating chemically induced mutations in somatic cells as well as germ lines of mice document the presumption that all mammalian species, including humans, are susceptible to environmentally generated mutation.[11] The ability to demonstrate genetic toxicity to human populations through epidemiological methods has been limited by the following:

- The rarity of induced mutations in a population. The sample size required to detect even a large increase in mutation is prohibitive.
- The small number of dominant sentinel genes (i.e., express a visible phenotype) that are uniquely identified with specific genetic diseases.
- The difficulty in identifying reproductively active populations exposed to biologically significant levels of suspected mutagenic agents.
- Variable expression of newly induced recessive mutations in the F_1 postexposure.

In 1992, the United Nations Environment Program reviewed the status of and methods available for genetic risk assessment.[21] A more recent classification scheme was

TABLE 23.9
Qualitative Classification Scheme for Human Mutation Risk

Classification	Somatic Cells	Germ Cells
Human mutagen	Positive mutagenicity data from human somatic cells exposed in vivo. These compounds may also be human carcinogens	Positive data derived from human in vivo germ cell mutagenicity studies
Probable human mutagen	Unequivocal evidence for genotoxicity from in vivo mammalian tests with supporting in vitro evidence	Sufficient evidence of interaction with mammalian germ cells and genotoxicity. Includes positive results from studies of heritable mutation effects in vivo or positive germ cell chromosomal aberrations. Supporting evidence that the agent can reach human germ cells
Possible human mutagen	Some evidence for genotoxicity in vitro or in vivo	Evidence suggesting interaction with mammalian germ cells and some genotoxicity
Not mutagenic or equivocal classification	Negative and/or equivocal test results from valid in vitro and in vivo tests for genotoxicity	Absence of the ability for interaction with germ cells

Source: Dearfield, K.L. et al., *Mutat. Res.*, 521, 121, 2002.

proposed by the U.S. EPA.[12] The system provides a qualitative risk classification for an agent that is derived from a four-level testing scheme that starts with in vitro methods and progresses through in vivo somatic tests in level 2 and finally into germ cell assays in levels 3 and 4 (see Table 23.7). Based on the compilation of test results, chemicals will fall into one of the classes ranging from *not mutagenic* to *human mutagen* as shown in Table 23.9. Some regulatory agencies have modified the four-level strategy shown in Table 23.7 by combining levels (e.g., some combine levels 3 and 4); however, the scheme shown in Table 23.7 provides a suitable road map for most situations. A comprehensive list of OECD and EPA protocols is found in the review by Cimino.[86] The complete genetic risk assessment process involves several steps:

- Hazard identification—The qualitative assessment of the intrinsic toxicity of an agent. This is based on the existing database and addresses the question of whether there is potential for human genotoxicity.
- Dose–response assessment—The relationship between the dose of an agent and the induction of an adverse (genotoxic) effect is determined.
- Exposure assessment—A determination of the extent of human exposure to the agent.
- Risk characterization—A description of the nature and potential for genotoxicity risk to humans using the information from the exposure assessment and the dose–response data.

Although the risk classifications provided in Table 23.9 are a qualitative guide, quantitative risk analysis for genetic damage can be performed by extrapolating dose–response results from animal models such as the mouse-specific locus test or the mouse heritable translocation assay to humans (Table 23.10). From the mouse data, the incidence of mutation induction can be calculated for documented exposure levels and expressed as the probability of new disease occurrence in the exposed population. Additional scaling factors must then be included to extrapolate from mouse to human (e.g., DNA repair capacity differences, metabolic differences, dose rate differences). The same problems that confound cancer risk estimates derived from animal studies also affect the ability to generate accurate genetic risk estimates. These include the ability to quantify the nature and shape of the dose–response kinetics, define species differences in susceptibility, and determine factors that influence the phenotypic expression of new mutations. A small number of quantitative genetic risk assessments have been made using data from mouse models for mutation and clastogenesis.[19,20,106]

Somatic Cell Risk in Human Populations

Unlike germ cell risk, the evidence is clear that humans are susceptible to mutagenic effects in somatic tissues. Genotoxicity biomarkers have been used to assess environmental, occupational, medical, and lifestyle exposures on the genome of somatic cells.[107,108] The vast majority of the biomarker studies of genotoxic damage involve the evaluation of individuals for chromosomal alterations, micronuclei, and SCEs. Other methods used include DNA adduct formation,[41] comet assay,[102] mutagenic urine,[77] and Hprt mutation in mouse splenic T cells.[109] These tools work well where traditional epidemiologic studies are not suited and can accurately identify high-risk populations.

Au[108] summarized genotoxicity biomarkers according to their applicability for human assessment. Some methods such as DNA adductions and SCEs are ideal for measuring

TABLE 23.10
Road Map for Calculating the Frequency of New Mutations (n_n) Induced in a Human Population Using Animal Experimentation Data

Step 1	Induced mutation frequency per target gene per unit dose, determined in mice (n_i)	Exposure dose in humans (i)	Expected induced mutation, per gene, frequency following mutagen exposure (m_i)	$(n_i) \times (i) = (m_i)$
Step 2	Expected per gene mutation frequency due to mutagen exposure (m_i)	Number of disease-causing genes in the human genome (n_d)	Expected frequency of disease-causing mutations in the F_1 population following mutagen exposure (m_t)	$(m_i) \times (n_d) = (m_t)$
Step 3	Expected frequency of disease-causing mutations in the F_1 population following mutagen exposure (m_t)	F_1 population size (n_e)	Expected number of new mutations causing disease in the F_1 offspring resulting from exposure to the parental population (n_m)	$(m_t) \times (n_e) = (n_m)$
Step 4	Introduce scaling factors into the calculation			(n_m) = scaling factors $\times (m_t) \times (n_e)$

Source: Favor, J. et al., *Mutat. Res.*, 330, 23, 1995.

exposure but carry little or no information regarding hazard potential or risk (Table 23.11). The majority of biomarkers identify biological effects indicative of possible genetic hazard. Evidence supporting the reliability of chromosome aberrations was derived from a European collaborative study assessing the predictive value of chromosome aberrations and SCEs.[107] The results showed that aberrations, particularly chromosome rather than chromatid alterations, were predictive of cancer but that SCEs were not.

Peters et al.[110] were able to demonstrate that heterocyclic amine carcinogens formed during the cooking of red meat could be detected as mutagens in urine of individuals consuming the meat. The results of their case-controlled study demonstrated that urinary mutagenicity was a valid biomarker for exposure to the consumption of cooked meat and also for colorectal adenoma risk.

TABLE 23.11
Biomarkers Applicable to the Identification of Human Genotoxic Hazard and Cancer Risk

Biomarker Function	Methods
Identifies exposure to genotoxic agent	DNA adducts
	Protein adducts
	SCE
Identifies hazard potential	DNA breaks (e.g., comet assay)
	DNA repair by-products in urine
Identifies genotoxic hazard	Chromosome breaks
	Micronuclei
	Mutagens in urine
	Gene mutation (HPRT or PIG-A) in lymphocytes
Indicates cancer risk	Mutations in specific codons of cancer genes (e.g., *ras*, p53, breast/colon cancer genes)
	Chromosome aberrations

While most of the genes responsible for human disease are located in nuclear DNA, mutations in somatic cell mitochondrial DNA (mtDNA) are also linked to a wide array of diseases and to aging.[111] Mitochondria are maternally inherited, and there are several hundred mitochondria per cell. mtDNA codes for about 13 proteins primarily involved in oxidative metabolism. Both nuclear and mitochondrial genes are necessary for the normal function of mitochondria. DNA repair processes such as long- and short-patch BER and MMR are active in mitochondria.[112] Mutations found in mtDNA appear as base substation mutations or deletion/insertion mutations. Mutant mitochondria are randomly distributed to daughter cells during cell division and increase the number of cells carrying mutant (dysfunctional) mitochondria in a particular tissue. When energy output in that tissue eventually becomes insufficient, clinical symptoms appear.[112] The types of diseases associated with mitochondrial mutations include metabolic disorders such as diabetes and degenerative disorders including neurophysiological and cardiovascular dysfunction. Studies have also shown that mtDNA mutations accumulate in postmitotic tissues over time in animals and contribute to the aging process.[111]

NEW DIRECTIONS IN GENETIC TOXICOLOGY

In 2008, the International Life Sciences Institute (ILSI), Health and Environmental Sciences Institute (HESI) Project Committee on the Relevance and Follow-up of Positive Results in in vitro Genetic Toxicology (IVGT) sponsored a workshop to identify promising technologies that would improve future testing and hazard assessment. The workshop (summarized in *Environmental and Molecular Mutagenesis*, 2001; 52:205–223) reviewed 16 methods or technologies and identified their stage of development. Some of the tests reviewed have since replaced existing tests and/or moved into more routine use since 2008, but the summary

does give some view into the possible application of technologies that still remain on the horizon. Among the areas that are considered in the emerging category are the following:

1. PIG-A gene mutation—Reviewed in an earlier section. This method could fill an important testing gap in current strategies. A substantial amount of research activity has been focused on the refinement and validation of this assay and a special issue of *Environmental and Molecular Mutagenesis* (2001; 52(9)) was devoted to the technique.
2. Humanized in vitro systems—Human cells may offer more relevant metabolism and genetic background for assessing mutation than rodent cells. Differences in gene expression and DNA repair capacities may also contribute to inaccurate responses with nonhuman in vitro systems.
3. Transgenic in vitro techniques—In vitro models may be derived from cells isolated from transgenic mice such as Muta™ Mouse.[103] Cell lines or primary cells carrying the recoverable transgene can be used to facilitate mutation detection and sequence analysis.
4. Thin film biosensors with DNA/enzyme coatings—Biosensors consisting of thin films of DNA and purified metabolic enzymes can be structured to detect agents with genotoxic properties. The combination on a thin film biosensor provides close proximity between the DNA and potential metabolite(s) formed when a test material is added to the biosensor. Proposed methods for detection of reaction products include electrochemiluminescent sensors. Biosensors offer advantages of automated large-scale testing.
5. Toxicogenomics—Analysis of gene expression profiles is not a new technology. Its application to genetic toxicology continues to evolve with improved reliability.[113] Toxicogenomic technologies are being actively pursued, and an expanded discussion of this topic is provided in a later section.

With the exception of the PIG-A mutation assay and toxicogenomics, the level of technology development is too limited to know how these methods may function in general genetic toxicology testing or research.

A technology that has long been used for the prediction of genotoxicity is SAR assessment. The existing SAR methods rely upon a combination of mathematical algorithms and qualitative experience rules to define the biological relationships between molecular structure and genotoxicity.[114] Commonly used SAR models (e.g., MultiCASE, Derek for Windows) are primarily used as tools to suggest potential for genotoxic hazard and need for follow-up testing. The guideline developed for evaluating genotoxic impurities in pharmaceutical ingredients is an example of how agencies rely upon SAR for this purpose. At the present time, there is no system with sufficient accuracy to replace the existing battery of bioassays.

TOXICOGENOMICS AND EMERGING TECHNOLOGIES IN TOXICOLOGICAL RESEARCH

Toxicological testing and biomarker discovery as well as drug development and patient therapy have been influenced by advances in molecular biological techniques such as *-omics* technologies. These technologies utilize a broad range of molecular tools to support the study of various macromolecules or subcellular components in response to exposures (i.e., environmental, chemical) and therapeutic treatments. The Human Genome Project (which involved sequencing the entire human genome) fostered the expansion of toxicological testing to include a new field termed *toxicogenomics*. Toxicogenomics combines genomics and bioinformatics to characterize and identify mechanisms of toxicity induced by chemicals and various exposures (i.e., pharmacological, environmental, toxicity protocols). Within this scientific discipline, genomics, transcriptomics, proteomics, and metabolomics may be applied to assess the toxicological responses of chemicals using in vitro and in vivo models[115–129] (Figure 23.7).

Data from toxicogenomic studies can be analyzed by tools combining biology, computer software, and statistics to generate biological information that support efforts in predictive and mechanistic toxicology, biomarker discovery, risk assessment, identification of disease mechanisms, and drug development. U.S. governmental research agencies such as the National Institute of Environmental Health Sciences (NIEHS) are actively studying toxicogenomic applications to toxicology. The Toxicogenomics Research Consortium (TRC) of the National Center for Toxicogenomics (NCT), a division of the NIEHS, is comprised of the NCT microarray group and five institutions (University of North Carolina at Chapel Hill, Fred Hutchinson Cancer Research Center, Oregon Health and Science University, Massachusetts Institute of Technology, and Duke University). The goals of the TRC are to (1) evaluate

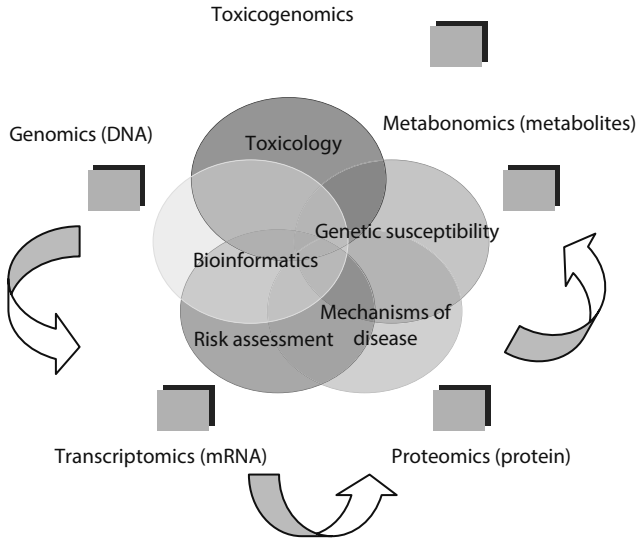

FIGURE 23.7 Interdisciplinary aspects of toxicological analyses.

toxicant-specific patterns of gene expression; (2) integrate gene expression profiling with proteomics, metabonomics, and phenotypic anchoring; (3) study the toxicological effects of chemical mixtures; and (4) contribute gene expression and proteomics data to the chemical effects in biological systems database (CEBS) (www.niehs.nih.gov).

In 2007, the National Research Council of the National Academy of Sciences issued a report, "Toxicity Testing in the 21st Century: A Vision and a Strategy." In this document, the authors suggest that the advances in fields such as molecular biology and biotechnology are offering enhanced methods for researchers to evaluate the health risks of potentially toxic chemicals in cellular systems of human origin. It is anticipated that knowledge from the initiatives of the governmental and academic consortiums will enhance the application of toxicogenomics to toxicological research and testing as detailed in the report.

This section summarizes the role of toxicogenomics in toxicology. Standard testing measures, challenges, and current advances including specific multiagency initiatives (toxicity forecaster [ToxCast] and Tox21) will be discussed.

OVERVIEW OF CURRENT TECHNOLOGIES

The four major -omics disciplines are defined as follows: (1) genomics (the study of gene sequences and genetic variability [humans possess ~30,000 genes]), (2) transcriptomics (the quantitative measurement of gene expression [messenger RNA, mRNA]), (3) proteomics (the measurement of protein production and levels in a cell, tissue, or biologic fluid), and (4) metabonomics (the multiparametric measurement of metabolites).

The development of technologies used in toxicology and toxicogenomics has evolved significantly over the last several decades. This review describes the current technologies used in toxicogenomics and provides examples where appropriate. Key technologies to be discussed include polymerase chain reaction (PCR) and quantitative reverse transcriptase/polymerase (qRT/PCR), microarrays (transcriptomics), methylation-specific PCR (MSP; DNA methylation), protein arrays (proteomics), and metabolic profiling (metabonomics) (Table 23.12). Extensions and applications of these technologies will be discussed as they relate to bioinformatics, transgenics, genomics, emerging technologies, and systems biology.

Polymerase Chain Reaction and Quantitative RT/PCR

PCR is a technique used to amplify specific DNA sequences with the aid of gene-specific primers (small sequences of DNA specifically designed for a gene target), heat-stable DNA polymerase enzymes, nucleotides, and other reagents. The process is often performed as a precursor to DNA isolation, forensic analysis, and disease diagnosis. It has been an integral part of in vitro toxicology and other scientific disciplines as applied to (1) gene expression analysis from in vitro and

TABLE 23.12
Technologies Used in Toxicogenomic Research

Technique	Application	Advantages	Limitations
Quantitative RT/PCR	Gene expression	Screen multiple samples; high-throughput screening	Limited gene targets per assay—even with multiplexing and gene cards; PCR array is improving this issue
SiRNA/RNAi	Gene silencing	In vitro assessment can be performed to test efficacy prior to in vivo assessments; drug discovery	Transfection efficiencies; toxicity in certain cases
Microarray (filter, chip)	Genomics/transcriptomics (transcripts, single-nucleotide polymorphisms [SNPS], DNA polymorphisms)	High-throughput screening of gene targets—thousands of genes	High-throughput analyses (i.e., many samples) are expensive; potentially large data set generation with limited understanding of biological impact
Next-generation sequencing	Genomics/transcriptomics (transcripts, SNPS, DNA polymorphisms)	High-throughput screening of gene targets—thousands of genes	High-throughput analyses (i.e., many samples) are expensive; potentially large data set generation with limited understanding of biological impact
Protein array (filter, chip, solution)	Proteomics	Profiling of multiple proteins	Some difficulties in correlating with mRNA changes; variations in time requirements for regulation of mRNA (transcription/stability) versus protein (translation, modifications)
Metabolic profiling (NMR, MS)	Metabonomics	Profiling of multiple metabolites; provides understanding of gene functions	Requires expensive/specialized equipment
MSP	Epigenetic DNA modification (i.e., methylation)	Provides gene-specific analysis	Multistep process leading to potential for sample lost; limited to gene-specific characterization—global methylation patterns must be assessed by other measures—advances are in progress

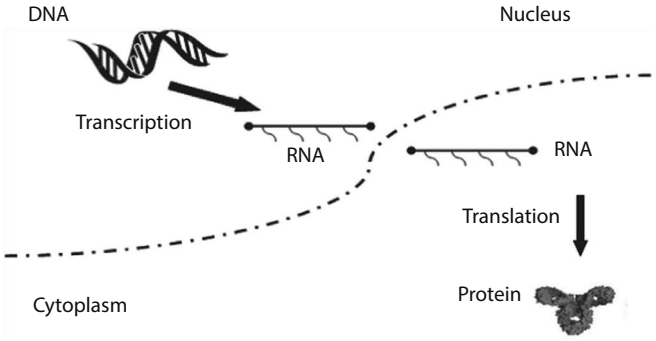

FIGURE 23.8 Cellular context of gene expression. RNA is synthesized in the nucleus through a process termed transcription in which the DNA code is transcribed to RNA. The RNA is subsequently transported to the cytoplasm where it is translated to protein, completing the chain for functional expression of the DNA (gene) following posttranslational processing.

in vivo samples, (2) in situ procedures of paraffin-embedded tissue, (3) gene cloning, and (4) genotyping since the 1990s.[130]

Gene expression is the process where the DNA code is expressed as RNA and ultimately protein, which serves as the functional product of the DNA code (Figure 23.8). Gene expression analysis requires the measurement of mRNA or protein. Since the late 1990s, qRT/PCR has dominated the field in assessing steady-state mRNA levels for individual/multiplex gene analyses. In this method, RNA is reverse transcribed to a complementary DNA copy, which is then amplified by PCR. Advances in chemical components developed to modify primers, probes, and reagents have resulted in significant improvements in quantitative and qualitative assessment of gene expression, thereby overcoming the prior limitations associated with detecting changes in mRNA levels[131,132] (Figure 23.9).

Advances in qRT–PCR facilitated the adoption of gene expression technology into toxicological testing and led to advanced methods of large-scale gene expression profiling via microarrays and gene chips.

Microarray Technology

Microarrays are grids of DNA fragments aligned on a chip to evaluate large amounts of biological information. Microarray technology has progressed from the use of nylon membranes formed to the size of an index card to gene chips designed as small as a letter-sized return address label. The arrays allow for the simultaneous assessment of genes/transcripts (i.e., 1000–30,000, entire genomes) involved in focused categories such as signal transduction (process that transfers the external cellular impact of chemical exposure to internal compartments and effects), stress response, cell cycle regulation, DNA synthesis/repair, inflammatory responses, and metabolism. Arrays are designed for different species (i.e., human, mouse, rat) and for disease and stress states (i.e., cancer, toxicology, inflammation). Array designs are aided by genome sequences from public databases and unique probe designs and are analyzed using bioinformatics techniques. Photolithographic techniques (adapted from the semiconductor industry) are used in the manufacturing process of microarray chips[133] (Figure 23.9). Aided by various data analysis

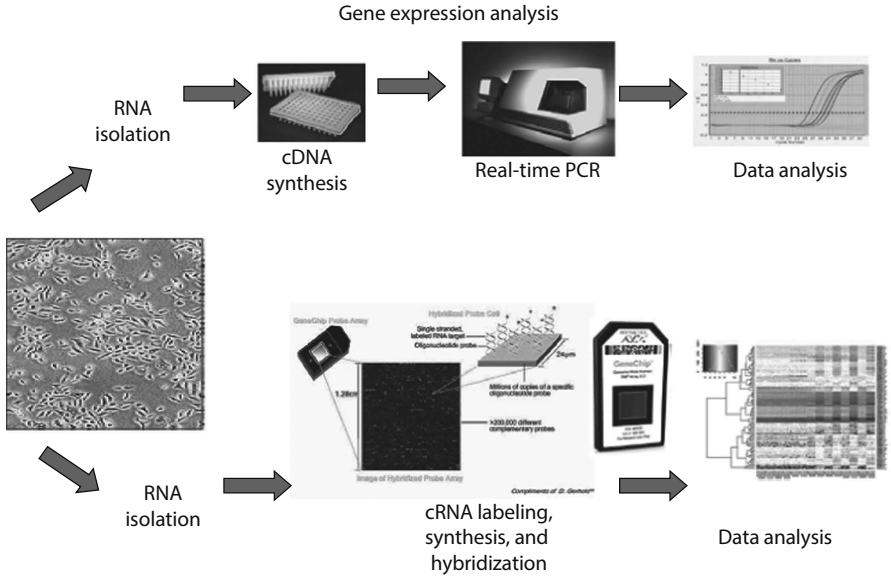

FIGURE 23.9 Advances in gene expression technologies. Upper panel. Real-time quantitative RT/PCR. RNA is reverse-transcribed to a complimentary DNA (cDNA) copy and subsequently amplified in the presence of buffers, enzymes, DNA bases, and fluorescent reagents via real time. Representative data analysis is depicted by the quantitation of mRNA facilitated by amplification of a relative standard curve prepared with positive control cDNA. Lower panel. Affymetrix gene chip expression analysis is conducted with RNA that is labeled, subjected to synthesis and hybridized to a specific gene chip. Various normalization, statistical and bioinformatic data analysis steps are conducted to simultaneously generate gene expression data from thousands of genes, which can be subsequently represented via hierarchical data comparisons (as depicted) for visual representation of differentially expressed genes.

methods including cluster and hierarchical analyses, researchers can obtain information on interactions in gene pathways.

Validation and standardization are important aspects of microarray research that are still under discussion. These issues addressed by Rockett and Hellmann[134] include measures for validation (quantitative RT/PCR, western or northern blots, in silico analyses), uniform formats for RNA amplification, normalization techniques, data analysis and representation, journal requirements for publication of array data and assay design, and standardizations for platform comparisons.[134,135] The minimum information about a microarray experiment (MIAME) checklist (www.mged.org) provides a good reference framework for microarray investigations.

DNA Methylation

Epigenetic modifications are changes in DNA that do not modify the gene sequence but may alter gene expression or phenotypes. DNA methylation is a process by which a methyl group is covalently added to the 5-carbon position of cytosine in a cytosine–guanine (CpG) sequence of base pairs. The methylation alters gene expression by preventing transcription of the gene. This process is referred to as gene silencing, is catalyzed by DNA methyltransferase,[136,137] and may be reversible. Since specific ratios of methylation in GC regions of DNA are required for normal maintenance of gene expression, global changes observed by hyper- and hypomethylation of genomic DNA can indicate epigenetic alterations induced by chemicals during disease progression.[138] Hyper- and hypomethylation patterns can lead to phenotypic changes that affect toxicological response to chemical exposure and may also define disease pathogenesis[138–142] (i.e., carcinogenesis, aging).

DNA methylation changes may be either global- or gene-specific (i.e., p16, GSTπ HOXA5). Global methylation—the overall level of methyl cytosines in a genome—may be determined via methyl-accepting capacity assays (SssI DNA methyltransferase) and chromatographic assays (high-performance liquid chromatography, thin-layer chromatography, or liquid chromatography/mass spectroscopy [LC/MS]). The latter assay requires digestion of the DNA into single nucleotides.

Numerous methods have been developed for analyzing gene-specific methylation including bisulfite-based methods. Bisulfite can selectively deaminate cytosine but not 5-methylcytosine to uracil. This leads to a sequence change in the DNA that discriminates cytosine from 5-methylcytosine. After the conversion has taken place, sequence differences between a methylated and unmethylated cytosine can be assessed by several methods including direct sequencing, restriction enzyme digestion, nucleotide extension assays, and MSP.

Cells can be exposed in vitro to chemicals causing demethylation and then analyzed for reversal patterns. Such investigations have led to the understanding of the efficacy of certain drug therapies and mechanisms of toxicity. Studies using in vivo models have shown methylation (either permanent or reversible) in genes controlling cell proliferation, cell cycling, and in disease states such as lung cancer and skin tumorigenesis.

Proteomics

Proteomics is the measurement of protein levels and states in a cell, tissue, or biological fluid. These measurements are important since proteins are the expressed characteristics of genes and function as effecter molecules in cells. A variety of protein alterations result from cellular responses to stimuli and stress. For example, cells may secrete cytokines or chemokines, proteins that play a role in immune response and inflammation. Alternatively, proteins may be modified by numerous posttranslational processes, which can be assessed by proteomic methods. For example, phosphorylation is a process that controls protein activity by the addition of phosphate molecules and supports the transfer of signaling through a cell; a change in the phosphorylation state of a protein can impact the function of the protein.

Tissue profiling by proteomic techniques has led to the detection of biomarkers as well as establishment of specific signal pathway profiling.[143,144] Early proteomic technologies consisted of 2D gel electrophoresis followed by LC/MS and subsequent biomarker validation with western blot (protein detection via antibodies on protein-binding membranes) or enzyme-linked immunosorbent assay (ELISA; protein detection in multiwelled plates coated with protein-binding matrix layered with antibodies). Recent technologies provide time-saving higher throughput and increased reproducibility (e.g., matrix-assisted laser desorption/ionization mass spectrometry [MALDI MS]- and surface-enhanced laser desorption/ionization [SELDI]-based protein chip technologies). These technologies allow for the identification of proteins in biological matrixes by analysis of proteins digested into smaller units (peptides), which can be distinguished on a mass (molecular weight) basis.

Protein arrays (filter, chip, and suspension) facilitate screening protein profiles of tissue samples and biological fluids on a smaller scale. Protein arrays are used to assess biomarkers of diseases and pathogenic states, including alterations in expression, cytokines/chemokines, and post-translational protein modifications (e.g., phosphorylation). They may be used to assess the effects of candidate drugs and environmental toxicants/irritants on the biology of cells, research animals, and human subjects and may be applied to functional genomics, validation requirements in drug discovery, and toxicology. Protein filter arrays are similar to microarrays with the screening process performed by antibody probes instead of labeled DNA probes. However, compared to microarrays, the number of targets is fewer due to difficulties related to protein chemistry of each antibody–protein interaction.

Proteomics has subdisciplines or categories as well. For example, *kinomics* combines genomics and proteomics for the study of kinases, proteins that function as enzymes to support phosphorylation of macromolecules. Kinases

promote the activation of proteins involved in transducing signals from external to internal cellular environments. This subdiscipline provides an important complement to genomic assessments since some gene products exhibit modified expression and activity past the mRNA state; hence, complete understanding of the cellular responses to treatments or chemical exposures requires a combination of technologies that measure gene and gene product changes across a continuum (e.g., exposure dose, time).

Proteomic and systems biology approaches have been used to assess and integrate toxicogenomic investigations on multiple chemical platforms such as benzene exposures, protein oxidation, drug toxicity, and MOA investigations as well as human risk assessments. The NTP issued a report in 2005, which described the advantages and challenges of using such data in cross-species data extrapolations. The report summarized the importance of properly designed experiments to achieve effective comparisons between species. It was recommended that proteomic profiles be established in vivo (i.e., rodent), reproduced in in vitro rodent cell cultures, and replicated in in vitro human cell cultures and subsequently link the correlation via computational modeling. In these cases, the most accurate assessment of the toxicological response would occur when common pathways are modified between organisms.

Metabonomics

The need to advance the understanding of the biological relevance of genomic data is ever present. Metabonomics is a seminal technology in functional genomics—a discipline that involves assessing gene function and its control mechanisms. As the genome represents all of the gene sequences of an organism, the metabolome represents all the low-molecular-weight molecules in a cell at a given time. Analysis of the metabolome was born from two similar yet distinct methods that have evolved from the need to assess animal and microbial/plant biochemistry. Metabonomics and metabolomics both involve characterization of metabolites through multiparametric measurements. Metabonomics deals with biological systems, including extracellular environments in an integrated and multicellular approach allowing for quantitative measurements of metabolic responses following a stimulus. Metabolomics predominately deals with concentrations of intracellular metabolites in simple cell systems. In recent years, the terms have been used interchangeably and most often referred to as metabonomics.

In concert with chemometric and bioinformatics tools, metabonomic data can generate biochemically based fingerprints (biomarkers) of drugs and toxicants and hence support linkages to biologically relevant end points from data obtained from various *-omics* technologies. Metabonomics offers the promise for obtaining drug effect and disease end points to support drug development, risk, and toxicological assessments in living systems[145,146] via analysis of biofluids, tissues, and cell lysates. This technology has been applied in ecotoxicological assessment of environmental contaminants,[147] physiological influences on biofluids,[148] systems biology in pharmaceutical research,[149] physiological monitoring, drug safety assessments and disease diagnosis,[150] toxicological assessments,[151–153] and cigarette smoke effects in cultured lung cells.[154]

APPLICATIONS AND EXTENSIONS OF TOXICOGENOMIC TECHNOLOGIES

Gene Expression and Genomics

Differential gene expression profiling and genomic evaluations via PCR, qRT/PCR, and microarrays have been conducted in in vitro, in vivo, and human samples to support chemical evaluations, risk assessment, drug discovery, genetic susceptibility, and analysis of tissue for disease pathogenesis (Table 23.13).

Complex mixtures of chemicals such as cigarette smoke condensate (CSC; also referred to as total particulate matter [TPM]) and diesel exhaust extracts can alter gene expression in lung cells as detected by qRT/PCR. For example, human bronchial epithelial cells exposed to CSC exhibited changes in interleukin (IL-8) mRNA and secreted cytokine levels, changes associated with inflammation.[155] Individual smoke constituents may also cause alterations. c-*myc* (a gene that promotes uncontrollable cellular growth) was evaluated in normal human bronchial epithelial (NHBE) cell cultures exposed to benzo[*a*]pyrene and benzo[*a*]pyrene diol epoxide (BPDE). Differential mRNA responses were observed between the chemicals and correlated with distinctions in DNA adduct accumulation and cell cycle regulation.[156]

Fukano et al. evaluated the effect of different cigarette filters on gene expression in in vitro lung cultures.[177] Cells exposed to smoke from cigarettes with a carbon filter exhibited reduced activation of heme oxygenase 1 (HO-1), a gene responsive to oxidative stress, compared to cells exposed to smoke from cigarettes with cellulose acetate filters.

Pathway-specific analyses have recently been enhanced by PCR array technologies and may be used in tobacco-related research. Typical platforms such as the PCR arrays from SA Biosciences offer 96- and 384-well formats to allow for quantitative and high-throughput screening for specific disease states, cellular conditions, signaling pathways, biological functions, gene regulation, and DNA methylation. The signal transduction pathway finder PCR array and inflammatory cytokine PCR array were used by Parsanejad et al. to determine the effect of tobacco smoke on selected molecular pathways.[172,173] Assessment of whole smoke in NHBE cells yielded responses in pathways associated with inflammation, apoptosis, and wound healing. The group subsequently evaluated a repetitive smoke exposure regimen to mimic a smoker's frequency of exposure[173] (Figure 23.10). The resulting cytokine responses exhibited adaptive, sustained, and chronic expression patterns over the time course of exposure.

Transcriptomic analyses with qRT/PCR and microarray have also facilitated numerous toxicological assessments

TABLE 23.13
Toxicological Assessments: Gene Expression and Methods of Detection

Agent/Disease	Model	Method	Reference
Acetaminophen/liver	Human and rat	Transcriptomics	Kerns and Bushel[157]
Multiple organs	In vivo (Balb/c mice)	Nanocapillary quantitative real-time PCR	Fabian et al.[158]
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	In vitro (HepG2)	Transcriptomics; metabonomics	Jennen et al.[153]
Peroxisome proliferator chemicals	In vivo (rat and mouse)	Transcriptomics	Ren et al.[159]
2,4-Diaminotoluene, benzo(*a*) pyrene, 2-acetylaminoflourene, or 3-methycholanthrene	In vitro (Balb/c 3T3 cells)	Transcriptomics	Rohrbeck et al.[160]
Endocrine-disrupting compound (bisphenol A)	In vivo (mouse)	Epigenetics	Bernal and Jirtle[161]
Diesel exhaust	In vitro (human airway cells; rat alveolar macrophages)	Semi-qRT/PCR	Baulig et al.,[162] and Koike et al.[163]
Bromobenzene	In vivo (rats)	Transcriptomics; proteomics	Heijne et al.[164]
Cytotoxic anti-inflammatory drugs; DNA-damaging agents	In vitro (HepG2)	cDNA microarray	Burczynski et al.[115]
Arsenic	In vivo (Tg.AC mice)	DNA methylation	Xie et al.[165]
Benzo[*a*]pyrene	In vitro	qRT/PCR	Fields et al.[156]
Cigarette smoke	In vivo	cDNA microarray; Gene Chip	Hackett et al.,[166] Shah et al.,[167] Gebel et al.,[168] and Beane et al.[169]
CSC/extracts; cigarette smoke	In vitro (human bronchial cells; SWISS 3T3 cells)	Real-time qRT/PCR; PCR array; cDNA microarray	Fields et al.,[170] Maunders et al.,[171] Parsanejad et al.,[172,173] and Bosio et al.[174]
Asbestos	In vitro	cDNA microarray, real-time PCR	Ramos-Nino et al.[175]
Oxidative stress	In vitro (Hep-G2)	cDNA microarray, real-time PCR	Morgan et al.[176]

associated with elucidating MOA, organ toxicity, reproductive toxicity, and chemical characterization.[157–160] Rohrbeck et al. applied toxicogenomic analysis to correlate in vitro carcinogenicity testing in Balb/c 3T3 cells to in vivo by identifying gene signatures predictive of select chemical carcinogens.[160] The testing strategy identified gene signatures and common regulated carcinogenic pathways that accurately predicted three previously classified carcinogens: 2,4-diaminotoluene, benzo(*a*)pyrene, and 3-methycholanthrene.

DNA Methylation

Recently, Liu et al. reported the effect of CSC on DNA methylation in normal small airway epithelial cells and immortalized bronchial epithelial cells following chronic exposure (9 months) to CSC.[178] Changes in expression profiles of genes associated with DNA methylation and altered hypo- and hyper-DNA methylation patterns along with growth of cells in soft agar (preneoplastic characteristic) were observed. The authors conclude that CSC induced cancer-related epigenetic changes along with the development of preneoplastic morphological transitions in the cells. The resulting model may support in vitro studies of lung cancer pathogenesis.

Development from the early stages in utero and throughout a life span is regulated through maintenance of the epigenome. Bisphenol A, an endocrine-disrupting compound used in the chemical industry in polycarbonate plastic production and in epoxy resin lining of metal beverage and food cans, can leach into the food supply from the container leading to exposures that impact development. Bernal and Jirtle evaluated the impact of bisphenol A on growth and development in a mouse model with biosensors helpful in determining the impact of in utero toxicant exposures on epigenetic programming in the offspring.[161] Bernal and Jirtle observed hypomethylation in the offspring, which led to distinguishing phenotypical differences.

Bioinformatics

Bioinformatics was developed out of a need to maintain, correlate, and evaluate large volumes of biological data generated from molecular and toxicogenomic studies. Bioinformatics is a multidisciplinary science that encompasses biology, computer programming, and statistics. The integration of data from toxicogenomic studies into databases supports investigations for risk assessment, mechanistic and predictive toxicology, and pattern recognitions in disease progression. Such efforts have been initiated by various agencies and research teams (Table 23.14) and provide valuable means for understanding toxicological responses at the molecular level. However, there are some challenges with database design and maintenance involving data integration (standardized data storage and exchange), uniform nomenclature, and standardized assay design.[179]

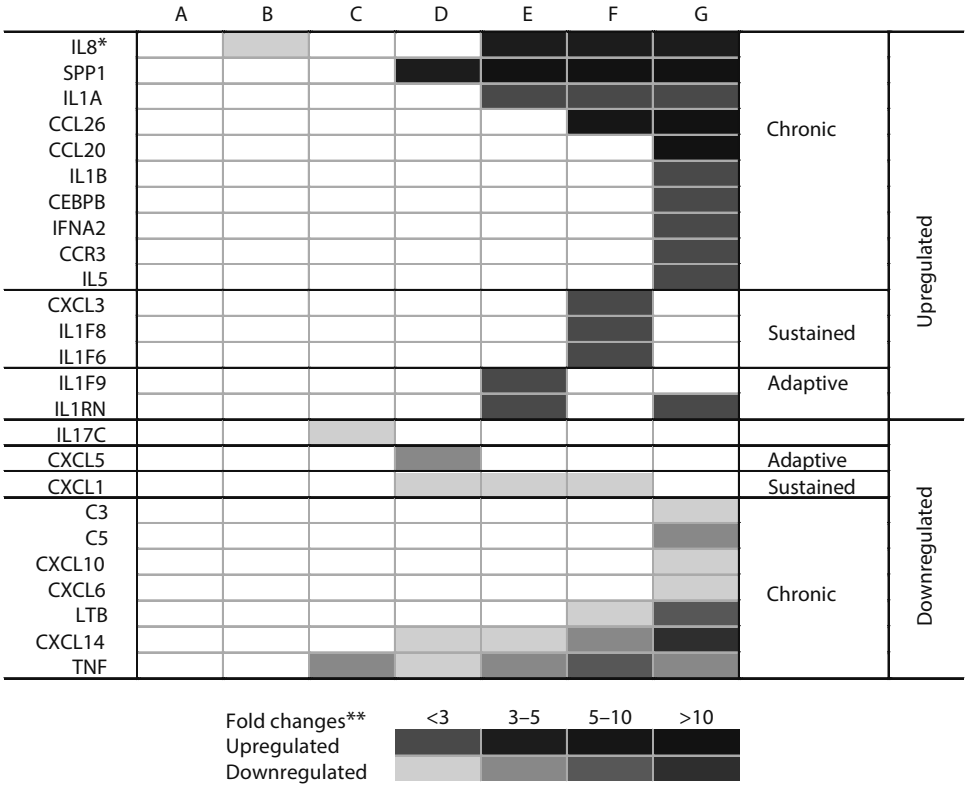

FIGURE 23.10 Inflammatory cytokine PCR array responses to CSC exposure in NHBE cells. Gene expression patterns in NHBE cells displaying adaptive, sustained, and chronic expression in response to 60 μg TPM/mL media. Groups A to G represent different treatment methodologies from 15 min to alternating repeated exposure and recovery times over a 24 h period. In this study, adaptive gene regulation refers to genes that adapted to the CSC exposure and short-term recovery (3 h) by returning to the baseline; sustained gene regulation refers to genes that remained up- or downregulated only during CSC exposure and short-term recovery; and chronic gene regulation refers to genes initiating or maintaining elevation or suppression patterns after a 12 h (long-term) recovery. *IL8 was the exception in expression pattern by exhibiting both up- and downregulation in the seven groups. **The fold changes are scaled by color intensity. Upregulation indicates gene expression is increased and is represented on the upper panel. Downregulation indicates that gene expression is deceased and is represented on the lower panel. The color scale shows enhanced color intensity as the fold change in expression as compared to the control samples gets larger either positively or negatively. The categories are <3, 3–5, 5–10, and >10. (From Parsanejad, R. et al., *J. Cytokine Interferon. Res.,* 28, 703, 2008.)

TABLE 23.14
Toxicogenomic Databases

Agency	Database Link
NIH Center for Bioinformatics	www.discover.nci.nih.gov/tools.jsp
NIEHS: NCT/TRC	www.niehs.nih.gov/research/supported/centers/trc/
FDA's National Center for Toxicological Research (NCTR)	www.fda.gov/ScienceResearch/BioinformaticsTools/Arraytrack/
EMBL-EBI (The European Bioinformatics Institute)	www.ebi.ac.uk/arrayexpress/
EGP	www.genome.utah.edu/genesnps/
Biocarta	www.biocarta.com

Additional web-based databases and software platforms are also useful resources. For example, the Biocarta system provides information on gene function and specific gene pathways as well as information on reagent/assay resources and links to pertinent scientific citations (PubMed). Software platforms such as Ingenuity, SpotFire, GeneSpring, GeneSifter, and Pathway Studio are also valuable tools for analyzing and supporting the interpretation of genomic data.

Transgenics

In vitro gene reporter assays serve as measures to detect toxicological effects of chemicals on promoters of genes involved in cellular regulation (i.e., transcription factors). Gene reporters consist of the promoter (controlling elements) for a gene of interest linked to DNA for luciferase or green fluorescence protein (e.g., proteins that liberate fluorescent light when induced or turned on). Gene reporter assays are facilitated by transfection (insertion) of gene reporter constructs into a variety of cell types, and subsequent quantitation of signal or protein generation in response to chemical treatment (Figure 23.11).

Several laboratories are investigating the use of luciferase models to evaluate transcription factor regulation of cell signaling pathways, including the nucleus-related factor 2 (Nrf2), AP-1, and NFκB pathways. Nrf2, AP-1, and NFκB

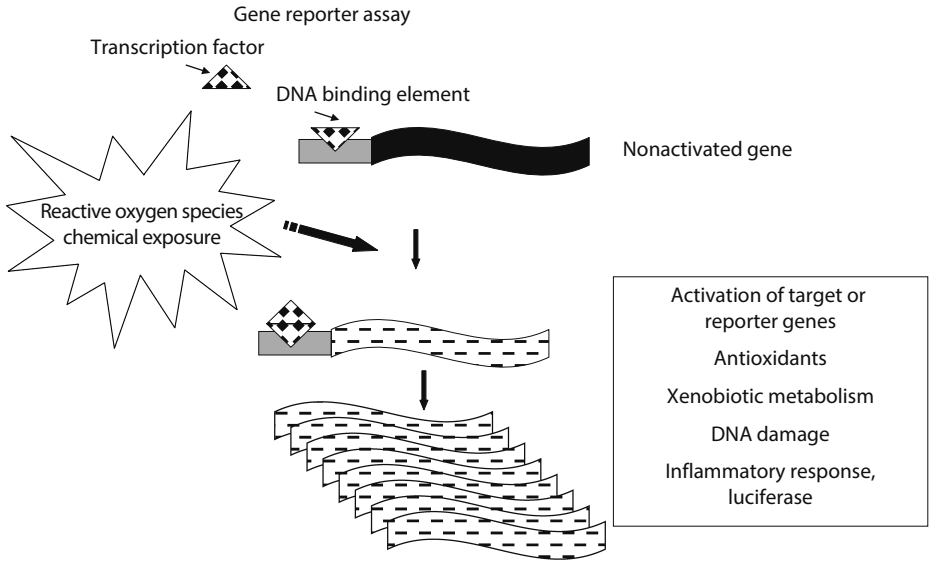

FIGURE 23.11 Gene reporter assay. Chemical exposures, processes that generate reactive oxygen species, DNA damage, or inflammatory mediators can direct transcription factors (such as Nrf2, AP-1, NFκB) to complimentary DNA-binding elements (bases) of a gene. After binding, the transcription factor can activate the expression of multiple target genes.

are transcription factors that play key roles in regulating the responses of genes involved in oxidative stress, cell proliferation/transformation, and inflammatory response via control of DNA transcription. BPDE has been observed to affect AP-1 and NFκB responsive genes, and the transcriptional regulation of these genes was confirmed via the dose-dependent activation of the luciferase gene through interactions with the AP-1 and NFκB promoters,[180] respectively (Figure 23.12). However, cotreatment with phenolic fraction of tobacco smoke condensate (TSC) (10 μg/mL or 30 μg/mL) resulted in a dose-dependent decrease in NFκB activation suggesting that fractions and mixtures of chemicals can differentially impact the response of individual chemical exposures.

Transgenic animals for mutation detection were developed in 1990 using shuttle vector technology.[181,182] A few models commercially available in the 1990s, such as the MutaMouse and BigBlue Mouse, measured *lac I* and *lac Z* gene activation.[183,184] These models laid the foundation for the development of genetically modified animals with altered gene expression patterns via either gene knockout or enhancement. Knockout (gene capability removed) and transgenically enhanced (gene capability increased) models are also being investigated to advance knowledge on the response of specific gene modifications on toxic responses and disease pathogenesis. Mouse models have been developed with enhanced tumorigenic potential due to genetic alterations. The Tg.AC mouse harbors increased ras gene activity and enhanced growth via transgene expression of oncogenic viral Harvey ras (v-Ha-ras) within the skin. UL53-3 X A/J mice harbor a dominant-negative p53 mutation, which inhibits cell cycle control. Such genetic alterations may support studies designed to evaluate tumorigenic potentials of chemical exposures in previously unresponsive

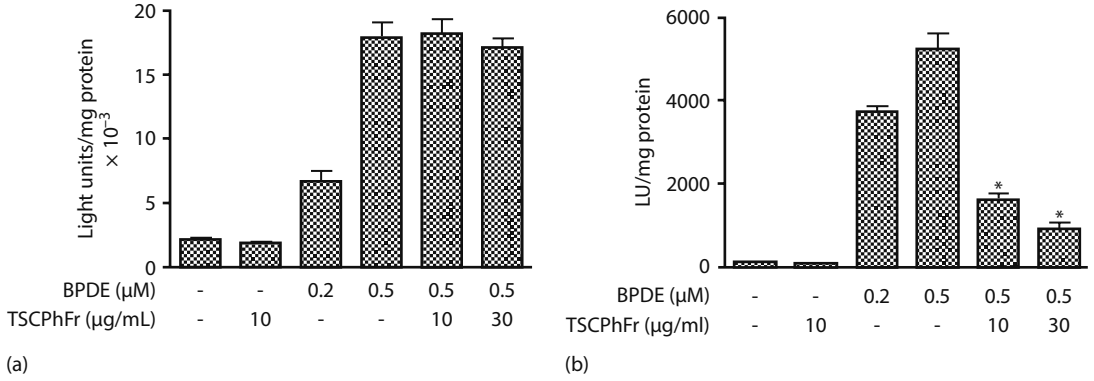

FIGURE 23.12 Effect of BPDE and TSC phenolic fraction on activation of AP-1 and NFκB. (Request for permission to reprint is in progress.) JB6 (P+) Cl 41 epidermal cells were transiently transfected with the AP1-luciferase and NFκB-luciferase reporter plasmids. Effect of TSC phenolic fraction on activation of AP-1 (a) and NFκB (b) was assessed following treatment with BPDE in the absence and presence of TSC phenolic fraction. Each bar indicates the mean ± SD of three parallel experiments. (*) indicates statistical analysis of significance with paired *t*-test ($p < 0.05$). (From Mukherjee, J.J. and Kumar, S., *Mutat. Res.*, 696, 89, 2010.)

models or models requiring long-term exposure regimens. These models have been used to assess the multistage aspects of tumorigenesis via exposure to 7,12-dimethylbenz(*a*)anthracene (DMBA), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or cigarette smoke.[185–187] Lung tumorigenicity has also been assessed to compare the A/J and *ras*H2 transgenic mouse models in response to mainstream tobacco smoke whole-body and nose-only exposure regimens.[188]

Transgenic animal research is used to facilitate understanding of disease progression and significance of DNA polymorphisms. For example, transgenic models for several research interests including apoptosis, cancer, diabetes and obesity, immunology and inflammation, cardiovascular diseases, and metabolism are commercially available. Additionally, efforts to assess the role of genotype variations in toxicology have been undertaken by the Environmental Genome Project (EGP). The EGP is a program initiated by the NIEHS, which has the mission to *improve understanding of human genetic susceptibility to environmental exposures*. To address this issue, EGP has undertaken several major research initiatives, including the Comparative Mouse Genomics Centers Consortium (CMGCC), Human DNA Polymorphisms Discovery and Characterization, and the GeneSNPs Database of Human and Mouse Environmental Responsive Genes. To improve understanding of the biological significance of human DNA polymorphisms, the CMGCC is charged with developing transgenic and knockout mouse models based on human DNA sequence variants in environmentally responsive genes. The candidate target genes include genes involved in DNA repair, cell cycle control, cell signaling, cell structure, gene expression, apoptosis, and metabolism. The models developed by the consortium are distributed by the Mutant Mouse Regional Repositories (http://www.mmrrc.org/) via arrangements with the National Center for Research Resources.

GENOMICS: PERSPECTIVES OF GOVERNMENTAL AGENCIES

Governmental research agencies such as the NIEHS are active in characterizing toxicogenomic applications in toxicological experiments. The TRC of the NCT, a division of the NIEHS, has goals to evaluate toxicant-specific patterns of gene expression as related to dose–response, molecular mechanisms, and biomarkers of human exposure. The consortium also plans to integrate gene expression profiling with proteomics, metabonomics, and phenotypic anchoring, study toxicological effects of chemical mixtures, and contribute gene expression and proteomics data to the CEBS (www.niehs.nih.gov). Due to these collective efforts, the genomic techniques in toxicological sciences are now more frequently employed and are anticipated to expand in the future.

Knowledge from these consortiums and agencies is anticipated to enhance the application of toxicogenomics to toxicological research as detailed in the report, "Toxicity Testing in the 21st Century: A Vision and a Strategy," prepared by the U.S. National Academy of Sciences (2007). The report provided a strategy by which in vitro testing using cell lines and human cells in concert with high-throughput screening of toxicity pathways can be a routine component of toxicity testing in the future.[189] The vision for toxicity testing for the twenty-first century consists of in vitro and short-term in vivo tests that are proposed to evaluate chemicals in a four-component framework: chemical characterization, toxicity pathways and target testing, dose–response and extrapolation modeling, and population-based and human exposure data. The toxicity testing tools consist of high-throughput screens, stem cell biology, functional genomics, bioinformatics, systems biology, computational systems biology, physiologically based pharmacokinetic models, SARs, and biomarkers. The goals of two specific multiagency initiatives, Tox21 and ToxCast, are in line with the vision.

Tox21

Tox21 is a collaboration between the U.S. EPA, National Institutes of Environmental Health Sciences (NTP), National Institutes of Health (National Human Genome Research Institute and NIH Chemical Genomics Center [NCGC]), and the FDA. The agencies are involved in a multidiscipline research initiative designed to develop and validate testing methods, which evaluate the impact of chemicals on toxicity pathways.[190] The charge of the consortium includes efforts to (1) research ways to use new tools to identify chemical-induced biological activity mechanisms, (2) prioritize which chemicals need more extensive toxicological evaluation, (3) develop models that can be used to more effectively predict how chemicals will affect biological responses, and (4) identify chemicals, assays, informatic analyses, and targeted testing needed for the innovative testing methods (www.epa.gov/ncct/tox21/; accessed March 22, 2012). Collectively, the work will support chemical prioritization, risk assessment, and hazard identification. The Tox21 chemical inventory consists of compounds with structural diversity and varying chemical characteristics such as industrial and food-use chemicals, pesticides, and drugs as well as chemicals and defined mixtures that have been tested by the NTP for genotoxicity, carcinogenicity, immunotoxicity, reproductive toxicity, and/or developmental toxicity. The complete Tox21 chemical inventory will undergo screening at the NCGC via multiple high-throughput screening assays. These data will be publically available via several databases ACToR (EPA), CEBS (NIEHS), and Tox21 Browser (NCGC) and PubChem.

ToxCast

ToxCast is a chemical screening tool that is part of the EPA's risk assessment program. ToxCast was initiated in 2007 in a phased manner to set priorities for chemicals requiring toxicity testing. The initiatives outlined to achieve this deliverable are detailed as follows:

- *ToxCast uses advanced science tools to help understand how human body processes are impacted by exposures to chemicals and helps determine which exposures are most likely to lead to adverse health effects.*

TABLE 23.15
Select ToxCast Publications

ToxCast Phase I data	In vitro screening of environmental chemicals for targeted testing prioritization—the ToxCast Project[191]	2010
Transcription regulators	Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPS's ToxCast program[192]	2010
BioMap cell systems	Profiling bioactivity of the ToxCast chemical library using BioMap primary human cell systems[193]	2009
Genotoxicity assays	Evaluation of high-throughput genotoxicity assays used in profiling the U.S. EPA ToxCast chemicals[194]	2009
ToxCast chemicals	Xenobiotic-metabolizing enzyme and transporter gene expression primary cultures of human hepatocytes modulated by ToxCast chemicals[195]	2010
Dosimetry and exposure	Integration of dosimetry, exposure and high-throughput screening data in chemical toxicity assessment[196]	2012
Chronic toxicity	Profiling chemicals based on chronic toxicity results from the U.S. EPA ToxRef database[197]	2009

- *ToxCast testing methods include over 650 state-of-the-art rapid tests (called high-throughput assays) that are screening 2000 environmental chemicals for potential toxicity.*
- *Phase I, proof of concept was completed in 2009, and it profiled over 300 well-studied chemicals (primarily pesticides).*
- *Phase I chemicals have over 30 years' worth of existing toxicity data since they have been tested already using traditional toxicology methods (primarily animal studies). Data from animal studies can be searched and queried using EPA's toxicity reference database (ToxRefDB) that stores nearly $2 billion worth of studies.*
- *Phase II was completed in 2013 and over 1800 chemicals were screened from a broad range of sources including industrial and consumer products, food additives, and drugs that never made it to the market to evaluate the predictive toxicity signatures developed in Phase I.*
- *Data from the high-throughput assays are available via the ToxCast database.*
- *Toxicity signatures from ToxCast are defined and evaluated by how well they predict outcomes from mammalian toxicity tests and identify toxicity pathways relevant to human health effects.*
- *ToxCast provides the Tox21 collaboration access to ToxCast's high-throughput screening data and chemical library to increase the data available on the nearly 10,000 chemicals being studied (www.epa.gov/ncct/toxcast/; accessed March 22, 2012 and March 6, 2013.)*

The tool combines several testing platforms including more than 500 high-throughput screening assays to assess the impact of chemical exposure in humans and the associated adverse health effects (e.g., chronic disease or reproductive problems). The initial *proof of concept* phase was completed in 2009 and included the evaluation of 300 chemicals consisting primarily of pesticides. These data were compared to prior animal testing of the chemicals in order to assess the concordance of the in vitro screens with the in vivo data. The second phase will incorporate the screening of 700 more chemicals including chemicals of consumer product, cosmetics, and drugs. The data from these studies are retained in the ToxCast database such that queries on the chemicals and assay used as well as affected end points, genes, and pathways can be conducted. Relevant publications from the program to date are listed in Table 23.15.

Collectively, the application of toxicogenomics in exposure assessment, hazard screening, risk assessment, human susceptibility, and mechanisms of action is showing promise and is becoming an integral part of toxicological research and in vitro testing. Current advances in drug discovery and personalized medicine and by the NCT support this assertion should provide key information to support the integration of toxicogenomics into toxicological test batteries.

QUESTIONS

23.1 Define epigenetic genotoxicity and identify the primary differences and similarities between epigenetic and nonepigenetic alterations to genes and gene expression processes.

23.2 What is the human *genetic burden* and what might be its origins?

23.3 While most testing strategies involve a battery of tests, what are the primary limitations associated with increasing the number of tests in a battery?

23.4 What were the reasons for expanding the testing options in the 2008 update to the original ICH genetic toxicology test battery for pharmaceutical products?

23.5 What factors make it difficult to perform quantitative risk estimates for heritable genetic effects in human populations?

23.6 What is transcription-coupled DNA repair and which of the various DNA repair processes is primarily involved?

KEYWORDS

Genotoxic, Mutagenesis, DNA repair, Safety assessment, Toxicogenomics, Gene expression, Toxicology, Genomics, Bioinformatics

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24 Short-Term, Subchronic, and Chronic Toxicology Studies

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CONTENTS

INTRODUCTION

Repeated-dose toxicity studies are conducted to screen for potential adverse effects of compounds such as pharmaceuticals (small molecules as well as biological agents), pesticides, food additives, or other chemicals using laboratory animals as surrogates for the intended exposed population or target species, most often the human. Repeated-dose studies may be of varying duration, generally 1–4 weeks for short-term studies, 3 months for subchronic studies, and 6–12 months for chronic studies. Many parameters indicative of the health of the test species are monitored in short-term, subchronic, and chronic toxicity studies, resulting in the ability to detect a variety of adverse effects, identify alterations due to exaggerated pharmacology, and/or assess the reversibility of findings. Sometime during their career, most toxicologists are involved in designing, performing, monitoring, or reviewing data from these types of toxicity studies as a result of the central role of these studies in safety assessment.

It has been suggested that subchronic data alone may be sufficient to predict the hazard of long-term low-dose exposure to a compound.[1] While this may be true for compounds where adequate structure/activity relationships exist and there is no indication of genetic toxicity, it generally is not true when compounds have completely unknown toxicity or when structure/activity relationships predict a potential adverse effect. For certain chemicals or mixtures, results from a short-term or a subchronic toxicity study may represent the most sophisticated toxicology data available. With many chemicals, a subchronic study is critical to the design of longer-term hazard assessment studies.

It is essential that toxicologists become familiar with the scientific principles upon which repeated-dose toxicity studies are based and understand the methodology used to perform these studies. This chapter provides an introduction to these studies and many of the principles upon which they are based. It focuses mainly on hazard assessment for chemicals that are small molecules. Today, biotechnology is a rapidly developing field. Hazard assessments for biologics, for example, recombinant proteins and conjugated antibodies, are special cases that will not be discussed in detail in this chapter.

The hazard assessment for a biologic is tailored specifically to the compound and may be more or less comprehensive than a classical assessment with a small molecule. Throughout this chapter, important differences between small molecule and biological development will be noted, when appropriate. Guidelines for testing biological materials have been drafted and can be consulted for more detailed information regarding considerations involved in the development of a biologic.[2] Despite some differences in requirements for development, many of the principles and methods of individual studies with small molecules are applicable to testing with biotechnology products. The toxicologist responsible for investigating the safety of these materials should become familiar with the current guidelines and suggestions for their testing.

REPEATED-DOSE STUDIES AND HAZARD (SAFETY) ASSESSMENT

The major steps performed in a typical hazard assessment are illustrated in Figure 24.1. However, in practice, there is no such thing as a *typical* hazard assessment program. This is particularly true in the case of pharmaceutical development where advancements in medical science have led to such things as targeted therapies, the use of nanotechnology, development of biological products, and gene therapy. Oftentimes, each hazard assessment program is unique and is based upon the characteristics of the individual material being tested, its intended use, and the intended patient/target population. Furthermore, the temporal sequence of the studies presented in Figure 24.1 may be modified depending on organizational practices. For example, some pharmaceutical companies may conduct the in vitro genotoxicity and/or screening reproductive toxicity assessments earlier in the development program in order to truncate the development of compounds with positive genotoxicity results or potential for teratogenicity, thus minimizing time and costs associated with the development of these *bad* drugs.

It may seem obvious, but the first step in any hazard assessment is determination of the material to be studied and the purpose of the assessment. Both of these factors will influence the final design of the program. At the time of selection of the material to be tested, its intended use in the marketplace will have been defined. In most cases, this dictates factors such as exposure route, test species, and the types of studies required. For example, a chemical company may need to assess the risk of a compound they are developing to be used in packaging of foods and to which humans could be exposed through the diet while a pharmaceutical

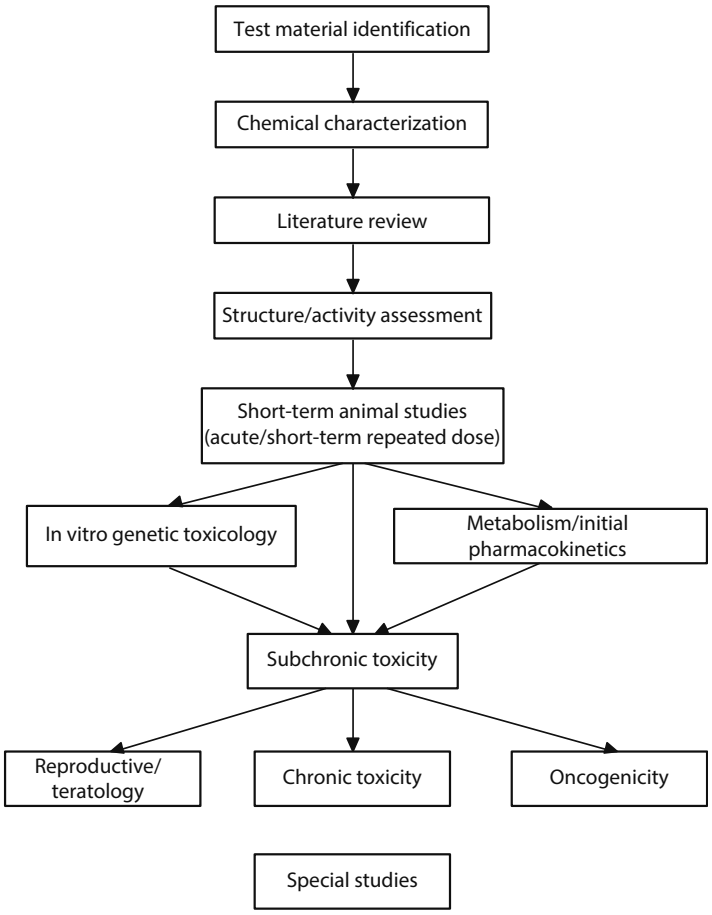

FIGURE 24.1 Schematic representation of a typical hazard assessment program illustrating a step-by-step, tiered approach and the interactions between the various elements. It should be noted that the approach presented here is not the only approach that could be used.

company may want to examine potential toxicity of a new drug administered intravenously that targets proteins in cell cycle pathways involved in cancer.

The next step is the chemical and physical characterization of the material or mixture to be investigated. Knowledge of the major component(s) and any associated contaminants is required for almost every phase of the hazard assessment program. The more detailed the chemical and physical characterization, the greater the likelihood that the entire program will be successful.

Once the toxicologist has information concerning the chemical nature of the test material, the scientific literature is searched to determine what is known, if anything, about its biological activity. If no information is available, the potential biological activity of chemicals with similar chemical structure should be ascertained. There are several computerized programs designed to search for structural alerts; two of the most commonly used databases are DEREK and MultiCASE.[3,4] Also, for targeted therapies, literature regarding effects of other compounds that target the same receptor as the novel compound should be reviewed to evaluate potential class effects. Based upon the chemical characterization and any toxicology information available in the scientific literature, the toxicologist can perform a structure/activity assessment. This will aid in developing the hazard assessment program and the specific designs for each of the toxicity studies.

Following test material characterization, literature evaluation, and structural analysis, in vivo testing is usually initiated. The first studies to be conducted are normally short-term toxicity studies. Historically, the initial short-term toxicity study was generally a single-dose acute toxicity study. Results from acute toxicity studies are used to estimate dosages to be used in short-term repeated-dose studies and to obtain initial data useful in assessing relative toxicity. A number of regulatory agencies have made recommendations concerning designs for acute studies as discussed in the acute toxicity chapter of this text. If warranted, additional endpoints may be added to these recommended designs based upon the structure/activity assessment and literature review. It should be noted, however, that in some cases, such as during pharmaceutical development, acute toxicity studies may not be required because it is currently believed that information from these studies can be garnered from dose escalation studies or other repeated-dose studies that establish a maximum tolerated dose.[5–7]

Data obtained from short-term repeated-dose toxicity studies are generally required for the successful design of subchronic toxicity studies. Similarly, data from subchronic studies are essential for the design of chronic studies. Table 24.1 lists objectives for short-term repeated-dose toxicity studies. Table 24.2 lists examples of data obtained from these studies that are useful in the design of subsequent studies. As will be discussed later, one of the difficult decisions for a toxicologist designing a toxicology study is the selection of the dose range to be used. Data on dose–response and the other toxicological endpoints evaluated in short-term toxicology studies are critical to the successful dose selection for subsequent toxicity studies.

TABLE 24.1
Objectives of Short-Term Repeated-Dose Studies

- Determine adverse effects of the test compound at doses low enough to allow survival of most animals, as opposed to acutely toxic doses.
- Determine adverse effects over a longer exposure (dosing) period than used in acute studies.
- Determine reversibility of adverse effects after discontinuation of treatment.
- Determine dose–response for adverse effects following repeated dosing and identify a no-observable-adverse-effect level (NOAEL).
- Identify organs affected by exposure to test material (target organs).
- Provide data concerning species differences, if any, in sensitivity to potential adverse effects.
- Provide initial data for comparative risk assessment.
- Determine the need for specialized endpoints to be assessed in longer-term studies.

TABLE 24.2
Data Obtained from Short-Term Repeated-Dose Studies That Are Useful in the Design of Subsequent Studies

- Palatability of test material/diet mixture, if dosing is by feed
- Body weight response patterns
- Physical observations
- Behavioral changes
- Clinical pathology
- Toxicokinetics
- Gross necropsy
- Histopathology
- Identification of target organs
- Dose–responses

Although not always available, data from genetic toxicity and metabolism studies are useful adjuncts to that from short-term repeated-dose studies. For instance, if the in vitro phase of the genetic toxicology program has indicated the test material has genotoxic potential, it may be dropped from consideration for further development and further testing may be unnecessary. It may also be possible to add in vivo genetic toxicity assessment to the design of subchronic or chronic toxicology studies. This can, in many cases, reduce the number of animals used in the overall safety assessment and conserve resources. Also, if information concerning the metabolism and pharmacokinetics of the test material is known, it should be used in the design of repeated-dose toxicity studies. For example, if pharmacokinetic or toxicokinetic studies indicate a potential for the accumulation of the test material after repeated dosing, this is an important consideration in deciding the most appropriate dose range. Furthermore, because different species may metabolize test materials differently, this information is important in the selection of appropriate animal species and strains for subsequent testing (i.e., a species that metabolizes the test material

TABLE 24.3
Objectives of Subchronic Toxicology Studies

- Identify adverse effects not detected in shorter-term acute or repeated-dose studies
- Provide additional information on adverse effects identified in short-term studies such as progression or reversibility of effects
- Identify observable effect level and the NOAEL
- Provide data for dose selection and other study design features for chronic toxicity and other longer-term studies
- Confirm and/or identify target organs or sites of action
- Provide data to determine if specialized endpoints are required
- Provide a basis for species selection for additional studies, if required, and for data extrapolation to humans
- Provide information for regulatory agencies in support of the safety of the test material
- Provide risk assessment data

in a manner similar to humans would be preferred over a species that metabolizes the test material by dissimilar metabolic pathways). The major objectives of subchronic toxicity studies are presented in Table 24.3.

In some cases, such as when environmental exposure will be limited/brief or during the development of cancer therapies intended to treat patients whose long-term survival is unlikely, subchronic toxicity testing may complete the data package required for a hazard assessment. In other cases, such as when drugs are being developed for non-life-threatening diseases or when the target species will be exposed to the test material for a significant portion of its life, data from subchronic studies are used to design additional studies, including chronic toxicity studies (as illustrated in Figure 24.1). Data from subchronic toxicity studies are useful in the design of oncogenicity and reproductive toxicity studies as well as any *special* studies that may be warranted based on the results of other studies and/or requested by a regulatory agency.

As noted earlier, data from a short-term repeated-dose study should be available before the initiation of subchronic toxicity testing and, generally, data should be available from a subchronic study before proceeding with chronic testing. The major utility in progression from shorter-term to longer-term studies is to ensure, to the extent possible, that proper dose ranges are selected. Requirements for a scientifically valid short-term repeated-dose study are similar to those of subchronic toxicity studies. Therefore, the general aspects of short-term studies will not be discussed separately. However, three aspects of short-term repeated-dose studies are especially important and need to be mentioned.

First, as implied by the description *short-term repeated-dose study*, these studies are of shorter duration than subchronic toxicity studies. Generally, the duration of these studies is either 14 or 28 days with the compound administered daily, although dosing frequency can vary and, in the case of pharmaceuticals, should be based on the intended dosing frequency/regimen to be used in the clinic, if known. A 28-day study can produce more robust information than a 14-day study, which, in turn, will provide more data than a 7-day study. The shorter-term studies may be valuable in identifying target organs due to exposure at high levels, while the longer studies provide more valuable information regarding adverse effects following prolonged exposure at lower dose levels.

Second, dose selection is somewhat dependent upon the purpose of the study. If the short-term study is designed to produce information to be used in the design of a subchronic study, dose levels should be selected to ensure any potential adverse effects are observed. Higher doses are generally used in short-term studies to determine target organs, and it may not be as critical to ascertain a no-observable-adverse-effect level (NOAEL). However, it is always useful to have a NOAEL in a toxicity study. If no further studies are anticipated with the compound, a dose range that includes a NOAEL becomes very important. Because little information other than acute toxicity data is generally available during early compound development, it may be necessary to run a more comprehensive range of dose levels in short-term repeated-dose studies. Four, five, or more dose groups are generally used in short-term studies. This increases the chances of characterizing the dose–response and should increase the confidence of dose range selection for subsequent studies.

A third aspect of short-term studies that is different from subchronic studies is the number of animals per group. Although fewer animals may be used, 5–10 animals of each gender in each dose group are recommended for short-term rodent studies, depending on which agency's recommendations are being followed. Additional animals may be required for nonroutine endpoints, such as a group added to ascertain the reversibility of an adverse effect upon cessation of dosing (often referred to as a recovery group). Also, additional animals may be placed in the high-dose group if potential compound-related mortality is expected.

To summarize, many factors influence the design of all repeated-dose toxicity studies. Before initiation of a study, it must be decided if the specific chemical or chemical mixture, that is, the test material, to be used in the study is appropriate. The appropriate animal model must be chosen, the correct route of exposure must be selected, and the study duration and dosing frequency must be decided. Control and treatment groups and their doses have to be selected. Parameters to be evaluated must be selected to maximize the probability of detecting potential adverse effects. By taking time to design a study carefully, the toxicologist can ensure the study will meet the regulatory requirements and provide adequate data to support the risk assessment.

Regulatory Requirements

National and international regulatory bodies have issued guidelines for the design and conduct of repeated-dose toxicity studies. Even though study designs described in these various guidelines have similar characteristics, some differences between the requirements have existed historically within and between regulatory agencies. This has resulted, in

some cases, in the duplication of studies to ensure guidelines of different agencies are satisfied. The agencies themselves and the regulated community agreed that this duplication of the use of animals and other resources was undesirable. As a result, regulatory authorities worldwide have harmonized many of their guidelines to ensure toxicity studies conducted under a single set of regulations will be universally acceptable. In the United States, the Environmental Protection Agency (EPA) has issued a single set of harmonized toxicity testing guidelines[8] to blend the requirements of the separate guidelines previously promulgated through the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), the Toxic Substances Control Act (TSCA), and the Organisation for Economic Co-operation and Development (OECD). OECD, an international organization of 30 countries including the United States, has issued its own toxicity testing guidelines.[9] Each member country has agreed to accept studies conducted according to these guidelines. In 1991, regulatory authorities and trade organizations from the United States, Japan, and the European Union initiated a cooperative effort through the International Conference on Harmonization (ICH) to produce guidance documents concerning requirements for safety studies with pharmaceutical products. ICH guidance documents generally address specific issues related to toxicity testing, for example, the duration of chronic toxicity testing,[10] handling of impurities,[11] and specific requirements for the testing of oncology agents[12] or biological products,[2] and are not detailed guidelines for toxicity study design.

Each regulatory harmonization effort discussed earlier has been useful, allowing for any well-planned, scientifically valid, adequately conducted, repeated-dose study to satisfy the requirements of multiple regulatory agencies. The scientific foundation for toxicological hazard assessment is continually expanding, and to compromise this foundation by pursuing standard checklist protocols is irrational, wasteful, and unscientific. Today, regulations generally recognize the importance of scientific judgment and encourage discussions between the regulated community and the regulator about alternative study designs. Investigators and regulators share in the obligation to conduct a scientifically sound study.

The remainder of this chapter relates to the typical design and conduct of subchronic and chronic repeated-dose toxicity studies. The information presented should provide the toxicologist with enough information to design a study to satisfy most regulatory guidelines. Parameters evaluated in subchronic and chronic toxicity studies are essentially identical, the major difference being the schedule for data collection. Table 24.4 compares the minimum requirements for subchronic and chronic toxicity studies using rodents as described in the EPA,[8] U.S. Food and Drug Administration (FDA)[13] and OECD[9] testing guidelines. For repeated-dose study designs intended to satisfy guidelines of a particular regulatory authority, it is recommended that the reader (1) use the testing guidelines of that authority as a starting point during the design of a study, (2) refine the design based upon characteristics of the compound of interest, (3) document the design in a detailed, written protocol, and (4) discuss the protocol with a representative of the regulatory authority prior to initiating a study.

Good Laboratory Practice Regulations

In addition to regulations and guidelines concerning the design of repeated-dose studies, the United States and international regulatory authorities have issued good laboratory practice (GLP) regulations, which are principles concerning the manner in which all nonclinical hazard assessment studies (e.g., animals studies) are to be conducted, documented, and reported.[14–21] These GLPs are designed to assure the quality of the study data and report. Requirements from each of the regulatory agencies are similar. GLPs set standards for the test system, laboratory organization, personnel, facilities, equipment, operations, and record keeping. They require that studies be conducted according to written protocols (see Table 24.5) and validated written standard operating procedures (SOPs). Chemical analyses are required to characterize the test article and control article administered during the studies. Study procedures and data must be clearly and completely documented and reported. Automated data collection systems must be qualified and validated prior to use.

Regulatory agencies inspect testing laboratories periodically to assure GLP compliance. Safety studies not conducted according to GLPs will not be accepted by regulatory agencies as pivotal studies to support registration; however, during early pharmaceutical development or during early stages of a risk assessment, studies may be conducted under non-GLP conditions in order to provide preliminary information regarding potential toxicities and can be valuable in dose selection for subsequent GLP-compliant studies. In addition, some versions of GLPs, including those of the U.S. regulatory agencies, provide for disqualification of laboratories that are in major noncompliance with GLP requirements. For these reasons, laboratories conducting studies for regulatory submission generally take great care to comply with GLPs. GLP regulations are generally similar between regulatory bodies. A comparison chart illustrating the requirements of FDA, EPA, and OECD GLP regulations can be downloaded from the FDA website at the address listed in Table 24.17.

STUDY DESIGN

Chemical and Physical Characterization of the Test Material

It may seem that selection of the test material for repeated-dose toxicity testing should require little involvement by the toxicologist because the test material is usually provided by a chemist or product manager. However, several important factors must be considered by the toxicologist to ensure that a study will adequately assess the potential toxicity of the chemical and be accepted by regulatory agencies. Obviously, one of these considerations is that the batch or lot of test material is representative of the chemical intended to be tested

TABLE 24.4
Subchronic and Chronic Rodent Oral Toxicity Studies Based on Various Regulatory Guidelines

	EPA OPPTS Guidelines		FDA Redbook 2000		OECD Guidelines	
	Subchronic [65]	**Chronic [66]**	**Subchronic [56]**	**Chronic [56]**	**Subchronic [89]**	**Chronic [90]**
Study duration	≥90 days	≥12 months	≥90 days	≥12 months	≥90 days	≥12 months
No. of treated groups						
Standard study	≥3	≥3	≥3	≥3	≥3	≥3
Limit test[a]	1	1	1	1	1	1
No. of negative control groups[b]						
Untreated control	1	1	1	1	1	1
Vehicle control	1	1	1	1	1	1
No. of animals/gender/group[c]	≥10	≥20	≥20	≥20	≥10	≥20
Age of animals (at start of study)	≤8–9 weeks	≤8 weeks	≤6–8 weeks	≤6–8 weeks	<9 weeks	≤8 weeks
Body weight measurement						
Frequency through 13 weeks	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly
Frequency after 13 weeks	NA	Every fourth week	NA	Monthly	NA	Monthly
Feed consumption measurement						
Frequency through 13 weeks	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly
Frequency after 13 weeks	NA	Monthly	NA	Monthly	NA	Monthly
Observations						
For mortality and morbidity (times/day)	2	2	2	2	2	2
For general condition (times/day)	1	1	n	n	1	1
For detailed clinical findings (frequency)	Weekly	Weekly	Daily	Daily	Weekly	Daily
Neurotoxicity evaluation (at term)[d]	y	y	y	n	y	y[f]
Ophthalmology						
No. animals pretest	AA	AA	AA	AA	AA	AA
No. animals/gender/high dose and control at term[e]	AS	10	AS	AS	AS	AS
Hematology and clinical chemistry (no./gender/group)	AS	10	10	AS	AS	10
Intermediate time(s)	n	6 months	≤14 days, 45 days	14 days, 3 and 6 months	n	3 and 6 months
Term	y	y	y	y	y	y
Urinalysis (no./gender/group)	AS	10	10	AS	AS	10
Intermediate time(s)	n	n	n	14 days, 3 and 6 months	n	3 and 6 months
Term	o	y	y	y	o	y
Gross necropsy and tissue collection	AA	AA	AA	AA	AA	AA
Organ weights (no./gender/group at term)	AS	AS	AS	AS	AS	AS
Adrenals, brain, epididymides, heart, kidneys, liver, spleen, testes/ovaries, uterus	y	y	y	y	y	y
Prostate	n	n	n	y	n	n
Thymus	y	n	y	y	y	n
Thyroid/parathyroid	n	y	y	y	n	y
Histopathology						
All tissues (all high-dose and control animals)	y	y	y	y	y	y

(*continued*)

TABLE 24.4 (continued)
Subchronic and Chronic Rodent Oral Toxicity Studies Based on Various Regulatory Guidelines

	EPA OPPTS Guidelines		FDA Redbook 2000		OECD Guidelines	
	Subchronic [65]	Chronic [66]	Subchronic [56]	Chronic [56]	Subchronic [89]	Chronic [90]
All tissues (all animals killed or died on study)	y	y	y	y	n	y
Target tissues and gross lesions (all animals)	y	y	y	y	y	y

NA, not applicable; y, yes/required; n, no/not required; o, optional; AA, all animals; AS, all survivors.

[a] If a test at one dose level of at least 1000 mg/kg body weight/day produces no observed adverse effects and if toxicity would not be expected based upon data from structurally related compounds, then a full study using three dose levels may not be considered necessary.

[b] Contingent upon the route of exposure in the test article–treated groups.

[c] Extra animals must be added for interim sacrifices and special determinations, for example, toxicokinetics and reversibility of effects.

[d] Not required if similar data are available from other studies or if other clinical signs are noted to an extent that would interfere with evaluation.

[e] Animals in intermediate groups are to be evaluated if compound-related findings are noted in the high-dose group.

[f] If previous studies indicate potential to cause neurotoxic effects.

TABLE 24.5
Protocol Contents Required by FDA Good Laboratory Practices Regulations

- Title and study objective
- Identification of test and control articles
- Identification of sponsor and testing facility
- Justification of test system (animal model)
- Test system information (number, body weight, gender, source, species/strain, age, method of identification, etc.)
- Description of study design and methods for control of bias, such as random assignment of animals to treatment groups, processing of clinical pathology samples in replicates, etc.
- Animal husbandry information
- Dosing information, including dose form preparation and route of administration
- Methods by which degree of absorption of the test and control articles by the test system will be determined, if necessary
- Types and frequencies of assays, analyses, and measurements to be made
- Description of statistical methods
- Records to be maintained

and eventually marketed. Adequate chemical and physical characterization is essential for this determination. Several regulatory agencies and organizations have issued guidelines to describe the information needed and some of the methodology to be used to characterize a chemical.[22–25]

A safety assessment should include a partnership between the toxicologist and a chemist that understands the chemical characteristics of the test material. A thorough chemical characterization of the test material should be provided to the toxicologist. This characterization should include methods of synthesis, precursors used in the synthesis, and any solvents and manufacturing aids used in the manufacturing process. It should also include a quantitative assessment of the major components, with associated accuracy and precision information, and at least a qualitative analysis of minor components. Ideally, the toxicologist should be provided with a mass balance for the test material. Also, the methods of purification of the test material should be provided. This enables the toxicologist to determine the potential for the occurrence of residues and impurities that could produce adverse effects. It also provides the toxicologist with information needed to determine if any specific residues or impurities should be specifically targeted for additional analytical determinations. Prior to initiation of a repeated-dose study, the toxicologist should carefully review the chemical and physical characterization data and applicable regulatory guidelines such as those issued by ICH dealing with residual solvents[26] and impurities.[11]

The specific characterization information required by the toxicologist may vary with each test material. The required data may be different from that used to establish manufacturing and quality control specifications. However, it is essential that the manufacturing and quality control specifications and procedures meet the specific regulatory requirements for toxicity testing. For instance, good manufacturing practices (GMPs), which are somewhat similar to GLPs, are required for some chemicals.[27–32] The chemist should ensure that adequate GMP records exist, if required.

Before initiation of a repeated-dose toxicity study, chemical analyses should be conducted to assure that the bulk test material is stable and that the test material is stable over the range of anticipated concentrations in the vehicle to be used in the study for the maximum period of use. According to GLPs, this evaluation can be conducted concurrently with the study, but detection of instability could invalidate the study results. If the material is stable only under certain conditions, for example, frozen storage, special arrangements for its storage should be made to eliminate instability problems. Instability of the test material may result in lower than expected doses and exposure of the animals to degradation products. The products of degradation may either have their

own unique toxicity or alter the toxicity of the test material. This may make it impossible to correctly interpret data from the study. Stability of the test material in different matrices may also need to be established to support a toxicity study. For example, stability in serum or plasma may need to be demonstrated if toxicokinetic sampling and analysis will be performed.

The toxicologist will have assessed the chemical characterization of the test material at the initial stages of the hazard assessment. However, more complete information may become available before the initiation of repeated-dose toxicity studies. Sometimes, the chemical synthesis or other production methods may change between the initial assessment and the start of subchronic or chronic studies. Therefore, it may be necessary to reassess the chemical characterization before the initiation of these studies. Unanticipated changes in the chemistry of the test material or mixture may necessitate changes in the toxicity study design and/or require additional studies to be performed in order to qualify newly identified impurities. As part of the test material evaluation process, the toxicologist also needs to be aware of any potential genotoxic impurities (GTIs) that arise during the synthesis process. Guidelines for dealing with GTIs and other impurities have been drafted[33,34] and should be consulted when setting limits for these contaminants.

Natural products of plant origin, for example, some of the herbal remedies that are popular today, are often complex mixtures, which present challenges to the chemist and toxicologist. The quantitative chemical composition can be quite variable based upon the environmental growing conditions of the plant, such as soil composition, rainfall, and temperature, and the particular cultivar. This variability results in a range of quantitative values for the components of the mixture and must be considered by the toxicologist.

The test material should be as similar as possible to the chemical to which humans will be exposed. Every attempt should be made to ensure that the test material is either identical to the final commercial product or representative of the anticipated final product. This may not always be possible with commercial products because large-scale production facilities are usually not available during early phases of a safety assessment. When such facilities become available, it may be possible to bridge between the final commercial product and the test material by chemical analysis. *Bridge chemistry* should identify any differences between the chemical that was tested in toxicity studies and the commercial product. This will allow a determination of the toxicological bioequivalence of the test material and final commercial product.

If a impurity of unknown toxicity (i.e., an impurity that was not identified in the test article batches used for toxicity testing) is detected in a drug substance or drug product after animal studies have been completed, the ICH requires that an evaluation be performed taking into account identification and qualification thresholds as described in the ICH quality guidance documents.[24] Based on this evaluation, additional testing may be required including genotoxicity studies, general toxicity studies, and/or other toxicity studies to address specific endpoints. Impurity identification and qualification should be done on a case-by-case basis, and it is suggested that the toxicologist familiarize him/herself with the recommendations and decision trees presented in the appropriate guidelines.

The test material should not only have the same chemical characteristics as the commercial material but also have the same physical characteristics, when possible. If the test material is a solid intended to be used as a powder, it should be administered to the test animals in powder form. Particle size of the powder should be similar for the test material and the material to which humans will be exposed. If humans will be exposed to the material in solution, a solution of the material should be used in the repeated-dose study. During drug development, the final drug product physical form (e.g., tablet, capsule, gel) is generally not defined and/or is not available for preclinical toxicity testing. For this reason, the form of test material used in these studies is oftentimes the neat material that is dissolved or suspended in some type of vehicle. Analyses should be conducted to ensure that the composition of the test material falls within the limits of anticipated or known product specifications. Physical specifications will allow the toxicologist, oftentimes in consultation with the chemist, to determine the most appropriate method of adding the test material to the dosing matrix, such as the diet or drinking water (also see route of administration section provided later).

It is important for the toxicologist to ensure that an adequate supply of test material is available before initiating a toxicity study. A single lot of the test material should be used throughout the subchronic or chronic study, whenever possible. Furthermore, if the repeated-dose study is part of a series of studies in a safety assessment program, it is desirable to use a single lot of test material for the entire program. This reduces the probability of encountering inconsistent results in different studies with the same test material, resulting from inter-lot differences in chemical/physical characteristics. If a single lot of test material is not available in sufficient quantity to complete a study or studies, multiple lots may be used. Chemical characterization of each new lot is required to ensure that it meets all specifications and reasonably duplicates previous lots.

The U.S. FDA has issued guidelines addressing the chemistry data requirements for direct food additive petitions.[23] ICH has issued guidelines for chemistry requirements for drug candidates during preclinical hazard assessment.[24] Although the toxicologist may not have as complete a data package as described earlier before the initiation of a repeated-dose study, sufficient data must be available to meet GLP requirements. FDA GLPs state that "The identity, strength, purity, and composition or other characteristics which will appropriately define the test or control article shall be determined for each batch and shall be documented. Methods of synthesis, fabrication, or derivation of the test and control articles shall be documented…."[35] U.S. EPA GLP statements concerning the requirements for chemical characterization of the test

material are essentially identical to those of the FDA.[36] The OECD GLP guidelines for chemical characterization state, "For each study, the identity, including batch number, purity, composition, concentrations, or other characterizations to appropriately define each batch of the test or reference items should be known."[20] Further information on the chemical characterization of the test material is provided by the FDA in its guidelines for toxicity studies, "The composition of the test substance should be known including the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials." The guidance also states that "the test substance used in toxicity studies should be the same substance that the petitioner/notifier intends to market."[37]

Route of Exposure and Method of Test Material Administration

The anticipated human route of exposure to the test material dictates the route of exposure for most subchronic and chronic toxicity studies. Nonintended routes of human exposure should also be considered during the selection of exposure route. The most common routes of exposure in these studies are dietary, oral (gavage or capsule), dermal, intravenous (IV), subcutaneous, and inhalation. Parenteral administration by infusion using implanted or external pumps or IV, subcutaneous, intraperitoneal (IP), or other types of injection is becoming increasingly common, especially with the increasing number of biological agents being developed that need to be injected because they are not absorbed from or broken down in the gastrointestinal (GI) tract. Less frequently, test materials are administered in the drinking water or by intraocular, intravaginal, or some other route.

Although the route of administration/exposure to pharmaceutical agents is usually clearly defined, there are several potential routes of human exposure to a single compound when dealing with compounds such as pesticides and industrial chemicals. For example, consumer exposure to a pesticide may occur by dietary consumption of food crops containing residues of the chemical. Farmworker exposure to the same pesticide may occur by either inhalation or dermal routes during application and harvesting. In such cases, subchronic testing may be required to assess the effects of exposure by all three routes. Emphasis is generally placed upon the route by which the most widespread human exposure would occur; chronic testing is usually conducted only using this route of exposure. Several of the more common routes of exposure are discussed below.

Capsule Administration

If expected human exposure is by the oral route, a solid test material (e.g., a dry powder) can be administered by capsule. Unformulated bulk test material can be given to some large animals, such as dogs and cats, in gelatin capsules inserted into the esophagus manually or with the aid of a mechanical device designed to prevent the animal from biting the individual administering the capsule. Capsules are available in a wide range of sizes and can hold, depending on the density of the test material, as much as 1.5 g. The amount of test material that can be administered is limited by the capacity of each capsule and the practical number of capsules that can be administered at one time. For smaller species such as rodents, small capsules are available; however, capsule dosing of rodents is oftentimes impractical, and the test material is usually administered orally as a solution or suspension in an appropriate vehicle.

Oral Gavage

Another common route of oral administration is oral gavage by intubation. This technique may be used for rodent and nonrodent species. For oral gavage, a solution or suspension of the test material is deposited into the stomach via the esophagus using an intubation tube attached to a graduated syringe or other device. A test material is added to an appropriate vehicle, usually aqueous. If the material is not readily soluble, a suspension may be prepared. For suspensions, a material such as methylcellulose, carboxymethylcellulose, ethylcellulose, or gum tragacanth is added to water to increase the viscosity, and the test material is homogeneously suspended in the vehicle. A wetting agent such as Tween 80 can be used to increase the suspendability of the material. Although aqueous vehicles are preferred, it is possible to use an oil vehicle for lipid-soluble materials. Food oils, such as corn oil, may be used at appropriate volumes, but mineral oils must be avoided. Also, absorption of the test material may be quite different from an oil vehicle compared with an aqueous vehicle, and absorption may be highly volume dependent. When a dosing formulation is a suspension, it is important to document that the suspension is homogeneous and stable. This can be accomplished by taking samples from the top, middle, and bottom of the formulation and assessing the concentration at each level. If the formulation is homogeneous, the variability between the concentrations at each level will be minimal.

The volume of test solution or suspension administered can influence gastric emptying time.[38,39] Since gastric emptying time may affect GI uptake and bioavailability, it generally is preferred that the doses of the test substance be administered on a constant-volume (mL/kg), variable-concentration (mg/mL) basis. For dosing using a constant volume, 10 mL/kg body weight is commonly used as the upper limit although the upper limit may vary by species. At 10 mL/kg, a 260 g rat would receive a dose volume of 2.6 mL, a 35 g mouse would receive 0.35 mL, and a 10 kg dog would receive 100 mL. Dosing usually is conducted once daily but may be performed more frequently in order to mimic the intended human dosing regimen or if concentration and/or pharmacokinetic considerations limit the achievable single-dose exposure to less than the amount desired. When multiple doses are administered each day, it is important not to exceed a reasonable maximum daily dose volume. Publications regarding maximum recommended daily dose volumes are available from various sources[40] and should be consulted when considering dose volumes to be used in a study. Generally, the maximum oral

gavage dose volumes for rats and dogs are 40 and 15 mL/kg, respectively, and maximum dose volumes for a bolus IV dose are 5 and 2.5 mL/kg for rats and dogs, respectively. There may be occasions when the maximum recommended dose volumes may be exceeded; however, these exceptions should be scientifically justified and should be discussed with veterinary personnel at the test facility prior to study initiation.

Daily exposure by oral gavage results in a bolus dose. Administration via the diet or drinking water results in a more constant exposure throughout the duration of the study that is more dependent on feeding patterns. Because rodents are nocturnal, the peak periods for activity and feeding behavior are the end of the light and dark periods (i.e., just before lights on or just after lights off). If high volumes of dosing solution (i.e., 20–50 mL/kg) are to be administered to rodents and/or if the presence of feed may interfere with the absorption of the test substance, consideration should be given to dosing animals during late morning or in the afternoon. For nonrodents, timing of dosing relative to feeding (i.e., before or at a certain time after daily feeding) will also affect test substance absorption and should be considered when designing a study. The timing of gavage dose(s) in relation to feeding should be stated clearly in the study protocol and report.

Administration of a test article as an undiluted liquid, as a diluted liquid, as a dissolved solid, or as a solid in suspension by oral gavage generally results in accurate delivery of the intended dose to each animal in a repeated-dose study. To deliver an accurate dose of test article in solution or suspension, the compound must be mixed with solvent or suspending agent at the proper concentration. The appropriate concentration of test solution or suspension can be calculated using the intended dose level and the dose volume of the solution/suspension to be administered to the animals. This calculation is illustrated in Figure 24.2. Obviously, this same calculation applies to solutions or suspensions for other exposure routes, for example, administration by capsule, dermally or parenterally.

Use of a vehicle to carry the test material into the animal has been mentioned several times in the preceding discussion. Choice of vehicle is a critical decision for toxicology studies. Obviously, the vehicle should be nontoxic at the dose (volume) administered. It should not act in an additive or synergistic manner to enhance the toxicity of the test material, nor should it interfere with the expression of any potential toxicity. Because such interactions are not always predictable, it is imperative that vehicle controls be used in all oral gavage toxicity studies. However, even including a vehicle control group may not always compensate for the administration of vehicle in the groups receiving the test material. Interactions between vehicle and test materials can be encountered and must be considered during the design of studies. Vehicles may produce physiological/nutritional alterations that may affect the toxicity of the test material, especially in longer-term studies. For instance, an oil vehicle may result in poor

Formula

$$\text{Concentration (mg test material/mL solution or suspension)} = \frac{\text{Intended dose level (mg test material/kg body weight/interval)}}{\text{Dose volume (mL solution or suspension/kg body weight/interval)}}$$

Example

The concentration of a solution intended to deliver a daily 1500 mg/kg dose level of test material A to rats at a dose volume of 5 mL/kg body weight (BW) is calculated as follows:

$$\text{Concentration (mg A / mL)} = \frac{1500 \text{ mg A/kg BW/day}}{5 \text{ mL solution/kg BW/day}}$$

$$= 300 \text{ mg A/mL solution}$$

As indicated in the following, a 300 g rat would receive 1.5 mL of this solution each day and, therefore, would receive the intended daily dose level. First, calculating the volume of solution administered to the rat,

$$0.3 \text{ kg BW} \times 5 \text{ mL solution/kg BW/day} = 1.5 \text{ mL/day}$$

Then, calculating the amount of test material A in that volume,

$$1.5 \text{ mL of solution/day} \times 300 \text{ mg A/mL solution} = 450 \text{ mg A/day}$$

Finally, calculating the dose level resulting from the administration of that amount of test material,

$$\frac{450 \text{ mg A / day}}{0.3 \text{ kg BW}} = 1500 \text{ mg A/kg BW/day}$$

FIGURE 24.2 Calculation of test material concentration in a solution or suspension.

absorption of fat-soluble nutrients. Food oil vehicles add to the caloric intake of the animal and may produce effects in longer-term studies. Absorption of fat-soluble materials from oil vehicles can be slower than when the same material is administered in an aqueous matrix. The resulting change in the toxicokinetics of the test material can produce profound differences in toxicity. Since toxicity testing generally uses exaggerated doses compared to human dose/exposure, it is sometimes difficult to formulate test article solutions of sufficient concentration to provide the necessary doses. In such cases, suspensions are sometimes used. The use of a suspension rather than a solution can also affect toxicokinetics and influence toxicity. For these reasons, among others, great care must be utilized in selecting an appropriate vehicle and formulation procedures for the administration of test materials to animals. Literature exists comparing the different types of vehicles commonly used and may be helpful when selecting an appropriate vehicle for toxicity studies.[41]

Dermal Application

For a drug, cosmetic, industrial chemical, or pesticide with dermal exposure potential for humans, dermal application is the most appropriate route of administration for repeated-dose studies. The test material is applied to a defined area of skin from which the hair has been removed. Shaving and/or depilatories are common methods for hair removal; however, both procedures can alter skin permeability. Because the test animal can ingest some of a dermal dose during grooming, it is common practice to cover the test material application site by wrapping the trunk of the animal with a semiocclusive material (e.g., gauze strips) during the exposure period. Occasionally, if exaggerated dermal absorption is desired, the application site is covered with an occlusive material (e.g., rubber dam). Either method of wrapping, that is, occlusion or semiocclusion, maintains the test material in contact with the skin. Wrapping also increases the accuracy of the administered dose since, on unoccluded animals, dry test materials may fall off the application site and liquid materials may run off or evaporate from the site. Restraint and/or *Elizabethan* collars are also sometimes used to prevent the animal from tampering with the wrap or to prevent oral ingestion if the application site is unoccluded. However, use of restraint or collars may stress the animals and potentially alter the outcome of a study. To avoid unnecessary stress, the animals should be acclimated to restraint devices and/or collars before dosing is initiated.

Parenteral Administration

A pharmaceutical intended for administration to humans by injection should be administered to the test animals by the same parenteral route. A common type of parenteral exposure is subcutaneous injection. To avoid irritation and other potential effects, such as fibrotic reactions, the specific site of injection should be changed daily. It is recommended that the dosing vehicle be aqueous since oil vehicles may track back along the needle path and be deposited in the lipophilic skin and hair. As with oral gavage dosing, absorption from an oil vehicle may differ compared to an aqueous vehicle. For subcutaneous administration, care must be taken to ensure that the material is deposited subcutaneously rather than intradermally or intramuscularly. This can be accomplished by lifting the skin of the animal to form a pocket and injecting the dosage into the subcutaneous space.

Other common routes of parenteral administration include IV, intramuscular (IM), and IP injections. Special considerations are required for each of these routes. For IV administration, the test material must be soluble in an aqueous vehicle because physiologic saline is the usual vehicle. Air bubbles must be cleared from the delivery system, that is, needle, catheter, and/or syringe, before injecting the material intravenously. The dosing solution for IV administration must be at a physiologic pH and must not be extremely irritating or corrosive. Repeated use of the same injection site must be avoided. To prevent inadvertent delivery of the test material into the muscle or other surrounding tissue, care must be taken to ensure that the needle tip is in the lumen of the vein before injecting the test material. Proper placement of the needle can be assessed by drawing a small amount of blood back into the delivery device, for example, catheter or syringe. If no blood is observed, the needle is not located in the lumen of the vein. For IM and IP routes of exposure, proper placement of the needle tip also must be assessed before administering the dose. In contrast to IV, drawing back on the delivery device without obtaining blood is required prior to administering material by the IM route. Similarly, drawing back on the device without obtaining blood or intestinal contents prior to injecting test material is necessary for correct IP administration. Most other considerations for IM and IP exposure are similar to those for IV administration, for example, the material must not be corrosive or irritating and repeated injections at the same site should be avoided.

Continuous infusion may be used to simulate a constant human exposure or, for IV infusion of poorly soluble test substances, to deliver a sufficiently large daily dose for safety assessment. Infusions may be administered using either implanted or external pumps. Implanted pumps may be battery-operated mechanical pumps (nonrodents) or osmotic pumps (rodents and nonrodents). These pumps permit unrestricted movement of the animal. Practical considerations of volume and weight of implanted pumps restrict their use to very soluble and/or potent test articles that can be administered slowly over a long period. External pumps may be connected to rodents and nonrodents using tethers and swivels or affixed to nonrodents as *back-packs*. External pumps not only allow for infusion of larger volumes of test substance but also require more waste substance because of the larger *dead space* of the tether catheter. Use of external pumps may be problematic for subchronic or chronic studies because of the relatively long study duration. The patency of the catheter for implanted and external pumps must be confirmed frequently. Infusion pumps should be calibrated prior to use on study to ensure that they will deliver the expected amount of test solution over the infusion period. The dose received by each animal should be

confirmed based on measurements of the actual amount of test solution infused during the dosing period. This is determined by measuring the weight of the pump or syringe and the solution prior to initiation and following completion of infusion.

Implantation

Subcutaneous or IM implantation is often used for evaluating biopolymers for medical devices or prostheses. In addition, test materials have been embedded in special matrices that allow continuous, sustained release for weeks or months after subcutaneous implantation. Demonstration of the stability of the test material in the matrix, both during preparation of the pellet and after implantation, is required when using this technology. The determination of plasma concentrations of the test material and/or its metabolites can be used to confirm proper dosing. Because of size limitations, these pellets are only useful for delivery of very potent substances, such as hormones and biological proteins.

Dietary Administration

Dietary administration is appropriate for a food additive or a pesticide that has potential to become a residue in or on food crops. This type of administration, although frequently used in subchronic and chronic toxicity studies, yields less accurate/more variable doses than most other routes of dosing. This is primarily because of differences between body weight and feed consumption of individual animals, which results in variable compound consumption. A further complication in rodent studies is that these animals tend to add body fat and not lean body mass as they age. Therefore, lipophilic compounds have a larger mass of fat into which they can partition as an animal grows older, thereby decreasing the effective dose. In addition, fat does not have significant detoxification enzymes. Therefore, as dose is increased to account for increased body weight in older rodents, detoxification capacity may be stressed, resulting in unanticipated toxicity. This complication is not unique to dietary exposure studies and will also affect the effective dose of a test material in rodents exposed by most other routes. While potential variability in effective dose is important to the interpretation of the results, no attempt is generally made to compensate for this variability during preparation of the test diet or calculation of the administered dose in repeated-dose dietary toxicity studies. Spillage or soiling of feed, which occurs fairly frequently with some test animals, is a factor that contributes variability to the calculation of dietary dose. Significant spillage or soiling must be considered when collecting feed consumption data to be used in the calculation of dietary concentration or compound consumption. Dietary concentrations used in repeated-dose studies can be determined by either of two methods: (1) attempting to keep a constant mg/kg dose level by adjusting the dietary concentration of the test material to account for changing body weight and feed consumption or (2) feeding a constant concentration in the diet (and therefore, the mg/kg dose level will decrease as animals gain weight).

Routinely adjusting the dietary concentration of a test material based upon the changing body weight and feed consumption of the animal provides reasonably good control over the delivered dose during a repeated-dose study. Dietary concentration is usually adjusted weekly during a subchronic study. In a chronic study, diet concentration is usually adjusted weekly during the first 13–14 weeks (especially for rodents because of the rapid growth of the animals during this period) and biweekly or sometimes monthly thereafter. Using this method, the mean feed consumption and body weight of a dose group during a given study interval are used to calculate the dietary concentration to be fed to that group during the following interval. There are several ways to calculate the concentration. One formula for this calculation and an example of its use are presented in Figure 24.3.

Feeding a constant concentration of the test material in the diet throughout the study is the second form of dietary administration. This method provides less control over the administered dose because it is a function of the amount of feed (and, therefore, test material) consumed and body weight gained by each animal. The consumed dose, or compound consumption, of each individual animal can be calculated using its feed consumption and body weight. The mean of the individual animal compound consumptions represents the compound consumption for each dose group. A commonly used formula for the calculation of individual compound consumption is illustrated in Figure 24.4.

When feeding a constant concentration in a rodent study, the toxicologist must be aware that the compound consumption may vary significantly for an individual animal or group of animals during the course of the study. For example, compound consumption during the first week of a 13-week rat study in which the test compound is fed at a constant dietary concentration is frequently more than twice the compound consumption during the last week of the study. This variation results from the rapid decrease in feed consumption relative to body weight (g feed/kg body weight/day) during the first several months of life. This variability in compound consumption is illustrated in the example given in Figure 24.4.

Drinking Water

Water-soluble test materials can be offered as a solution in the drinking water, providing adequate dosage can be achieved. This route may be preferred when it mimics human exposure conditions, compared with event-oriented exposure such as pill taking. Spillage can be a significant problem when administering material in drinking water, and recovery of spilled water is usually not feasible. Another complication with drinking water administration is that evaporation of water and/or volatilization of the test material can occur from the tip of the drinking (sipper) tube, resulting in the alteration of the concentration of the test material in the water. The use of a sipper tube containing a ball-bearing tip minimizes this problem. Finally, consideration should be given to the type/composition of water bottles, stoppers, and sipper tubes used in a toxicity study. The bottles, stoppers, and tubes should be made of material that will not degrade when exposed to water containing the test material.

Formula

$$\text{Concentration (mg test material / kg diet)} = \frac{\text{Intended dose level (mg test material/kg body weight/day)}}{\text{Projected feed consumption (kg feed/kg body weight/day)}}$$

where projected feed consumption (PFC) for a study week is based on body weight (BW) and absolute feed consumption (AFC) data from the previous week and is calculated as follows:

$$\text{PFC (kg/kg/day) for week}_n = \frac{\text{AFC week}_{n-1}\text{ (kg)}}{\text{7 days}} \div \left[\text{BW end of week}_{n-1}\text{ (kg)} + \frac{\text{BW gain during week}_{n-1}\text{ (kg)}}{2}\right]$$

Example

In a subchronic rat study, the mean body weight of the males in the 15 mg/kg/day dose group at the beginning of week 11 is 520 g. At the end of week 11, the mean weight for these males is 540 g. Mean feed consumption of these animals is 154 g during the 7 days of week 11. The dietary concentration intended to deliver a 15 mg/kg/day dose level of compound A to this group of rats during week 12 of the study is calculated as follows:

$$\text{PFC (week 12)} = \frac{0.154\text{ kg feed}}{\text{7 days}} \div \left[0.540\text{ kg BW} + \frac{0.020\text{ kg BW gain}}{2}\right]$$

$$= 0.04\text{ kg feed/kg BW/day}$$

and, therefore,

$$\text{Concentration (week 12)} = \frac{15\text{ mg A/kg BW/day}}{0.04\text{ kg feed/kg BW/day}}$$

$$= 375\text{ mg A/kg feed}$$

Note that, because their body weight and feed consumption differ, the diet concentrations for males and females in the same dose group will generally differ throughout the study.

FIGURE 24.3 Calculation of adjusted diet concentration to yield constant dose level.

Assessment of the Adequacy of Test Material Preparations

Whatever the route of administration, it is critical to confirm that the test material is delivered to the animals at the intended doses. For dietary studies, test diets should be prepared before the initiation of a repeated-dose study using the intended diet preparation method. It must be shown that this preparation method yields diets containing the appropriate amounts of homogeneously mixed test material. Chemical analysis of samples taken from several locations in each test diet preparation should be conducted to determine if the proper concentrations of test material have been achieved and to assess the homogeneity of the dietary admixtures. If the results of these analyses indicate that the anticipated concentrations were not achieved or the distribution of test material in the diet was not homogeneous, the diet preparation method should be revised and retested. Diet preparation must be validated before the study can be initiated.

During the prestudy homogeneity determinations, additional diet samples should be collected and analyzed to show that, within the range of concentrations to be used in the study, the test material is stable in the diet. These samples should be stored under animal room conditions and under frozen conditions for the maximum period of time during which the diet will be used or stored. For a study in which dietary admixtures will be prepared and fed once per week, stability of the test material in the diet would commonly be assessed for samples stored under animal room conditions for at least 7 and 14 days. This allows the estimation of the degradation rate at room temperature. Analysis of frozen diet samples stored for several intervals is also advisable. Demonstration of stability under frozen storage conditions makes chemical analysis of diet samples immediately after collection during the toxicity study unnecessary. It also validates the possibility of confirming analytical results by reanalysis of stored frozen samples, if needed.

Even though the adequacy of the diet preparation method and stability of the test material in the diet have been demonstrated, it is important to monitor diet concentrations during the study. For each diet preparation during the first several weeks of the study, concentrations of the test material in the diets should be assessed. Subsequent analysis of diet preparations every 2–4 weeks will add assurance that diets were prepared properly. More frequent analysis, for example, weekly throughout the study, is even more desirable.

For routes of administration other than dietary, the principles cited earlier also apply. Concentration, homogeneity, and stability of the test material in solvents or suspending agents must be determined for studies using oral, dermal, inhalation,

Formula

Compound consumption (mg test material/kg body weight/day) = Concentration (mg test material/kg feed) × Relative feed consumption (kg feed/kg body weight/day)

where relative feed consumption (RFC) for a study week is based on body weight (BW) and absolute feed consumption (AFC) during that week and is calculated as follows:

$$\text{RFC (kg/kg/day)} = \frac{\text{AFC (kg)}}{\text{7 days}} \div \text{BW start of week (kg)} + \frac{\text{BW gain during week (kg)}}{2}$$

Example

In a subchronic rat study, a group of males is fed test material A at a constant dietary concentration of 2% (w/w). The body weight of one animal in this group is 175 g at the start of week 1 and 225 g at the end of week 1. Its feed consumption during the 7 days of week 1 is 168 g. Subsequently, this rat weighs 490 g at the start and 510 g at the end of week 13 and its feed consumption during that week is 196 g. The compound consumption of this rat for each week is calculated as follows:

$$\text{RFC (week 1)} = \frac{0.168\,\text{kg feed}}{\text{7 days}} \div \left[0.175\,\text{kg BW} + \frac{0.050\,\text{kg BW gain}}{2}\right]$$

$$= 0.120\,\text{kg feed/kg BW/day}$$

$$\text{RFC (week 13)} = \frac{0.196\ \text{kg feed}}{\text{7 days}} \div \left[0.490\,\text{kg BW} + \frac{0.020\,\text{kg BW gain}}{2}\right]$$

$$= 0.056\,\text{kg feed/kg BW/day}$$

and, therefore,

$$\begin{aligned}
\text{Compound consumption (week 1)} &= \text{2\% test material A} \times \text{RFC (week 1)} \\
&= \text{2 g A/100 g feed} \times \text{0.120 kg feed/kg BW/day} \\
&= \text{20,000 mg A/kg feed} \times \text{0.120 kg feed/kg BW/day} \\
&= \text{2400 mg A/kg BW/day} \\
\text{Compound consumption (week 13)} &= \text{2\% test material A} \times \text{RFC (week 13)} \\
&= \text{20,000 mg A/kg feed} \times \text{0.056 kg feed/kg BW/day} \\
&= \text{1120 mg A/kg BW/day}
\end{aligned}$$

FIGURE 24.4 Calculation of compound consumption resulting from constant diet concentration.

or other routes of administration when the test material is to be administered in solution, in suspension, or as an aerosol. Suspensions represent a special case because care must be taken to assure that the suspensions do not settle and become nonhomogeneous during administration to the test species. For inhalation studies, the concentration of gas, aerosol, or particulates to which the animals are exposed and the homogeneity of the atmospheres within the exposure chambers should also be assessed using appropriate analytical methods.

Duration of Exposure

As stated previously, subchronic toxicity studies involve the administration of the test material to the test species during a significant portion of its lifetime. Classically, these studies are conducted for 90 consecutive days or approximately 13 weeks. Chronic toxicity studies in rodents most commonly involve dose administration for a major portion of their life span, generally 12 months, although studies of shorter duration (i.e., 6–9 months) are considered acceptable by some groups.[10] Rodent chronic toxicity studies are sometimes combined with lifetime oncogenicity studies in order to achieve efficiencies during some of the study procedures, for example, diet preparation. During these combination studies, the animals in the chronic toxicity segment are generally studied during the first 6–12 months and then are euthanized and evaluated. Those in the oncogenicity segment continue on study generally for at least 24 months. In nonrodents, for example, dogs and nonhuman primates, which are longer lived than rodents, 6–12 months represents a significantly smaller portion of their life span but is currently considered an adequate duration of exposure to detect chronic effects.

Test material administration during a repeated-dose toxicity study can be continuous, intermittent, or repeated. In most dietary and drinking water studies, the animals have free access to diets or water containing the test material throughout the study and exposure is essentially continuous, although influenced by diurnal patterns of consumption. In dermal or inhalation studies, exposure to the test material is intermittent, generally 4–6 h/day. When the route of administration is IV infusion, exposure may be either continuous or intermittent. With bolus dose parenteral administration or oral gavage, test material administration is generally once or, at most, a few times each day. In some cases, such as with some biological agents, dose administration will only be performed once each week or even once monthly due to the prolonged pharmacologic response of these compounds. In the past, labor-intensive methods of administration, such as oral gavage, were sometimes done only during the standard work week, that is, 5 days/week. This is not recommended because 2 days of nonexposure each week during the study may be sufficient to allow modification or reversal of toxic responses.

Dose Groups

The minimum number of groups receiving test material in a repeated-dose toxicity study is generally three (low, mid, and high dose). According to OECD guidelines, "the highest dose level should be chosen with the aim of inducing toxic effects but not death or severe suffering" while FDA guidelines simply state "the high dose should be sufficiently high to induce toxic responses in test animals." Wording in the guidelines differs slightly regarding mid- and low-dose levels. Essentially, the mid-dose level should produce no more than slight toxicity and the low-dose level should produce no toxicity yielding a NOAEL. As previously stated, a short-term (2–4 weeks) repeated-dose study should be conducted to aid in the selection of doses for subchronic testing. For test materials where a dose–response has not been well defined during a short-term repeated-dose study, additional dose groups may be required in the subchronic study to ensure that the range of desired responses, that is, no toxicity to significant toxicity, is achieved. However, it is sometimes difficult to completely satisfy these criteria. Before selecting doses for a chronic toxicity study, a subchronic study that defines no-effect and effect levels should be completed.

Limit Studies

For test materials that possess very low potential for toxicity, the inclusion of only one test material dose group in a repeated-dose study is sometimes acceptable. A study with this design is termed a limit study. Limit testing is inappropriate for materials with anticipated high human exposure. The dose level for the test material group in dietary and dermal limit studies is normally at least 1000 mg/kg/day. Another type of limit study involves utilization of the maximal exposure level under the conditions of the study. For example, suppose the majority of toxicology data concerning a lipophilic drinking water contaminant has been collected using oral administration in a corn oil vehicle and additional data are desired using a water vehicle. The limited water solubility of the test material may result in the maximal dose being significantly lower than the dose used in the corn oil gavage studies. However, the test material can be tested as a saturated water solution. Such a study may reveal the test material to be either more or less toxic in water than in the oil vehicle. The data are relevant to the assessment of hazard associated with exposure in the drinking water because the maximal possible exposure by this route of administration was tested. Thus, while a limit study may not define the *complete* toxicology of a test material, it can define the *practical* toxicology of the material.

Control Groups

Adequate controls are essential to successful toxicity studies of all types, including repeated-dose studies. Studies should contain at least one control group for comparison with the groups receiving the test material. The control group should be treated identically to the treated groups except the control group should receive no test material. Control groups can be either negative or positive controls.

Negative control groups are intended to demonstrate the normal state of the animal for comparison to data from the groups treated with the test material. They also provide an opportunity to compare baseline data for the current study to baseline data from previous studies. There are several types of negative controls. If the test material is dissolved or suspended in a vehicle for administration, a vehicle control group should receive, by the same route of exposure, the maximum amount of solvent or suspending agent administered to any of the test material groups. If the test material is administered in the diet, an untreated control group should receive the same diet without test material. For test materials administered undiluted, a sham control group should receive the same physical treatment as the treated groups, for example, insertion of an intubation tube with or without the delivery of an innocuous substance like water, administration of empty capsules, and injection of physiological saline.

Positive control groups are intended either to demonstrate susceptibility of the animal to a specific toxicity or to compare the response of test material–treated animals with that of animals treated with a chemical that produces a known toxicity similar to the test material. If a positive control group is included in a study design, at least one negative control group should also be included. Positive control groups are infrequently used in repeated-dose toxicity testing. However, if the chemical structure of a test material suggests that it may possess a specific toxicity, for example, neurotoxicity, it may be important to demonstrate that the species and the strain selected for testing are susceptible to that toxicity.

A positive control that is sometimes useful in repeated-dose studies is the reference control. This control consists of a material that is chemically or physically similar to the test material but has either a comprehensive toxicology database associated with it or a history of use without adverse effects.

Inclusion of a reference control group allows a comparison between reference and test material within the same study. This can assist in identifying any effects related to the general characteristics of the reference material. For instance, oral administration of a poorly absorbed oil can decrease the absorption of fat-soluble vitamins. If the test material is known or suspected to produce this effect, use of a reference material, such as mineral oil, can be useful. This would distinguish effects related to vitamin depletion from effects produced directly by the test material. Additionally, if the test material were to add substantially to the caloric intake, a reference control diet isocaloric to the test diet would be useful, especially in longer-term studies. A reference control group also may be useful to compare the degree of anticipated toxicity of the test material to a reference material of known toxicity. For example, it could be important to demonstrate that the hepatotoxicity of a test material intended for use as an anesthetic is significantly less severe than that produced by an anesthetic already in use.

Animal Models

To increase the probability of testing in a species that may respond to the test material in a manner similar to humans, two species are generally used for repeated-dose toxicity studies. Routinely, one rodent species and one nonrodent species are utilized. Rats and dogs are the generally preferred species for most routes of administration. The rabbit is the preferred nonrodent for dermal administration. Mice, hamsters, miniature swine, guinea pigs, nonhuman primates, and a few other species are used on occasion in these studies. Many factors should be carefully considered during the selection of the most appropriate species and strain for testing with a specific chemical. Some of these factors are summarized in Table 24.6.

Toxicokinetics

Ideally, the selection of an animal model for repeated-dose toxicity studies should be based upon the similarity between toxicokinetics/metabolism of the test chemical in that species and strain to its toxicokinetics/metabolism in humans. This selection criterion assumes that these factors are known in potential test animals and in humans. Often, these data are unavailable during the initial phase of a hazard assessment.

TABLE 24.6
Selection Criteria for Species and Strain in Repeated-Dose Studies

- Requirements by regulatory agencies
- Metabolism of test material in a manner similar to humans
- Availability of historical control data
- Most sensitive species and strain
- Responsiveness of particular organs and tissues to specific toxicities
- Availability of the species and strain
- Availability of appropriate animal housing and husbandry
- Experience of the laboratory in the use of the species and strain

Although the metabolism of a chemical may be understood in one strain of one species of laboratory animal before the initiation of repeated-dose testing, it is seldom known in several species and strains. With the exception of pharmaceuticals, the metabolism of a chemical in humans is almost never known before the initiation of a subchronic or chronic study. Consequently, similarity in metabolism between humans and animal models is seldom the initial basis for the selection of test species and strain. This may change, however. Currently, human microsomes and systems that express specific human detoxification enzymes are commercially available. Therefore, in vitro metabolism in human hepatocytes and hepatocytes isolated from various test species can be compared before initiating a hazard assessment.[42]

Sensitivity to Test Material

Another commonly used criterion for the selection of the animal species and strain for repeated-dose testing is sensitivity to the test material. As a conservative approach to the extrapolation of toxic effects seen in animals to humans, the animal model selected should be the most sensitive to the effects of the chemical. Data required for this decision are often not available until a significant portion of the total hazard assessment program for the chemical has been completed. Acute and short-term repeated-dose studies may reveal information concerning species sensitivity. However, relative sensitivity of different species and strains frequently only can be determined following the completion of longer-term studies with their more comprehensive endpoints.

In any event, sensitivity to the chemical should be considered during the selection of the test animal. For example, differences in sensitivity of particular organs and tissues to toxic compounds among different species should be considered. Strains that have aberrant metabolic pathways, especially those associated with detoxification, should not be used except in special cases. For instance, the Gunn rat does not produce certain glucuronides[43] and would not be an appropriate animal model for a hazard assessment. Cats are deficient in their ability to produce glucuronides but can produce sulfate conjugates.

After the determination of which species in repeated-dose toxicity studies is the more sensitive species, it is appropriate to use the data gathered for that species (e.g., NOAEL values) in the hazard assessment, whether it will be used for setting exposure limits for environmental contaminants or setting human doses in pharmaceutical trials. It is also important to base the evaluation of the most sensitive species on internal exposure rather than simply using dose level to compare sensitivity. It may also be appropriate, in some cases (e.g., oncology compounds), to convert doses from dose based on body weight (mg/kg) to dose based on surface area (mg/m^2) when evaluating sensitivity to test articles.

Historical Control Information

Availability of historical control data for the parameters evaluated during repeated-dose toxicity testing is an important consideration in selecting the test species and strain.

These data are frequently useful in determining the toxicological relevance of a finding when comparison of data from treated and concurrent control groups suggests a potential compound-related effect. Historical data concerning growth, feed consumption, clinical pathology, and other parameters are often useful in interpreting findings from a subchronic or chronic study. Historical histopathology data are of particular importance due to the subjective nature of these data. Although published data can be useful, historical data from the laboratory at which the study is being conducted are most relevant. Most laboratories have historical databases for commonly used species and strains. If less common species are being considered, the availability of historical data should be assessed before final selection.

Other Animal Model Considerations Involved in Study Design

After consideration of the earlier criteria, pragmatic considerations are necessary during the selection of a species and strain. The animals should be obtained from a reputable, reliable supplier who will guarantee the health of the animals and will arrange expeditious and controlled shipment of the animals to the laboratory. It should be ensured that the supplier maintains careful records concerning the animal colony and maintains a healthy colony. The supplier should provide disease-free animals because it is often not desirable/possible to treat for disease once a study has initiated. Furthermore, the use of diseased animals will ultimately affect the outcome of the study and could cause results of the study to be inaccurate and misleading. Animal health issues can be minimized, in part, by receiving animals from suppliers that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and that follow animal care procedures as described in the Institute of Laboratory Animal Resources (ILAR) *Guide for the Care and Use of Laboratory Animals* (aka "The Guide").[44]

The quantity of available test material may influence the selection of the animal model. For example, it may be necessary to select a rodent species if the amount of test material available is insufficient for long-term administration to a larger species such as the dog.

Capabilities of the testing laboratory should be considered during test animal selection. The laboratory must have appropriate caging and other equipment and must be able to maintain the proper environmental conditions in the animal room. In addition, the laboratory conducting the study should have experience with the use of the chosen species in toxicology studies to avoid problems associated with species-specific physiology and anatomy.

Age of Animals

The age of animals used in subchronic and chronic toxicity studies is relatively standard. For rodents, initiation of test material administration at 6 weeks of age will satisfy virtually all guidelines for testing. Dogs approximately 4–6 months of age at initiation of exposure to the chemical are usually acceptable, but if the test material is expected to produce toxicity in reproductive organs, older dogs should be used in order to avoid confusing results due to the variability in the degree of sexual maturity noted in 4- to 6-month-old dogs. Precise age of nonhuman primates is frequently not known; however, age can be approximated by experienced suppliers. For nonhuman primates and other less commonly used species, young animals should be used, keeping in mind that sexually mature primates must be used for reproductive and developmental toxicity studies and/or when effects on the reproductive organs are suspected.

Prestudy Health Assessment

To the extent possible, it should be assured that each animal included in a repeated-dose study is in good health. The animals must not have been previously used for any other type of experimental procedures. An exception is sometimes made for nonhuman primates, which may occasionally be used for more than one study, with a reasonable period between studies to ensure that any residual test material is absent. These animals should undergo extensive health screening, including clinical pathology evaluations, between studies.

For rodent studies, enough animals of each gender should be obtained to allow exclusion of those with conditions that could interfere with completing the study or be interpreted as compound related at completion of the study. It is good practice to obtain 5%–10% more animals than will be required to fill the study groups. Minimally, pretest physical examination and body weight measurement should be conducted to assess the health of each animal before study initiation. Pretest ophthalmologic examination and clinical pathology evaluations are advisable. Animals in poor health or exhibiting ocular or other defects should be eliminated from consideration for the study.

To ensure that the toxicologist is aware of any infection that the animals may be exposed to during the study, a sentinel group is often maintained in the room with the study animals. For rodents, this group normally contains 5–10 animals of each gender. Serum antibody titers are assessed in these sentinels at the initiation of the study and at the termination of the in-life phase of the study. If necessary, antibody titers and/or other evidence of infection can also be obtained from these animals during the study without disturbing the test animals. A relatively complete list of antibody analyses used in rodent species is presented in Table 24.7. In addition to serology testing, samples (tissue, feces, bronchial lavage) can also be collected for the evaluation of the presence of microorganisms and/or parasites that may be present in test animals.

Health assessment of nonrodent species by the supplier is generally more comprehensive than rodents. This reduces the need to obtain many extra nonrodents. However, it is good practice to conduct procedures after receipt of nonrodents to assure that their health status has not changed before use in a subchronic or chronic toxicity study.

TABLE 24.7
Serum Antibody Analyses in Rodents

Rat	Mouse
Sendai virus	Sendai virus
Pneumonia virus of mice	Pneumonia virus of mice
Reovirus type III	Reovirus type III
Mycoplasma pulmonis	*Mycoplasma pulmonis*
Lymphocytic choriomeningitis virus	Lymphocytic choriomeningitis virus
Mouse adenovirus FL/K87	Mouse adenovirus FL/K87
Mouse polio virus	Mouse polio virus
Hantaan virus	Hantaan virus
Encephalitozoon cuniculi	*Encephalitozoon cuniculi*
Cilia-associated respiratory bacillus	Cilia-associated respiratory bacillus
Rat parvovirus—IFA	Mouse parvovirus—IFA
Rat coronavirus/sialodacryoadenitis virus	Murine hepatitis virus
Kilham rat virus	Minute virus of mice
Toolan H-1 virus	Ectromelia virus
Rat minute virus	Mouse pneumonitis virus
Rat theilovirus	Polyomavirus
Pneumocystis carinii	Mouse thymic virus
Infectious diarrhea of infant rats	Epizootic diarrhea of infant mice virus
	Mouse cytomegalovirus
	Mouse norovirus
	Mouse rotavirus

Number of Animals

Regardless of the study type/design, the minimum number of animals necessary to achieve the study objectives should be used. The *3Rs* principles (replacement, reduction, and refinement), first described by Russell and Burch,[45] should be employed when determining ways to minimize animal usage in toxicity studies. To satisfy most regulatory guidelines, a minimum of 5–20 rodents of each gender should be included in each control and test material dosed group, depending on the duration and specific study design in a repeated-dose study. For nonrodents, the minimum number of animals of each gender in each group is four. However, the minimum number of animals is frequently exceeded in an attempt to compensate for unexpected mortality or to increase the sensitivity of the study. Twenty rodents or five to six nonrodents of each gender per group are often used as the base number of animals for the study. Some study designs include an interim necropsy at one or more intervals for the detection and evaluation of the progression of potential effects during the study. Other designs may contain treated animals that will be maintained without exposure after the termination of the main study groups to determine the reversibility of any adverse effects. Still other designs include satellite groups for special purposes, for example, toxicokinetic determinations or untreated sentinel animals used to monitor the health of the study animals. The base number of animals placed on study at its initiation should be increased by the number of animals to be used for these enhanced study designs.

Individual Animal Identification

Before assignment to study, each animal must be assigned a unique identification number. This number will be associated with the animal throughout the study and will be used to identify specimens, tissues, and data from the animal after the in-life portion of the study is completed. Therefore, this number must stay with the animal continuously during the study so that there is no chance of misidentification. It is not adequate to simply attach the animal identification to the animal's cage. Animals may escape from their cage or may be placed in the wrong cage during cage-changing operations. Unique identification numbers can be placed on the animals by a number of methods. Whatever the identification method, it should remain permanent and readable for the duration of the study. Older identification methods used for rodents included toe clipping, where a small portion of the toe was removed in a specified coded manner, and ear punching, where holes were punched through the ears in a specified coded manner. These methods are not acceptable today as more precise and humane methods have become available. Currently, the use of a numbered tag attached to an ear or tattoos placed on the tail or ear are commonly used methods for large and small animal identification. Another commonly used method involves subcutaneous implantation of a microchip that can be read by a hand-held scanner. In many cases, these *chip readers* can be interfaced with computerized on-line data collection systems that can help ensure the proper data are collected and procedures are performed on the animals.

Randomization of Animals

After excluding all animals that do not meet the study acceptance criteria (e.g., animals that do not pass physical examination or are not within specified body weight boundaries) and assigning unique identification numbers to the remaining animals, the next step is to randomly place them into study groups. This is a critical step in the study to ensure the greatest ability to detect statistical differences between the groups in the study without bias. A number of randomization methods have been devised, some more appropriate than others. Historically, one of the most popular methods was the use of manual random number tables. However, as technology has advanced, the use of computerized randomization procedures has become the norm. After randomization into the various study groups, some method should be employed to determine if the animals are truly randomized based upon a parameter critical to the study. The most commonly used parameter is body weight. Statistical analysis of mean body weight data is conducted to show that there are no statistically significant intergroup differences in mean body weight at the initiation of the study. It is not uncommon to find that the mean body weight

in one of the study groups is significantly different from one or more of the other groups. In cases where there is a significant difference between the mean body weights of any of the study groups, the randomization process is repeated until no statistically significant differences exist. Randomization must be conducted independently for each gender because of body weight differences between males and females.

Animal Husbandry

Proper care and maintenance of animals in a repeated-dose toxicity study is essential not only for ethical reasons but also to minimize mistakenly attributing adverse findings due to poor husbandry to the test material. The Animal Welfare Act (AWA), enforced by the Animal and Plant and Health Inspection Service of the U.S. Department of Agriculture (USDA), mandates standards for acceptable handling, care, treatment, and transportation of many species, including most laboratory species except rodents.[46] In its *Guide for the Care and Use of Laboratory Animals*, the Institute of Laboratory Resources (ILAR) of the National Research Council has published guidelines that are widely accepted as standards for laboratory animal husbandry.[44] From their arrival at the laboratory, animals must be maintained in an appropriately controlled environment. They must be provided an adequate quantity and quality of feed and water and be housed in clean cages of appropriate design. Although there may be occasional exceptions based on specific study requirements, animals should be acclimated to the study room conditions for at least 1 week before study initiation.

Environmental Factors

Temperature and humidity should be controlled within limits specified in the documents referenced earlier. Table 24.8 is taken from the ILAR document and contains the recommended temperature ranges for various laboratory animal species.[44] Low humidity can result in drying of the mucous membranes and eyes of laboratory animals. High humidity can result in the growth of bacterial and fungal populations that result in respiratory distress and dermal involvement such as ringworm. In addition, urine and excreta may not dry as readily, thereby increasing room odor. Relative humidity of 30%–70% is considered acceptable by ILAR for most laboratory species.[44]

TABLE 24.8
ILAR-Recommended Dry-Bulb Temperatures for Common Laboratory Species

	Dry-Bulb Temperature	
	°C	°F
Mouse, rat, hamster, gerbil, guinea pig	20–26	68–79
Rabbit	16–22	61–72
Cat, dog, nonhuman primate	18–29	64–84
Farm animals and poultry	16–27	61–81

Adequate ventilation is a key factor in maintaining good animal health during a toxicity study. Establishing a positive room air pressure reduces possible exposure of animals to test materials being used in other animal rooms. When more air is forced into a room than can be completely cleared by exhaust systems, air flows through the cracks, around the door, and the partial pressure of air in the room becomes positive with respect to the hallway or area outside the room. Ventilation should be homogeneous throughout the room; this generally is controlled by adjustable diffusers, and the ventilation of all rooms in a facility must be *balanced* periodically to provide the same relative air flow and positive pressure with regard to hallways. In general, 10–15 fresh air changes per hour is considered acceptable but this range is highly dependent on a number of factors, for example, the number of animals/cages in the room.

Common lighting schedules used are 12 h of continuous light and 12 h of darkness for rats, mice, dogs, and monkeys and 14 h of light and 10 h of dark for hamsters. This schedule allows the animals to become acclimated to a consistent light cycle. This stimulates a constant pattern of secretion of thyroid hormones, ACTH, and growth hormone. Regulated lighting cycles are necessary in reproduction studies because rodents enter continuous estrus under conditions of constant light phases without darkness.[47] Because high-intensity fluorescent light can cause blindness in albino rodents, current practice is to limit their exposure to high-intensity light to times when observations are collected by providing dual-intensity (high-low) lighting systems.

Animal Caging

Historically, in the United States, rodents were commonly housed one per cage during subchronic and chronic toxicity studies. In other countries, rodents are frequently housed in groups during these studies because it is believed that multiple housing increases survival and decreases background pathology. Although single housing of rodents is still preferred in some situations, many companies in the United States have adopted group housing procedures similar to other countries based on animal welfare considerations and recommendations in "The Guide." There are benefits and drawbacks to each housing option, which should be kept in mind when designing a study. On the positive side, multiple housing of rodents allows for greater social interaction. Animals that are group housed tend to gain less weight and may live longer than their individually housed counterparts. The negative aspects of multiple caging include problems associated with unique identification and trauma to the animals from fighting. Multiply housed rodents are more susceptible to transmitted disease and other health concerns. Furthermore, a multiply caged rodent that dies on study may sustain tissue destruction from cannibalism. It is also not possible to determine individual feed consumption when multiple animals are housed in a single cage, resulting in the inability to correlate body weight with individual feed intake. Additionally, if the test material is fed as part of the diet, it is not possible to calculate actual doses for individual

rodents in the absence of individual feed consumption data. Although some of the same problems exist for nonrodents, multiple housing of some species (e.g., nonhuman primates and dogs) on a regular or continuous basis during the study is a globally accepted practice to permit the social interaction and exercise considered necessary for these species.

Rodents generally are housed in metal (stainless or galvanized steel) or plastic (polyethylene, polypropylene, or polycarbonate) cages. Metal caging or floor pens are used for dogs. Minimum cage sizes for all species are stipulated in the ILAR publication[44] and, for nonrodents, in the AWA.[46] Compliance is monitored by federal and state health agencies. Since minimum sizes for cages are stipulated, only caging type remains to be decided. The two major types of caging are solid floor cages or pens and suspended-floor cages; both have advantages and disadvantages.

Solid floor caging requires bedding to be added to the cage to absorb and contain waste materials and may introduce dust. Sawdust and chips of some conifers induce hepatic cytochrome P450 monooxygenase activity, which may affect the outcome of the study and are, therefore, not recommended. In addition, solid floor caging allows animals to have access to their waste. Solid bottom shoebox-style cages used for rodents may restrict airflow and clear the atmosphere of potentially harmful gasses at a slower rate than suspended wire cages. Finally, special consideration should be given to the design of shoebox cages equipped with automatic watering systems. If designed improperly (and sometimes regardless of the design of the cages), all animals housed in a cage may be lost if the watering system malfunctions and fills the cage with water.

Cages with suspended wire floors also have disadvantages. Traumatic foot and leg injuries can occur, particularly with smaller animals. Plantar foot pad lesions are common in long-term studies of rats housed in wire-bottom cages. As a result, "The Guide" now recommends "Flooring should be solid, perforated, or slatted with a slip-resistant surface. In the case of perforated or slatted floors, the holes and slats should have smooth edges. Their size and spacing need to be commensurate with the size of the housed animal to minimize injury and the development of foot lesions. If wire-mesh flooring is used, a solid resting area may be beneficial…" Finally, wire-mesh cages also expose the animals to room drafts.

Most gradients in light, temperature, or airborne products in an animal room will occur vertically. Animals within groups should be distributed in cage racks so that members of each study group are present equally at all vertical caging levels. This practice, and the practice of periodically changing the relative position of each cage rack within the room, avoids confounding treatment group with cage position. Documentation of environmental conditions and of cage/rack rotation is essential.

Cleaning of cages at frequent intervals is also essential. Generally, sanitization of cages every 1–2 weeks depending on cage type and husbandry procedures is sufficient. Poor husbandry practices may result in skin lesions, alopecia, or the appearance of signs and behavior that may be interpreted as possible effects of the test article.

Diets

The influence of diet and nutrition on the toxicity of test materials is another important aspect of the design of toxicity studies. The diet fed to the animals during toxicology studies can influence the results. Therefore, the decision made by the toxicologist and/or animal husbandry/veterinary staff at a test facility concerning what diet to feed the animals may have a profound impact upon the outcome of the studies. The diet fed during a study should be designed to meet the nutritional needs of the test species. Although diets can be custom-made, they are generally obtained from commercial suppliers. The supplier should be reputable and capable of supplying information concerning basic diet composition and nutritional information. Although not feasible for a long-term study (e.g., 12 months), the same lot of diet should be used for the entire study whenever possible. Diets should be used before their expiration dates and should be stored under appropriate conditions to maintain their nutritional value, prevent insect and rodent infestations, and ensure they are not contaminated by environmental chemicals.

Commercial diets are available in either ground *powder* or pelleted form. When the test material is to be incorporated into the feed, a powdered diet is generally used. Use of powdered diet also facilitates the determination of feed consumption. Pelleted diets are most frequently used when test material administration is by routes other than dietary. A powdered diet can be pelleted after a test material has been added, which reduces the dust from the diets. Pelleted diets also decrease the potential exposure of animal room personnel to the test material. However, the heat and pressure involved during the pelleting process may cause degradation of test materials sensitive to these conditions.

Diets used in toxicity studies are of two basic compositions. Currently, diets made from natural ingredients are most commonly used. However, semipurified diets made from refined macronutrients, such as protein and carbohydrate, and micronutrients, such as vitamin and mineral mixes, are sometimes used. Each of these diets has advantages and disadvantages that must be carefully considered by the toxicologist.

Natural diets are formulated from unrefined plant and animal products to meet the nutritional requirements of a particular species. In closed-formula diets, the manufacturer does not provide the exact proportions of the constituents. The diets are formulated based upon nutritional specifications without emphasis on the consistency of specific ingredients between lots. Plant materials contain a number of *nonnutritive* components that can affect various physiological and biochemical functions, including detoxification and metabolic activation, in the test animal. These components may vary with plant species, strain, growing conditions, and site. Therefore, individual lots of closed-formula diets may differ in these constituents. Open-formula diets are formulated with constant quantities of specified ingredients. An example of an open-formula diet is the NIH-07 rodent diet, which has been relatively well characterized.[48] Open-formula diets have advantages for long-term studies because of their consistent

formulation and may be preferred by research institutions over closed-formula diets.[49] An additional consideration concerning both open- and closed-formula natural diets is their potential to contain contaminants, such as pesticides, heavy metals, and mycotoxins. To overcome this problem, some manufacturers provide diets that have been assayed for certain potential contaminants to ensure that they are below stated specifications. It is highly recommended that these *certified* diets be used in toxicity studies. A disadvantage of natural commercial diets is that their nutritional composition cannot be readily altered. It is possible to supplement these diets but not possible to remove constituents. An important advantage of natural diets is their long history of use and the resulting large quantities of historical control data.

As noted earlier, semipurified diets are made from refined macroconstituents, such as protein, carbohydrate, and fiber, and micronutrient mixes containing individual minerals and vitamins and a defined fat source, such as corn oil. Their constituents can be varied to design diets for specific nutritional purposes and allow for the inclusion of test materials that may provide nutrient activity or result in nutritional deficits. Nutrient composition can be reproduced exactly from lot to lot of semipurified diet. As opposed to natural ingredient diets, semipurified diets do not contain pesticides, mycotoxins, and other constituents that may alter the animal's response to the test material. However, a major problem with semipurified diets is a lack of historical data from their use in long-term studies. A large number of different dietary compositions are currently in use and data obtained from one semipurified diet may not extrapolate to another semipurified diet. Even with commonly used semipurified diets, such as the AIN-76A[50] and the AIN-93[51] diets, there are insufficient data to determine its impact on long-term toxicity studies, especially carcinogenicity studies.[52] Although these diets can be utilized in subchronic toxicity studies, the data obtained may not be as useful as that from studies with natural diets, especially when these data are used to design longer-term studies with natural diets. While it may be necessary to use semipurified diets with specific test materials, care must be taken if these studies are to be used in a safety assessment.

Drinking Water

Drinking water free of contaminants that could interfere with the objectives of the study should be available to the animals during repeated-dose toxicity studies. Water is frequently provided to the rodent and nonrodent animals through automatic watering systems. In these systems, a common water supply is piped to the animal cages and each cage contains a valve that allows the animal ad libitum access to water. Water bottles are another more labor-intensive method of providing water to rodents and some nonrodents. Each bottle is fitted with a stopper containing a sipper tube through which the animal can drink and the bottle is attached to the cage. A third method of water delivery, generally only used with nonrodents, is to provide the animals with water in a drinking bowl. Any of these methods is acceptable as long as procedures are in place to ensure that the animals are provided an adequate supply of potable water.

In-Life Evaluations

Physical Examination

Several parameters are routinely evaluated during the treatment and recovery phases of subchronic toxicity studies. Each animal should be observed twice daily at least 4 h apart (a.m. and p.m.) for overt signs of toxicity, moribundity, and mortality. During these a.m. and p.m. observations, the cage of each animal should be opened to permit unobstructed observation. In addition, each animal should be removed from its cage for a complete physical examination at least once per week. These examinations should include detailed observations to allow for the approximation of the time of onset of any changes as well as the severity and persistence of changes involving the skin, fur, eyes, mucous membranes, respiratory function, circulatory system, autonomic and central nervous system, somatomotor function, and general behavior. A study-specific or SOP-specific glossary of clinical terms and descriptive criteria for each finding is recommended. The terms should be simple and descriptive, using a minimum of medical or diagnostic terminology.

Body Weight Measurement

It is recommended that body weight be determined at least once per week, even though biweekly or monthly measurement after the first 3 months of a chronic toxicity study is acceptable to most regulatory agencies. Weekly measurement is recommended because body weight is one of the most sensitive indicators of the condition of an animal if it is monitored frequently and carefully. Rapid and/or marked body weight loss is usually a harbinger of ill health or death. Rapid body weight loss can be due to decreased feed and/or water consumption, disease, and/or specific toxic effects.

Feed Consumption

In rodents, feed consumption generally is measured once per week during subchronic studies and weekly the first 3 months of chronic studies. After the third month of a rodent chronic study, feed consumption may be measured less frequently, that is, biweekly or monthly. For nonrodents, in which the quantity of feed required usually does not allow weekly feeding, feed consumption is evaluated for shorter intervals, often once or twice per day. Accurate measurement of feed consumption is essential for studies in which the test material is administered in the diet. As discussed earlier in this chapter, feed consumption and the dietary concentration of the test material are used to calculate the dose of test material consumed by the animals in such studies. Some species, especially the mouse and the nonhuman primate, frequently soil or waste feed. This makes accurate measurement of consumption difficult. In these species, feed consumption measurement can be attempted using either a feed container designed to minimize wastage or by attempting to estimate feed wastage. Limitations of such data should be considered in evaluating test material consumption and the significance of any apparent differences between feed consumption in

test material–treated and control animals. Feed consumption measurement is another means of monitoring animal well-being. Animals that are ill or suffering adverse effects from exposure to the test material frequently may exhibit significantly decreased feed intake.

Ophthalmologic Examination

Ophthalmologic examination of all test animals should be conducted before the initiation of dose administration and at the completion of the dose administration period. Examination may also be performed at the end of a treatment-free recovery period (if included in the study design) based on findings at the end of dosing. Slit lamp examination and use of an indirect ophthalmoscope are two common methods of ophthalmologic examination during toxicity testing. These evaluations should be conducted by a veterinary ophthalmologist or other personnel experienced in the observation of the species used for the study.

Clinical Pathology

Clinical pathology parameters such as hematology, clinical chemistry, and urinalysis are important indicators of general health and toxicity and are assessed at the termination of a subchronic or chronic study. In addition, pretest and interim (typically at 4 weeks in subchronic studies and at 13 weeks in chronic studies) clinical pathology may be conducted to allow the evaluation of progression of any effects noted during the study. In rodents, clinical pathology determinations are usually conducted for 10 animals of each gender in each group. For nonrodents, clinical pathology should be done for all animals.

Sample Collection

Proper sample collection and handling are critical to completion of a meaningful clinical pathology evaluation. Whenever possible, the method of sample collection should be the same throughout the study and should be one that distributes variance, such as run-to-run variation in an enzyme assay, equally across groups. Samples generally are collected according to either a totally random design or a stratified random design. A stratified random design ensures that approximately the same numbers of animals of each gender and from each group are sampled within any block of time or during any set of assays.

Repeated blood sampling of rodents can be accomplished by serial collection from the same animals by nonterminal procedures (such as puncture of the jugular vein) or by collection from the abdominal aorta or vena cava at the termination of subgroups of animals. Because of practical restraints on the frequency and volume of blood collection in rodents, pretest studies often are not performed. Repeated collection of adequate volumes of blood is usually not a problem for nonrodents, and pretest clinical pathology is often included in studies using these animals.

The effect of repeated blood sampling on the animals and on the sample volume that can be reliably obtained at each sampling interval should be considered. Sample volumes should be sufficient both to conduct the assays indicated in the protocol and, if possible, to provide a reserve sample for any necessary repeat test. However, significant reduction of total blood volume (more than 10%) by blood collection should be avoided and a sufficient amount of *recovery* time should be allowed between sampling intervals. Publications regarding maximum blood sampling volumes and recovery periods are available and should be consulted when designing a toxicity study.[40]

Plasma or serum to be used for clinical evaluation should be clear and straw-colored. Red or pink plasma suggests that some hemolysis has occurred either as a result of pathology or, more commonly, as an artifact of the sample collection/preparation procedures. Severe artifactual hemolysis may alter the results for some of the clinical parameters to be evaluated. Slight hemolysis, commonly observed in serum and plasma collected from rodents by jugular venipuncture, generally is acceptable for clinical pathology studies as long as historical laboratory ranges have been established for blood collected by this method. If unusual or unexpected results are obtained, aliquots of serum or plasma can be *spiked* with ascending amounts of test material to determine if it interferes with the assay.

Clinical Chemistry, Hematology, and Urinalysis

Clinical pathology should include the determination of a number of serum or plasma chemistry and hematology parameters. The parameters included in the clinical chemistry evaluation should assess electrolyte balance, protein and carbohydrate metabolism, and organ function. An acceptable list of clinical chemistry parameters is shown in Table 24.9. Additional parameters should be assessed, as appropriate, to address other anticipated effects of the test material, for example, serum cholinesterase levels in the case of carbamate or organophosphate insecticides. It goes without saying that assays designed for the assessment of clinical chemistry

TABLE 24.9
Clinical Chemistry Parameters Normally Obtained in Repeated-Dose Studies[a,b]

- Glucose*
- Urea nitrogen*
- Creatinine*
- Total protein*
- Albumin
- Globulin
- Albumin/globulin ratio
- Inorganic phosphorus
- Calcium
- Sodium*
- Potassium*
- Chloride*
- Bilirubin (total)
- Cholesterol
- Triglycerides
- Alkaline phosphatase*
- Aspartate aminotransferase
- Alanine aminotransferase*
- Gamma glutamyl transferase*
- Ornithine carbamyl transferase*

[a] Based, in part, upon the recommendations of the U.S. FDA [23].

[b] This list does not include all clinical chemistry parameters that could be obtained. Additional parameters could be added depending upon the test material. When the blood volume obtained for analysis is small, the FDA recommends priority be given to those assays marked with an asterisk (*).

TABLE 24.10
Hematology and Urinalysis Parameters Generally Determined in a Repeated-Dose Study

Hematology	Urinalysis
Hematocrit	Appearance
Hemoglobin	Urine volume
Erythrocyte count	Specific gravity
Mean corpuscular volume	pH
Mean corpuscular hemoglobin	Glucose
Mean corpuscular hemoglobin concentration	Protein
Total leukocyte count	Microscopic evaluation of urinary sediment
Differential leukocyte count	
Reticulocyte count	
Platelet count	
Prothrombin time	

in humans must be validated for use with the species used in the toxicology studies. Typical hematologic parameters assessed during repeated-dose testing are shown in Table 24.10. The reader is referred to the chapter concerning clinical pathology in this text for a more detailed discussion of these clinical chemistry and hematology determinations. In addition, two excellent veterinary texts are available for further reference.[53,54]

Urinalysis is often included in the clinical pathology evaluation and may be important, especially for test materials that are nephrotoxins. Urinalysis parameters typically evaluated are listed in Table 24.10. However, urinalysis is frequently of limited value because collection of satisfactory urine samples is fraught with technical difficulties. Urine generally is collected in containers or tubes from troughs or trays placed below the cages in which the animals are housed and, therefore, steps must be taken to minimize fecal contamination. Because urine is frequently collected during an extended period, for example, overnight, bacterial growth in the sample is a concern. Collection of the sample on ice can reduce bacterial growth but presents technical challenges as well. Care should be taken that water be either freely available to the animals throughout the urine collection period or withdrawn at the appropriate time before collection. Care also must be taken to ensure that the sample is not inadvertently contaminated by feed or drinking water spilled by the animal. Because of these difficulties, the utility of urinalysis should be discussed with an experienced veterinary clinical pathologist prior to its inclusion in the design of a repeated-dose toxicity study. If urinalysis is conducted, its limitations must be kept in mind when the data are reviewed.

Postmortem Evaluations

One of the more definitive assessments of toxicological effects conducted during a repeated-dose study is the macroscopic and microscopic examination of tissues and organs from treated and control animals. In typical subchronic and chronic studies, samples of approximately 50 tissues and organs are collected during the necropsy of each animal. Table 24.11 presents a list of tissues that are commonly collected for potential histopathologic examination.

TABLE 24.11
Tissues Collected for Histopathology in Repeated-Dose Studies

Adrenals	Mammary gland (females)
Aorta	
Bone marrow smear	Nose
Brain	Ovaries
Cecum	Pancreas
Colon	Pharynx
Duodenum	Pituitary
Epididymides	Prostate
Esophagus	Rectum
Eyes	Salivary gland (submandibular)
Femur and bone marrow	Sciatic nerve
Gall bladder (when present)	Seminal vesicles
Heart	Skin
Ileum	Spinal cord (cervical, thoracic, lumbar)
Jejunum	Spleen
Kidneys	Sternum and bone marrow
Lacrimal gland	Stomach
Larynx	Testes
Lesions	Thymus
Liver	Thyroid with parathyroid
Lungs	Trachea
Lymph nodes (mandibular and mesenteric)	Urinary bladder
	Uterus

Necropsy

Necropsy of an animal is conducted when it dies during the study, when it is euthanized during the study for humane reasons (e.g., in cases of moribundity), or when it is euthanized at a scheduled interval (interim sacrifice or termination of the study). For animals that are euthanized, exsanguination should be included in the termination procedure to facilitate the subsequent fixation of the tissues. Necropsy should be completed as quickly as possible after the death of an animal to avoid autolysis that can interfere with the subsequent microscopic examination of tissues. Autolysis is an especially important consideration for animals that die during the study because their death may not be discovered for a significant period of time. Animals found to be moribund (i.e., about to die) during the study should be euthanized for humane reasons and to avoid tissue autolysis.

During necropsy, tissues and organs are systematically removed and macroscopically visible abnormalities are noted. These abnormalities include changes in color, shape, size, or consistency of a tissue. The documentation of an abnormality in the necropsy records should include its location and a clear description of the change, using nondiagnostic terminology. Completeness of the examination during

necropsy and the quality of the description of abnormalities are critical to the determination of pathologic effects. An abnormality in a tissue can only be prepared for microscopic examination if it was collected and accurately described during necropsy. Because of the central role that the necropsy plays in detecting effects in a repeated-dose toxicity study, it is extremely important that necropsy technicians are highly trained and experienced in the necessary techniques.

After collection, tissue samples are usually preserved by immersion in an appropriate fixative, commonly 10% neutral buffered formalin. In some cases, particularly for organs such as testes or eyes, special fixatives should be used.[55] In order to ensure adequate fixation, the volume of fixative should be at least 10 times the volume of tissues. Certain organs, for example, the lung and urinary bladder, are frequently perfused with fixative prior to immersion to improve fixation and the microscopic appearance of the tissue when sectioned. During the collection of large numbers of tissues from many animals, it is possible to inadvertently miss a tissue. Therefore, it is highly advisable to inventory and document the samples as they are placed into the fixative containers. This inventory will be invaluable during subsequent preparation of the tissues for microscopic examination and in reconstructing the study during poststudy auditing of the data. In addition, it must be ensured that the identity of each tissue is clearly maintained while in fixative. This is not a problem for tissues that are large or have distinctive morphology. In order to ensure subsequent identification of extremely small tissues and those with indistinct morphology, they are frequently placed in labeled plastic cassettes or cloth bags prior to being placed in the fixative container.

Organ Weights

Collection of terminal body weight and organ weights for all animals during necropsy is normal practice in repeated-dose toxicity studies. Minimally, weights should be recorded for the brain, liver, kidneys, testes, and adrenal glands. Frequently, other organs such as the thyroid/parathyroid, ovaries, spleen, thymus, uterus, epididymis, heart, or lungs are also weighed. Consideration should be given to the residual blood that remains in organs such as the spleen, heart, and lungs. Residual blood may be variable between animals due to the method of euthanasia and blood collection. Organs should be weighed as soon as possible after removal from the animal. They should be trimmed free of fat and connective tissue prior to weighing and placed into fixative immediately thereafter.

It is common practice to normalize organ weights by expressing them relative to body weight and brain weight. Relative organ weights are used to eliminate the influence of normal variation in animal growth on the interpretation of organ weight data. However, normalized organ weight data should be reviewed with the knowledge that they have limitations. Expressing organ weights relative to body weight can yield apparent, but artificial, compound-related effects on organ weights in studies where the test material affects body weight gain. Organ weights normalized to brain weight help overcome this problem because test materials that alter body weight generally do not alter brain weight. The best practice is to consider all three types of data, that is, actual organ weight and organ weight relative to body and brain weight. Histopathologic data are often used to help assess the significance of apparent differences between organ weights of test material–treated and control animals. Organ weight changes without correlative microscopic findings are given less credence than organ weight changes in tissues that also have microscopic abnormalities.

Microscopic Pathology

Microscopic examination of the tissues and organs of treated and control animals is one of the most time-consuming laboratory functions in toxicity studies. In nonrodent studies, sections of all protocol-specified tissues and organs from all animals should be prepared for microscopic examination. Generally, in rodent studies, only tissues and organs from the control and high-dose group animals and animals that were euthanized or died during the study are examined microscopically. For the other treatment groups, only a few major organs (e.g., liver and kidney, and any other organs in which macroscopic abnormalities were noted at necropsy or in which test material–related effects are detected in high-dose animals) are examined. Although initial histopathologic examination of control and high-dose tissues followed by examination of other doses is typical in rodent studies, simultaneous histopathologic examination of all tissues from all animals is not uncommon. This practice yields the most expeditious completion of the histopathologic evaluation phase of a study because sequential examinations are not required. Simultaneous examination also reduces the intergroup variability in diagnoses that might occur when tissues from intermediate groups are examined considerably after completion of the evaluation of the control and high-dose groups. Such variability can lead to incorrect conclusions concerning compound relationship of lesions noted in the intermediate-dose groups. The U.S. FDA has proposed that tissues from the high-dose and control animals initially be subjected to histopathologic examination with subsequent evaluation of lower-dose-group tissues based on findings in the high-dose animals.[56]

Routinely, tissues are prepared for light microscopic examination by embedding in paraffin, sectioning at 5–7 μm, and staining with hematoxylin and eosin (H&E). Special stains, such as stains for the presence of fat (e.g., Oil Red O) or connective tissue (trichrome stain) may be used for some tissues. The use of a protein-specific stain (Mallory–Heidenhain) to confirm suspected alpha-2-microglobulin nephropathy in male rats is illustrated in Figure 24.6. If desired, representative samples of selected tissues may be frozen at necropsy and stored for biochemical or immunohistochemical analyses. Tissues may be specially prepared for electron microscopy by fixation in paraformaldehyde or Karnovsky's fixative.[57] In the histology laboratory, it is important that tissues be prepared according to standardized procedures especially with respect to the type of section (i.e., cross, longitudinal), location, and orientation on the microscope slide. The histology technician must review the observations recorded during

necropsy of the animals to ensure that all grossly observed lesions are properly sectioned and mounted on the slide for subsequent microscopic examination. Whenever possible, samples of lesions prepared for examination should include the lesion and portions of surrounding *normal* tissue.

Histopathologic examination of the tissues requires specialized training and is performed by a pathologist trained and experienced in toxicological pathology. This training and experience provides the pathologist with the knowledge of the normal features and naturally occurring lesions that can be observed microscopically in tissues from laboratory animals. However, the pathologist's responsibility is more than evaluation of the tissues and accounting for all the lesions reported at necropsy. The pathologist should be an integral part of the protocol design team, providing input into many factors, for example, selection of the species/strain to be used and clinical pathology parameters to be evaluated. The pathologist should also provide guidance concerning the list of tissues to be collected in order to ensure that they are processed, stained, and evaluated in a manner that satisfies the study objective.

It is critical that the pathologist review the data generated during the in-life and necropsy phases of the study before proceeding with the histopathologic evaluation. Results of clinical observations, clinical chemistry and hematology determinations, organ weights, and necropsy examinations can lead the pathologist to focus on particular organs as potential targets for toxicity during the microscopic examination. For example, increased liver weight should lead to a more careful examination for hepatocellular hyperplasia or hypertrophy, while elevated serum creatinine along with a necropsy description of the surface of the kidneys as *rough* should result in a more thorough examination for nephropathy.

Some have suggested that knowledge of in-life and necropsy findings will bias the pathologist, causing a more stringent examination of the potentially affected tissue. Similarly, some are concerned that knowledge of the dose level administered to the animal during the study will bias the pathologist, resulting in a more thorough examination of the tissues from animals that received the test material. The second situation in particular could result in a higher incidence and/or severity of microscopic findings in treated animals compared with controls, a higher incidence that is simply an artifact of the thoroughness of the microscopic examination. One way to prevent potential bias is to keep the pathologist uninformed of other study findings and of the identity of the animal until histopathologic examination of the tissues has been completed. This so-called blinded reading does prevent bias but may also prevent the pathologist from identifying certain subtle, dose-related changes. Therefore, *blinded reading* is not recommended for routine histopathologic evaluations and should only be conducted in special situations.

Pathologic changes in cellular or subcellular structure can occur either spontaneously, such as with aging, or as a result of exposure to a chemical. Deciding which changes are toxicologically relevant and what severity should be assigned to a change during histopathologic examination is quite subjective. Because of this, it is possible that different pathologists looking at the same tissues will produce different diagnoses. It is even possible that, during histopathologic evaluation of a large number of tissues that extends over a number of months, the criteria for diagnosis of the same finding by a single pathologist will change somewhat. The variability that results from the subjective nature of histopathologic evaluation is unavoidable. In order to minimize its effect on the results of toxicity studies, several procedures can be useful. First, during each *reading period*, the pathologist should examine tissues from a small subset of the study animals. Each subset should contain approximately equal numbers from each control and treated group to be examined. Second, if a potential target organ is identified over an extended period of time, the pathologist should reexamine that organ from all animals during a compressed *reading period* of a few days or less in order to assess to what extent, if any, *diagnostic drift* occurred over time. Third, informal peer consultation concerning unusual or subtle tissue changes observed during the examination of the tissues can be conducted to arrive at a consensus diagnosis. Additionally, a formal peer review process involving (1) reexamination of target organs, (2) reexamination of a representative percentage of other tissues from the study, and (3) review of interpretation of the pathology findings can be conducted by a second pathologist in order to ensure consistency in diagnosis and grading of tissue changes and accuracy of the pathology conclusions.[58]

The objective of the histopathologic evaluation is the same as the objective of all other determinations during a repeated-dose toxicity study, that is, to detect adverse effects that could be relevant to humans or any other target species exposed to the test compound. Because of certain idiosyncrasies, some animals and strains are not useful for this purpose. In some cases, the animal exhibits a high spontaneous rate of pathology in a particular organ that prevents detection of any compound-induced increase in the background rate. For example, severe testicular pathology occurs spontaneously in a very high percentage of old male Fischer 344 rats (see Figure 24.5). Therefore, this strain of rat is generally not useful for the detection of testicular effects. In other cases, a pathologic change may occur in the laboratory animal in response to compound exposure, but that pathology would not be expected to occur in the target species. An example of this is light hydrocarbon nephropathy that occurs only in male rats. Many hydrocarbons produce this nephropathy, for example, D-limonene and unleaded gasoline. The pathology is caused by accumulation of a male rat specific protein, alpha-2-microglobulin, in the renal tubule following exposure to these chemicals. This nephropathy is characterized by hyaline droplets in the cytoplasm (Figure 24.6), granular casts in the lumen (Figure 24.7), and cellular regeneration in the tubules. Because humans do not produce alpha-2-microglobulin, this gender- and species-specific pathologic finding has no relevance to human hazard assessment, and the male rat is not a suitable model for human renal effects related to light hydrocarbon exposure. It should be noted, however, that for both examples cited, the test animal is perfectly acceptable for use as a human surrogate in toxicity testing as long as the limitations imposed by their idiosyncrasies are taken into account.

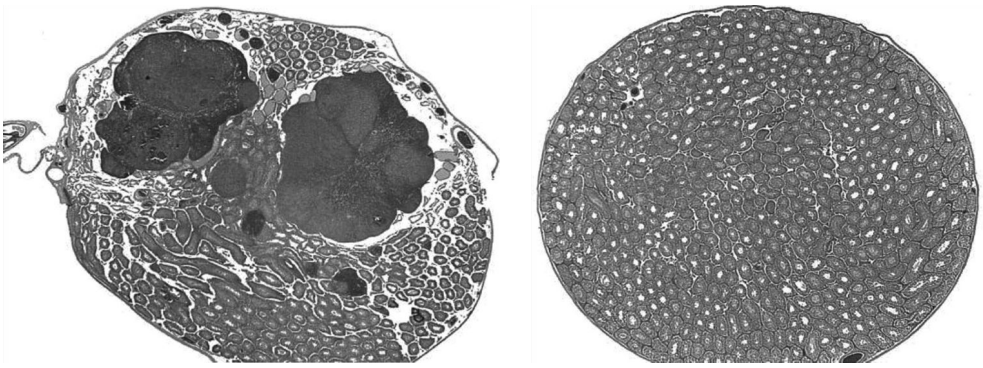

FIGURE 24.5 Photomicrographs of hematoxylin-and-eosin-stained testes from Fischer 344 rats (2.5× magnification). The testis on the left exhibits an interstitial cell tumor. The testis on the right is without pathologic changes.

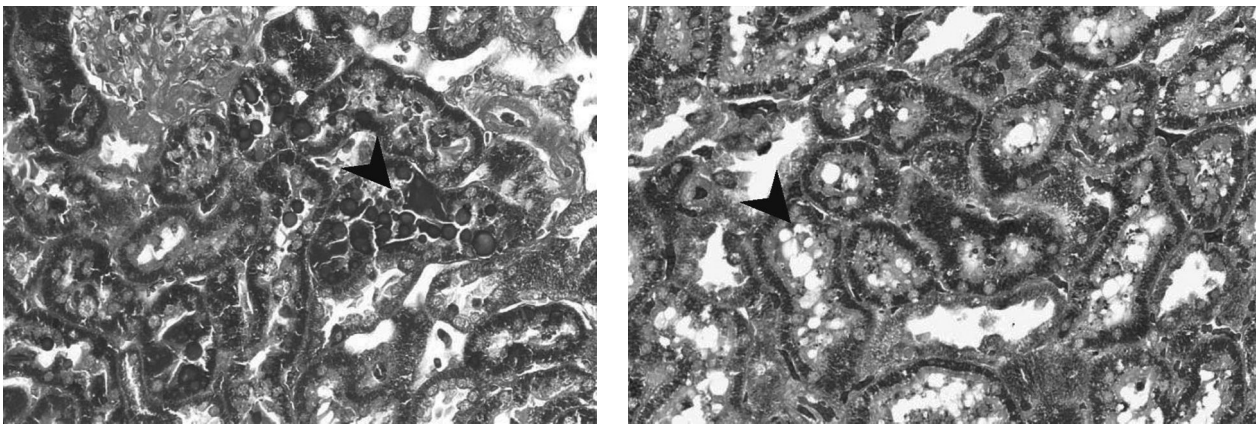

FIGURE 24.6 Photomicrographs of kidneys from Fischer 344 male rats stained for protein using Mallory–Heidenhain stain (100× magnification). The kidney tubules on the left are stained heavily and contain many hyaline droplets (arrow). The kidney tubules on the right exhibit normal levels of protein staining (arrow).

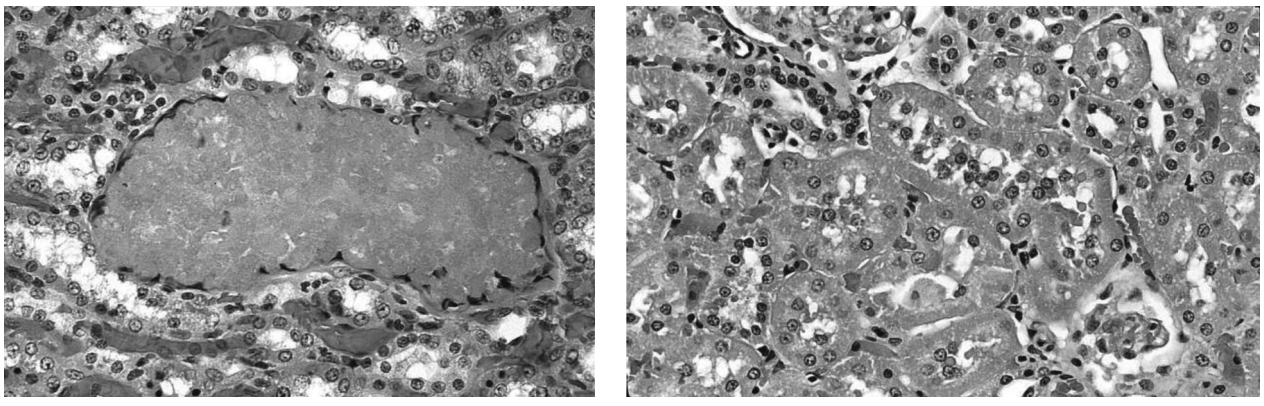

FIGURE 24.7 Photomicrographs of hematoxylin-and-eosin-stained kidneys from Fischer 344 male rats (100× magnification). The lumen of one tubule in the kidney on the left is obstructed by a large accumulation of granular casts. The kidney on the right is without pathologic changes.

Even though the idiosyncratic situations discussed earlier do occur occasionally, the pathology that occurs in most laboratory animals and most tissues is considered relevant to the assessment of hazard in humans. The histopathologic examination of tissues in a subchronic or chronic toxicity study can yield a vast array of diagnoses. A detailed discussion of possible chemical-related pathologic findings is beyond the scope of this chapter. For detailed descriptions of methods for and diagnosis of veterinary toxicological pathology, the reader is referred to a review by Hardisty[58] and two comprehensive texts on the subject.[59,60]

Additional Endpoints for Repeated-Dose Toxicology Studies

Evaluation of special endpoints can be added to repeated-dose toxicology studies to maximize the utilization of animal resources, minimize the time and cost of a hazard assessment, and obtain additional data. Care should be used in selecting these endpoints to ensure that valid methodology is used and that the data will be accepted by regulatory agencies. Guidelines for safety assessment of food ingredients suggest data concerning the immunotoxic and neurotoxic potential of the test material be generated during subchronic toxicity studies.[56] To ensure the methodology and data presentation is acceptable to the regulatory agencies, meetings should be held with the appropriate agency during design of the study.

It is possible, through the addition of special endpoints, to make a subchronic or chronic toxicity study so complicated that the main objectives are jeopardized. All the ramifications of addition of special endpoints to a study design, including practical considerations such as daily workload, should be considered to ensure that basic study endpoints are not compromised. Rather than overwhelming the capabilities of the testing laboratory, conducting a separate study designed to evaluate the special endpoints may be preferable. This is not to say that special evaluations should never be added to subchronic or chronic studies. With appropriate consideration of the possible complications, they can be and often are successfully added to standard study designs.

If a question still remains whether additional endpoints should be added to short-term repeated-dose studies or to longer-term studies, the following should be considered. If these endpoints were added to short-term studies, the data would be available to aid in the design of longer-term studies. However, if the potential change in the parameter to be evaluated occurs only after longer exposure periods, a change might not be observed in short-term studies. The most conservative approach is to add the endpoints to each study type. The following paragraphs provide examples of nonstandard toxicological endpoints that may be added to classical repeated-dose toxicity study designs.

Genetic Toxicology

Certain in vivo and in vivo/in vitro genetic toxicology data may be obtained during or at the termination of a repeated-dose toxicity study. Addition of these endpoints to a study could decrease the number of animals used in a hazard assessment and shorten its duration. In vivo genetic toxicology studies increase the value of a hazard assessment. They use the route of administration by which humans are/will be exposed to the test material and all processes of absorption, distribution, metabolism, excretion, and DNA repair are intact. Therefore, these in vivo studies provide important information that cannot be obtained from in vitro studies using cellular systems.

A number of in vivo genetic toxicology assays are currently in use, some of which are suitable for incorporation into repeated-dose toxicity assays. For example, the authors have incorporated the bone marrow micronucleus assay into a classic subchronic study design. During many repeated-dose toxicity studies, bone marrow smears are made (see Table 24.11). These bone marrow smears are made essentially in the same manner as those used for the in vivo micronucleus assay.[61] The slides are stained with acridine orange and the polychromatic erythrocytes analyzed for micronuclei. The use of bone marrow slides from the repeated-dose study has several advantages: (1) it incurs no additional time and cost for collecting the samples, (2) allows the assay to be conducted on animals exposed to the test material for long periods of time, (3) eliminates the need for resources to conduct an independent study, and (4) does not interfere with the histopathologic assessment of the tissues.

It may be possible to use bone marrow slides from repeated-dose toxicity studies for the bone marrow chromosomal aberration assay.[62] This is another in vivo genetic toxicology assay that could provide additional data without conducting an independent study. Another such genetic toxicology assay is the in vivo/in vitro unscheduled DNA synthesis assay.[63] In this assay, freshly isolated hepatocytes are used to determine unscheduled DNA synthesis. Although this assay is not compatible with histopathologic use of the liver, as few as 2–3 extra animals per group are all that is required. One problem associated with incorporation of additional endpoints, such as genetic toxicology, into a repeated-dose study design is that the laboratory conducting the study must have valid assay methodology. This is not always the case.

Recently, regulatory agencies have approved the evaluation of micronuclei in rat and mouse blood samples.[64] Although not common practice at this point, the ability to assess genotoxic potential in vivo using blood samples may become more prevalent as the pressure to reduce animal use and refine experimental techniques intensifies.

Not all in vivo genetic toxicology assays are completely suitable for incorporation into repeated-dose studies. For instance, the in vivo sister chromatid exchange assay requires the administration of deoxybromouridine to the animals. This compound can compromise the classical endpoints used in toxicity studies, including histopathology. However, it is possible to incorporate extra animals to assess specific endpoints while not compromising the main study animals.

Neurotoxicity

It is possible to incorporate neurotoxicity screening into repeated-dose toxicity study designs. In fact, the FDA states that neurotoxicity screening should be incorporated into these study designs.[56] EPA has similar recommendations.[65,66]

Neurotoxicity screening is designed to determine if the test material has the potential to produce adverse effects on the nervous system. Screening is conducted to determine if additional, more sophisticated, neurotoxicity testing is required. The first indication of a requirement for neurotoxicity testing may come from the structure/activity assessments. However, the neurotoxicity database is not as extensive as those for certain other types of toxicity and may not provide useful insight into the need for neurotoxicity testing. Drugs

and pesticides that target the nervous system are a well-known exception. For instance, there is no question that a new organophosphate insecticide will require neurotoxicity testing. For most compounds, it will be necessary to develop data through empirical testing.

Most classical repeated-dose study designs contain elements that may provide some information on neurotoxicity potential. These include cage-side observations of the animals, physical examinations, and measurement of parameters, such as food consumption, that may relate to behavior modifications during the study. Additional information is obtained during histopathologic examination of the nervous system tissues collected at necropsy, such as the brain and spinal cord. The FDA believes that these procedures, as well as others, should be specifically included into the design of repeated-dose toxicity protocols.[56] Table 24.12 lists the design elements recommended by the FDA for a neurotoxicity screen. Specific behavioral and neurotoxicity tests exist to provide most of the requested data. The particular test designs should be chosen based upon their validity, history of use, lack of undue stress to the animal, and the experience of the laboratory with the specific test. Care should be taken to perform these procedures on all the test article groups and controls in the study. A concern is that some of these tests may stress the animals and produce changes in the traditional parameters measured during a toxicity study. If this were to happen, it is assumed that the control group would also demonstrate these changes. This may or may not be true. Therefore, a conservative approach would be the addition of extra animals to the study that would be subjected to the manipulative procedures for neurotoxicity screening and not be used in the traditional phases of the study. For a more complete discussion of the FDA recommendations, the reader is referred to the guidelines for Toxicological Principles for the Safety Assessment of Food Ingredients.[56]

TABLE 24.12
FDA Criteria for a Neurotoxicity Screen as a Component of Short-Term and Subchronic Studies

- Histopathologic examination of tissues representative of the nervous system, including the brain, spinal cord, and peripheral nervous system
- Quantitative observations and manipulative tests to detect neurological, behavioral, and physiological dysfunctions. These may include
  - Physical appearance
  - Body weight
  - Body posture
  - Incidence and severity of seizure, tremor, paralysis, or other signs of neurological disorder
  - Level of motor activity and alertness
  - Level of reactivity to handling or other stimuli
  - Motor coordination and strength
  - Gait
  - Sensorimotor response to primary sensory stimuli
  - Excessive lacrimation or salivation
  - Piloerection
  - Diarrhea
  - Polyuria
  - Ptosis
  - Abnormal consummatory behavior
  - Any other signs of abnormal behavior or nervous system toxicity

Immunotoxicity

Rapid advances have been made during the last 20–30 years with respect to the detection of immunotoxicity. The two major forms of immunotoxicity are immunosuppression and hyperactivity of the immune system. Immunosuppression results in a reduction in the animal's resistance to infection and potential increase in susceptibility to tumorigenesis by a suppression of critical immunological responses. Hyperactivity of the immune system can result in autoimmune diseases and increased sensitivity to allergic disorders. Determination of the mechanisms associated with these disorders can be extremely complex because of the large number of biochemical, cellular, and physiological factors that can be affected as well as the cellular interactions required to mount an immunological defense. The detection of potential immunological changes is less complex, and a number of tests exist that can provide warning of a potentially immunotoxic compound.

The FDA has published recommendations for the inclusion of immunotoxicity evaluations in repeated-dose toxicity studies.[56] The agency suggests that such evaluations be conducted in rodents. Immunotoxicology testing procedures are divided into two broad categories. Type I tests are those assays that do not require study animals to be treated with an agent that presents an immunological challenge. Type II tests are assays that require study animals to be challenged with an agent that elicits an immune response, such as antigens, vaccines, infectious agents, or tumor cells. Because type I tests do not require manipulation of animals, they can be included in the routine assays done during a repeated-dose toxicity study. Because type II tests require treatment of the animals with an immunological challenge, these animals are not suitable for evaluations conducted during toxicity studies. Therefore, additional animals must be included in the study design if type II assessments are to be included.

Table 24.13 lists the immunotoxicology evaluations the FDA suggests be included in type I tests. Generally, these evaluations are those currently recommended for repeated-dose toxicity studies with the exception of more comprehensive histopathology of lymphoid tissues. Inclusion of these evaluations into standard study designs should have no impact on the validity of the study. Inclusion of the expanded type I tests listed in Table 24.14 would require more planning during the study design phase and assurance that the laboratory performing the study would be capable of performing the assays. Generally, the expanded type I test would only be conducted after consultation with the FDA.

Type II immunotoxicity tests recommended by FDA could be done as a component of a repeated-dose toxicity study but appear to be more appropriately conducted as independent studies and are beyond the scope of this chapter.

TABLE 24.13
FDA Draft Recommendation for Type I Immunotoxicity Test That Can Be Included in Repeated-Dose Toxicity Studies

Type I Test

- Hematology
  - White blood cell counts
  - Differential white blood cell counts
  - Lymphocytosis
  - Lymphopenia
  - Eosinophilia
- Histopathology
  - Lymphoid tissues
    - Spleen
    - Lymph nodes
    - Thymus
    - Peyer's patches in gut
    - Bone marrow
  - Cytology (if needed)[a]
    - Prevalence of activated macrophages
    - Tissue prevalence and location of lymphocytes
    - Evidence of B-cell germinal centers
    - Evidence of T-cell germinal centers
  - Necrotic or proliferative changes in lymphoid tissues
- Clinical chemistry
  - Total serum protein
  - Albumin
  - Albumin-to-globulin ratio
  - Serum transaminases

[a] More comprehensive cytological evaluation of the tissues would not be done unless there is evidence of potential immunotoxicity from the preceding evaluations.

TABLE 24.14
FDA Draft Recommendation for Expanded Type I Immunotoxicity Test That Can Be Included in Repeated-Dose Toxicity Studies

- Hematology
  - Flow cytometric analysis
    - B-lymphocytes
    - T-lymphocytes
    - T-lymphocyte subsets
      - TH and TS
    - Immunostaining of blood or spleen
      - B-lymphocytes
      - T-lymphocytes
- Serum chemistry
  - Serum protein electrophoresis
    - Albumin
    - α-Globulin
    - β-Globulin
    - γ-Globulin
  - Quantification of γ-globulin fraction
    - IgG, IgM, IgA, IgE
  - Complement
  - Cytokines
    - IL-2, IL-1, γ-interferon
  - Autoantibodies
  - Antiparietal cell antibodies
- Histopathology
  - Immunostaining of B-lymphocytes in spleen and lymph nodes with polyclonal antibodies to IgG
  - Immunostaining of T-lymphocytes and subsets with monoclonal or polyclonal antibodies
  - Micrometric measurements of germinal centers and periarteriolar lymphocyte sheath of the spleen and follicles and germinal centers of lymph nodes
- In vitro analysis of functional capacity of specific immune cells
  - Activity of natural killer cells
  - Mitogenic stimulation of B- and T-lymphocytes
  - Macrophage phagocytic index
  - Stem cell assays

Safety Pharmacology

The core battery of safety pharmacology studies includes studies to evaluate effects of test materials on the central nervous system, the cardiovascular system, and the respiratory system. In some cases, evaluations can be added to standard repeated-dose study designs to provide information regarding adverse effects on these systems.[67] In other cases, such as the development of oncology drugs intended to treat late-stage cancers, stand-alone safety pharmacology studies may not be required and data obtained in toxicity studies are considered sufficient.[12] A functional observational battery or Irwin screen may be added to a repeated-dose toxicity study in order to evaluate effects on CNS function. The addition of CNS evaluation is relatively noninvasive, while adding endpoints to evaluate respiratory and/or cardiovascular function can be more challenging. With the advent of telemetry and/or jacketed data collection systems, many of the complications involving respiratory and cardiovascular parameter assessment can be overcome. It is always important to keep in mind that adding too many additional endpoints to a study can compromise the overall objective of the study, which is to identify potential toxicity and target organs. Several publications regarding the integration of safety pharmacology parameters into toxicity studies and the potential challenges in doing so are available.[68]

Toxicokinetics

Advances in modern analytical chemistry, especially high-performance/mass spectroscopy, have provided the toxicologist with the capability of obtaining toxicokinetic data in the early stages of a hazard assessment. These data can be important in the interpretation of data from a current study as well as in the design of subsequent studies. Demonstration of systemic exposure is also important and may provide insight into factors that may influence the toxicity of the test material (see the toxicokinetic chapter in this volume). For these reasons, toxicokinetic assessments are increasingly being incorporated in more protocols for short-term and long-term repeated dosing studies. According to ICH guidelines, toxicokinetic evaluation should be performed in studies of pharmaceutical products because "Toxicokinetics is thus an integral part of the nonclinical testing programme; it should enhance the value of the toxicological data generated, both in terms of understanding the toxicity tests and in comparison with clinical data as part of the assessment of risk and safety in humans."[69]

It is currently possible to obtain in vitro metabolism and preliminary in vivo metabolism data before the initiation of short-term repeated-dose studies. These data can be useful in the design of 14-day repeated-dose studies and aid in the integration of toxicokinetic procedures into short-term protocols. Because of the small quantities of blood required with the current analytical procedures, blood collection from studies with larger animals can be done using the main study animals and eliminates the need for animals devoted to toxicokinetics alone. However, with rodent species, it still may be necessary to have an extra set of animals in the study for the assessment of toxicokinetics. Using a separate set of animals in rodent studies to obtain toxicokinetic data still has advantages over doing an independent toxicokinetic study. It ensures that the animals are similar to those in the toxicology study with respect to age, environmental factors, and dosing parameters. Recent advances in analytical and sampling procedures such as dried blood spot technology have reduced the number of separate toxicokinetic animals needed in rodent studies and may, depending on the number of sampling time points and other considerations, enable blood collection from main study rodents.

Whereas the initial in vivo studies may be single-dose studies, repeated dosing studies provide the opportunity to obtain data following multiple doses. They can, therefore, provide information concerning induction and inhibition of the test material's metabolism associated with the previous exposures. Toxicokinetic data obtained during different time periods in a subchronic study provide information concerning the effects of prolonged dosing on the absorption, distribution, metabolism, and excretion of the test article and also information concerning the disposition of metabolites and may provide explanations for unexpected results in the toxicology study. Data obtained at the initiation, midpoint, and near study termination may indicate important changes in toxicokinetics based upon biochemical or toxicological alterations of biodisposition of the test article and its metabolites. For instance, hepatotoxicity that is manifested after several weeks of dosing may significantly alter the metabolism of the test article. Toxicokinetic data can also provide information useful in the design of longer-term studies. For instance, gender differences in toxicokinetics may indicate a need for differential dosing of males and females. Species differences in the toxicokinetics may indicate the most suitable animal model for longer-term studies and aid in the extrapolation of the animal data to humans.

Miscellaneous Other Endpoints

There are a wide variety of additional endpoints that could be evaluated in repeated-dose studies to address specific issues. In part, any additional endpoints would depend upon the questions to be addressed and the creativity of the toxicologist designing the studies. For instance, the increased availability of electron microscopy makes this endpoint a more viable option today than in previous decades. The development of an increased number of histochemical assays, especially those employing specific antibodies, makes the preserved wet tissues, embedded tissues, and the histopathology slides obtained from these studies valuable for future use. In many cases, the need for additional endpoints is unknown until the initial results of the study are available. The preserved tissues then become a valuable resource. For instance, if it is found that the liver from a study contains vacuoles and the toxicologist suspects these to be fat vacuoles, it is possible to use special lipid stains to determine if the vacuoles are lipid. Other special stains exist for a variety of purposes. An example of a protein-specific stain, Mallory–Heidenhain, is illustrated in Figure 24.6. If the toxicologist believes that the histopathologic data indicate a particular compound is producing cellular proliferation, there are a number of approaches to investigate this hypothesis. A standard method to determine cellular proliferation is by injecting the animal with ^{3}H-thymidine and measuring the incorporation of the radiolabel into cellular DNA by methods such as autoradiography. However, if the study has been terminated, this is not possible. Also, the toxicologist may not want to administer radiolabel to the animals for a number of reasons, especially if the in-life portion of the study has not been terminated. If the evidence of increased cellular proliferation occurs in tissues with a relatively high rate of normal proliferation, such as the gut mucosa, it is possible to determine the mitotic index by counting mitotic figures in cells from slides previously prepared for histopathologic analysis. Alternatively, it is now possible to immunostain for specific proteins associated with cellular proliferation in preserved tissues.

In cases where potential effects and/or target organs are suspected prior to study conduct, the use of bioimaging techniques such as ultrasound, magnetic resonance imaging (MRI), and computed tomography (CT) scanning is becoming increasingly common. These imaging techniques can be useful because they allow longitudinal assessments of internal structures/organs. For example, imaging techniques can be used to evaluate tumor onset and progression in long-term studies and the ability of cancer treatments to shrink tumors, to evaluate hypertrophy of tissues in response to test article treatment, to examine bone density, or, in the case of medical devices, to verify the placement of the device. There is a growing amount of information regarding bioimaging techniques and their application in toxicity testing, and the reader is encouraged to review this information when evaluating potential inclusion of imaging methodology in their studies.[70,71]

Lastly, in certain situations, valuable information can be obtained by adding evaluation of specific biomarkers to a repeated-dose toxicity study in addition to the routine assessments that are normally performed. For example, if a test article is suspected to be cardiotoxic, evaluation of serum troponin T levels may be useful. Release of this protein from cardiomyocytes (resulting in elevated serum troponin T levels) can occur at dose levels that may not produce histological alterations. There has been an increase in interest in identifying biomarkers that would serve as early indicators of potential toxicity, and efforts have been initiated to identify sensitive biomarkers to predict liver, kidney, and testicular toxicity, to name a few.

DATA ANALYSIS AND INTERPRETATION

Compilation and Summarization of Study Data

Once the data have been collected from a repeated-dose toxicity study, the next steps involve data analysis, interpretation, and reporting. These data are derived either from the measurement of a parameter associated with the test animal, for example, body weight, feed consumption, and serum enzyme activity, or from the observation of the animal, for example, physical examination findings and macroscopic and microscopic pathology. The frequency, number, and variety of these measurements and observations in repeated-dose toxicity studies yield an extremely large volume of data that must be organized and summarized prior to analysis. Historically, individual animal data were recorded manually and then were compiled either manually or following entry into a computer. Some specialized data are still handled manually today; however, most routine data are collected, compiled, and statistically analyzed electronically using custom or commercially available computer programs.

For quantitative data such as body weights and feed consumption, individual animal data are used to calculate the mean values with a measure of statistical variation for each treated or control group. For other types of quantitative data such as the number of animals exhibiting a behavioral effect or the number of animals in which a particular finding is determined histopathologically, the incidence of the observation in each treated and control group, that is, the number of animals affected as a fraction of the total number of animals observed, is presented. Some data, like results of microscopic examination of urinary sediment, cannot be effectively summarized using a group mean or incidence value. Summarization of these types of data involves listing the individual animal data in their appropriate groups.

Despite advancements in technology such as on-line data collection systems and computerized table and report generation systems, the final evaluation of the vast amount of data gathered from even short-term repeated-dose toxicity studies and writing the study report still rely on the knowledge and judgment of the toxicologist. Data interpretation and report writing styles differ between toxicologists and evolve with experience. One approach to data evaluation is to first look at summary tables for each type of data and note mean values, sample size, variability, trends in the data over time, and any statistically significant findings. Differences from the control group values should be evaluated for dose responsiveness, and persistent and interrelated data should be evaluated for consistency (e.g., are there decreases in body weight gain that correlate with decreased feed consumption, and are there elevations in liver enzymes that correlate with microscopic findings?). After reviewing summary tables and identifying potential effects, it is helpful to evaluate the individual data in order to identify outliers that may be contributing to excessive variability in the data (if observed). After evaluating the individual and summary data, the toxicologist can then identify test article–related effects versus spurious findings. The use of scientific judgment coupled with the results of statistical evaluations and comparisons to historical control data will then allow the toxicologist to make a final decision regarding the relationship of changes in the data to the test material as well as the toxicological significance of the findings.

Determination of Compound-Related Effects and Adversity of Effects

Data from the test article groups are compared with data from the control group to determine if any compound-related effects have occurred. In virtually all cases, the data from one group of animals will differ somewhat from the data for any other group. Therefore, differences between the sets of data that are potentially related to the compound must be differentiated from spurious occurrences and from normal biological variation. This is accomplished by two methods. The first is simple examination of the data and detection of differences worthy of further consideration based upon the experience of the toxicologist and comparison with historical data. The second method uses statistical tests to detect differences for which the probability that the difference occurred by chance is low. These methods should always be used in combination. Although it is an extremely powerful and useful tool, statistical analysis alone should not be used to detect compound-related effects since, as stated by the FDA, "… statistical outliers are not always biological outliers, and a significant statistical test ($p < 0.05$) does not always indicate biological significance."[56]

Differences between the data from the control and treated animals that are detected using the methods cited earlier may indicate either an adverse effect associated with the test material, physiological adaptation to the test material, or normal biological variation unrelated to the test material. Determination of the relationship of test article treatment to differences observed during a study as well as the toxicological relevance of differences between treated and control groups is based on a number of factors that are frequently considered in combination with each other. These factors are listed in Table 24.15 and discussed in the following paragraphs.

TABLE 24.15
Factors Evaluated to Determine the Test Article Relatedness and Toxicological Relevance of Differences between Treated and Control Groups

- Dose-related trends
- Reproducibility
- Related findings
- Magnitude and type of difference
- Occurrence in both sexes

Dose-Related Trend

One of the best indicators of whether an effect is related to a test article is the presence of a dose-related trend in the data, that is, the magnitude of the effect varies directly with the dose level. Such an effect is reflective of the basic principle of toxicology stated by Paracelsus in the sixteenth century and often paraphrased as, "The dose makes the poison." If a difference from controls is noted in two or more dose groups and the severity or incidence of the difference increases as the dose level of the test material increases, it is probably a compound-related effect. When a difference from controls is noted only in animals receiving the highest dose level of the test material, it may or may not be compound related and other factors must be considered in determining its significance. Differences from control data in test material–treated animals are probably not associated with the compound if a dose-related trend is not observed. Because of this, as stated previously, selection of an appropriate range of dose levels is extremely important and facilitates data interpretation. One important consideration when evaluating dose–response relationships is the internal exposures (plasma concentrations) of the test material at each dose level. In some cases, absorption may be saturated at, for example, the mid and high doses. If saturation occurs, the internal exposure of the mid- and high-dose groups would not differ. In this case, effects on parameters in the study would be expected to be similar between the two groups and no dose–response would be evident. For this reason, it is important to examine the toxicokinetic data for dose proportionality in conjunction with the dose responsiveness of other findings.

Reproducibility of Effect

Another reliable indicator of a compound-related effect is its reproducibility. If a difference from controls is noted in the test article–treated animals at multiple intervals during a study, the difference is likely related to the compound. Further weight is given to the determination of the compound relationship if the same difference is noted in other independent studies in which the test material was administered to the same species and, even more weight is given, if the difference is observed in a second species. The absence of reproducibility, especially in the same species, is an indication that the difference may have occurred by chance.

Correlated Findings

Another consideration in the assessment of test article relatedness of an intergroup difference is the presence of related findings. For example, an elevation in the activity of serum alanine aminotransferase in treated animals when compared with the control group is probably related to the compound if there is an elevation in serum aspartate aminotransferase with concomitant hepatic necrosis. If no correlation with other findings is observed, the elevation may be unrelated to the test article or, at least, its relationship to test article exposure must be determined considering other factors. Furthermore, test article–related effects may be considered adverse or nonadverse based on correlative findings. For example, an increase in liver weight (or other organ weight changes) may not be considered adverse in the absence of microscopic findings in the tissue.

Magnitude and Type of Intergroup Difference

The magnitude and type of difference observed between treated and control data may also give an indication of its potential association with test material administration and also impact the assessment of whether or not findings in a study are considered adverse. For example, a doubling of an organ weight in treated animals compared to controls should be considered more likely to be compound related than a 10% increase, even if the smaller increase is statistically significant. Furthermore, a fairly large decrease in the activity of serum alanine aminotransferase in test article–treated animals is generally assigned limited clinical significance whereas an increase in the same magnitude in the activity of this enzyme may be considered indicative of a toxic effect. It is obvious that the assessment of the significance of a change on the basis of its magnitude or type requires knowledge of normal trends and ranges for the data.

Gender Differences

The determination if an effect is compound related is also influenced by whether or not the difference occurs in animals of both genders. Since compound-related effects often occur in both genders, a difference from controls that is noted only in treated animals of one gender may not be associated with the test material. It must be remembered, however, that there are cases where one gender is more sensitive to the toxicity of a chemical and, therefore, only the sensitive gender will exhibit the effect at a given dose. For this reason, a difference should not be considered insignificant solely on the basis of its absence in one gender. Male rats are well known for their greater capacity to detoxify certain compounds because of their higher activity of P450-dependent monooxygenases. Therefore, they may demonstrate less toxicity to these compounds than females. However, if the metabolic product is more toxic than the parent compound, they may demonstrate higher sensitivity than females. This gender difference is not seen in a number of other species, including humans.[72]

STUDY REPORT

After data from a study have been analyzed, a report is prepared. Depending upon the intended use of the report, it may contain various levels of detail. For example, a report prepared for submission to a regulatory agency in support of the safety of a chemical will generally contain much more detail (including all individual animal data) than a research report intended to be used by an organization or individual only to give guidance for future testing or a manuscript prepared for publication in the scientific literature. Whatever the purpose of the report, it should be written in sufficient detail to permit peer review of the conduct and conclusions of the study and to allow the study to be reproduced exactly, if required.

Report Content

All reports, regardless of their purpose, should contain certain common elements that are essential to adequately describe the conduct and results of a study. The report should clearly state the objective(s) of the study. It should precisely define the test material, indicate the test species used, and describe the methods and equipment employed to collect and analyze the data. Protocol deviations and an assessment of their impact upon the study should be presented. The report should present the data pertinent to the study objective(s) in a form that facilitates its review and should discuss these data in the depth required to support the conclusion(s) of the study. The discussion should describe any compound-related effects observed and should explain how the various factors described earlier were used to determine the toxicological relevance of any differences between treated and control animals in the study. Finally, the conclusion(s) drawn from the results of the study should be clearly and concisely stated.

Retrospective Report Audits

After the study has been completed and reported, retrospective audits of the study are frequently conducted. The manufacturer of the test material may audit the study prior to submitting it to a regulatory agency in support of registration or approval of the test material for its intended use. The regulatory agency to which the study report was submitted may also conduct an audit. Regulators also audit study reports to assess the compliance of the testing laboratory with GLP regulations. Whatever the reason for retrospective auditing of the study, the process is essentially the same. The raw data are inventoried to ensure that they have been properly maintained. The data are reviewed and compared with the study report to ensure the report accurately and completely presents the methods used and the data collected. Any deviations from laboratory SOPs, the study protocol, or GLP regulations that occurred during conduct and documentation of the study should have been clearly explained in the report. Individual animal data should support summary tables and discussion of the results should be consistent with the data. Retrospective auditing is of great value to all parties involved. The manufacturer of the test material can feel comfortable that they will receive no surprises during a subsequent audit by the regulatory agency. The regulator will be more confident about the quality of the study if the data have been audited. If the regulator is confident about the data, regulatory review will proceed more smoothly and, therefore, regulatory decisions will be expedited.

Data and Specimen Collection and Retention

The preceding sections of this chapter describe the design of repeated-dose studies and the main parameters to be evaluated during those studies. Knowledge of this information is critical to study conduct. Just as important to registration or acceptance of the data from these studies is proper documentation of the procedures and results. All GLP regulations require complete and accurate documentation of the study, documentation that would allow an appropriately trained and experienced individual to repeat the study with little variation from the original design.

GLPs require that documentation of study procedures and the resulting data be recorded in indelible ink that is suitable for photocopying. The individual responsible for creating the records must be documented along with the date the record was created. Any change or correction to the documentation must be made as soon as possible after the original entry and must clearly explain the reason for the change, identify the individual making the change, and indicate the date the change was made. If portions of the data are to be recorded electronically, the accuracy and reliability of any system used to collect and manipulate the data must be validated prior to its use. Electronic data collection systems are subject to the same basic requirements as hand-recorded data. That is, the individual responsible for entry or revision of the data must be identified, the date of these entries must be detailed, and an explanation of the reason for any changes to the original entry must be documented.

While the study is in progress, records and specimens must be stored in an orderly and secure manner that will facilitate report preparation once the study has been completed. At completion of the study, GLP regulations require that study documentation and specimens be placed into an archive facility that will ensure their integrity and security. The study protocol, all study records, test article samples, fixed tissues, paraffin blocks, microscopic slides, and the study report(s) must be archived following completion of data collection. Specimens with a limited useful life span such as blood samples for clinical pathology determinations and samples from mutagenicity assays do not need to be retained.

Access to the archive facility should be controlled by a designated archivist and limited to authorized individuals. The conditions of storage must prevent deterioration of the data and specimens. Archives must be temperature and humidity controlled and should have provisions for fire suppression and vermin control. The removal of any data or specimens from the archives should require documentation of the transfer, and procedures should be in place for retrieval of the items if they have not been returned to the archives in a timely manner.

The duration of retention for archived materials is dependent upon the regulatory agency to which the data will be submitted and the reason for the submission. Archival requirements for several regulatory agencies and types of submissions are shown in Table 24.16. After the materials have been archived for the required period, the data and specimens may be discarded without compromising the regulatory status of the study. However, many companies effectively archive data from pivotal studies indefinitely even though there is no regulatory requirement to do so. This extra precaution may prove useful in addressing future

TABLE 24.16
GLP Requirements for Duration of Data and Specimen Archiving

Regulatory Agency	Retention Requirement
FDA	The shorter of At least 2 years following date of approval of application for research or marketing permit At least 5 years following date of nonclinical laboratory study submission supporting application for research or marketing permit At least 2 years following date of study submission supporting application for research or marketing permit if submission is not approved or in other similar situations
EPA FIFRA	The longer of For the life of the approval for any study submitted in support of an approved research or marketing permit At least 5 years following date study submitted in support of a research or marketing permit At least 2 years following date of study submission if application for research or marketing permit is not approved or after the decision has been reached not to seek a research or marketing permit
EPA TSCA	At least 10 years following effective date of final test rule or publication of the acceptance of a negotiated test agreement
OECD	For the period specified by the appropriate authorities

liability issues related to the test material or in designing additional studies if new safety questions for the test material arise at some time in the future.

REGULATIONS CONCERNING GENERATION AND USE OF DATA FROM REPEATED-DOSE TOXICITY STUDIES

Almost every industrialized country in the world has regulations governing the introduction, transportation and use of new pesticides, food additives, human and animal drugs, and other chemicals. Many of these countries also have regulations governing medical devices, workplace exposure to chemicals, the introduction of industrial chemicals into commerce, and the disposition of chemical waste.

There is general uniformity in the objectives of these laws, that is, not to impede the beneficial use of chemicals, but at the same time to ensure their safety in use. Even though the regulatory agencies can agree, in broad terms, on one framework of toxicity testing guidelines, the toxicologist must become familiar with the details of particular guidelines to fulfill the role as a bridge between scientific and regulatory concerns. Several regulations governing repeated-dose toxicity and other types of toxicity testing are briefly described in the following text.

U.S. Laws and Regulatory Guidelines

Federal Food, Drug, and Cosmetic Act

Federal Food, Drug, and Cosmetic Act (FFDCA)[73] as amended by the FDA Modernization Act of 1997[74] controls the introduction of human and animal drugs, direct food additives, indirect food additives (such as packaging materials), and components of cosmetics. In the case of new human or animal drugs, safety and efficacy must be established for a particular therapeutic application before the FDA grants approval for marketing. The approval process is comprehensive and involves two sequential phases. For the investigational new drug (IND) phase, the industry is required to file preclinical toxicity data with the FDA before investigation of the safety and potential therapeutic value of a drug in limited numbers of humans. When the efficacy and safety of the drug in the treatment of a particular disease is established through extensive clinical trials, these data together with additional animal toxicity data are provided to the agency as part of a new drug application (NDA) or new animal drug application (NADA). The NDA is reviewed by the FDA with respect to safety and efficacy of the drug. As a consequence of the FDA review, the NDA is either approved or disapproved or has deficiencies in the data cited.

The summary of an NDA must address benefit/risk relationships, clinical data, nonclinical pharmacology and toxicology, human pharmacokinetics, bioavailability, and microbiology. It must also contain information on pharmacologic class, scientific rationale and clinical benefits, as well as chemistry and manufacturing.[75]

With respect to food additives, industry must show that a material either intended for direct addition to food, such as a preservative or flavoring agent, or having indirect contact with food, such as a packaging or can-coating material, is safe for its intended use. Results from a hazard assessment are submitted to the FDA for review as part of a Food Additive Petition. If the data demonstrate the safety of the chemical, a regulation is published allowing the chemical to be used for a particular purpose in food or in contact with food. In 1982, the FDA published *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*.[13] This so-called Redbook delineated the nature of evaluations necessary to determine food additive safety. It provided a basic scheme for scientifically sound decisions for the development of the safety assessment. In 2007, the Redbook was replaced by a revised version, *Toxicological Principles for the Safety Assessment of Food Ingredients "Redbook 2000."*[56] These guidelines include a priority setting system that increases the efficiency of the food additive safety assessment.

FFDCA also provides an alternative method by which materials can be approved for use in or on food, that is, the Generally Recognized as Safe (GRAS) process.[76] For a material to be considered GRAS, its safety evaluation must satisfy three basic requirements. First, a group of experts qualified by scientific training and experience must conclude that the material, when used as intended, is safe for human

consumption. The sponsor of the potential additive convenes this expert group, and the group operates without regulatory oversight. Second, the information considered by the expert group during the GRAS review must be common knowledge, that is, available in the scientific literature. Third, there must be general agreement within the scientific community with the conclusion of the expert group. Final GRAS approval does not require regulatory review; however, many producers of such materials petition FDA to affirm their GRAS status prior to marketing them.

At the present time, there are no requirements for the FDA to review cosmetic formulations for safety prior to marketing. While the FFDCA only requires that the cosmetics be free of any *poisonous and deleterious* substances, responsible suppliers of ingredients for use in cosmetics and manufacturers of final products conduct toxicity studies relevant to the specific cosmetic.

The FFDCA also addresses the concentrations of pesticides permitted in foods in the United States. Under FFDCA Section 408, the EPA establishes maximum allowable concentrations for pesticide residues in raw agricultural commodities, that is, food crops, eggs, raw milk, and meat. Under FFDCA Section 409, EPA also establishes a maximum allowable concentration for a pesticide in processed food, if processing concentrates the residue of the pesticide in the raw agricultural commodity. These maximum concentrations are referred to as tolerance levels or tolerances. Human exposure resulting from consumption of foods containing tolerance levels of a pesticide must not exceed the maximum permissible intake of the pesticide established by EPA under the FIFRA. Tolerances for most foods are enforced by FDA, while those for meat, poultry, and some egg products are enforced by the Food Safety and Inspection Service within the USDA.

Federal Insecticide, Fungicide, and Rodenticide Act

The FIFRA was administered initially by the USDA and is now administered by the EPA.[77] Under FIFRA, EPA is responsible for the registration of pesticides for use in the United States. This act requires extensive toxicity testing to be conducted in mammalian, avian, and aquatic species to support the safety of a pesticide. Detailed guidelines for toxicity study design and reporting have been issued by the EPA.[8] Toxicity data submitted in an application for registration of a pesticide are reviewed by toxicologists in the Office of Pesticide Programs at EPA. Other data specifically required by FIFRA as a condition of pesticide registration include product chemistry, residue chemistry, environmental fate, reentry protection, spray drift, plant protection, nontarget insects, and product performance.

Food Quality Protection Act

The Food Quality Protection Act (FQPA) was signed into law in 1996 and amends sections of both FFDCA and FIFRA.[78] It is intended to establish more consistent regulation of pesticides and to protect human health using an approach that places increased emphasis on the scientific evaluation of pesticide safety data. Among other provisions affecting pesticide registration and tolerance setting, it (1) mandates special considerations for protection of infants and children, (2) requires determination of aggregate pesticide exposure from all sources (e.g., food, home use, and drinking water), (3) requires summation of exposures from multiple chemicals that exhibit a common mechanism of toxicity, (4) expedites approval of pesticides considered to be most safe, and (5) requires periodic reevaluation of tolerances for registered pesticides to be certain that the data supporting registration remain acceptable and complete by current standards.

Toxic Substances Control Act

TSCA is administered by the Office of Pollution Prevention and Toxics within EPA and is a complex and far-reaching law that affects industrial chemicals existing in commerce as well as new chemicals in the United States.[79] One of the first requirements of TSCA was the compilation of an inventory of chemicals that were active in commerce in the years 1977–1979 and the determination of the need for toxicity testing of these existing chemicals.

Manufacturers or importers of industrial chemicals that are considered new under the TSCA definition are required to notify the EPA at least 90 days before the manufacture or import of a new chemical. The act requires that certain information regarding the new chemical be submitted to the EPA for review in a premanufacture notification (PMN). While the act does not require toxicity testing to be conducted on a new chemical prior to manufacture, it does require submission of all existing health and safety data for the new chemical so that its risk to health or the environment can be assessed. If the EPA determines in its review that the new chemical does present an unreasonable risk, one of several actions it may take is to require that the chemical be tested for specific toxic effects. The EPA may also issue a testing rule requiring that a specified chemical or chemical mixture be tested for certain toxic effects. The EPA has issued test standards for the conduct of toxicity studies on chemicals or chemical mixtures for which testing will be required under TSCA. These standards are the same guidelines used for pesticide testing under FIFRA.[8]

Transportation Act

Regulations promulgated by the Department of Transportation (DOT) require that materials shipped in interstate commerce be labeled and contained in a manner consistent with the degree of hazard they present.[80] The DOT requires that acute toxicity data for chemicals be used to place them into *packing groups*. Labeling requirements for chemicals are based upon this packing group.

Coast Guard

Prior to importing a chemical into the United States, the Coast Guard requires a set of acute mammalian toxicity data for the chemical.[81] This acute toxicity profile should minimally include the following: acute oral toxicity, acute dermal toxicity, and skin and eye irritation studies.

Consumer Product Safety Act

Prior to passage of the Consumer Product Safety Act, the classification and testing for acute toxicity of household products was conducted under regulations promulgated by the FDA, which administered the Federal Hazardous Substances Act (FHSA). The function of administering FHSA now resides with the Consumer Product Safety Commission. If results obtained in acute oral or dermal toxicity tests conducted using methods outlined in the Code of Federal Regulations (CFR) for hazardous substances meet prescribed criteria of toxicity, labeling and packaging as prescribed in the regulation must be used.[82]

Occupational Safety and Health Act

This law, administered by the Occupational Safety and Health Administration (OSHA) of the Department of Labor, is designed to ensure safety in the workplace.[83] No requirements exist in this law for manufacturers to test substances for toxicity prior to their use in the workplace. The impact of the TSCA has an overlapping effect in that occupational exposure to new chemicals is considered in premanufacture notices. As indicated previously, specific test requirements under TSCA affect new or existing chemicals that are manufactured or processed in the United States.

Resource Conservation and Recovery Act

Resource Conservation and Recovery Act (RCRA) authorizes the EPA to institute a national program to control hazardous waste defined as "solid, liquid, semi-solid or gaseous waste that may cause increased mortality or serious illness, or may cause substantial hazard to the health or the environment when improperly managed."[84] The main purpose of these regulations is to control the generation, storage, treatment, transportation, disposal, record keeping, and reporting of hazardous waste. RCRA places the primary responsibility of identifying and managing hazardous waste on the waste generators. Other persons or institutions involved in waste disposal and management also have an obligation to know if the waste is hazardous. The degree of toxicity, concentration, migration to the environment, persistence or degradation in the environment, bioaccumulation in the ecosystem, types of improper management, quantities of waste, past human and environmental damage records, and other factors are all considered.

International Laws and Regulations Concerning Hazard Assessment and Toxicity Testing Guidelines

The United States has not been alone in developing laws to protect humans and their environment from possibly dangerous effects of new industrial chemicals in the marketplace. In the European Union, the Council of Ministers of the European Economic Community (EEC) has issued a directive concerning laws, regulations, and administrative procedures that relate to the classification, packaging, and labeling of dangerous substances.[85] An amendment to this directive was adopted later to protect humans and their environment from potential risks that might arise through the marketing of new chemicals.[86] It requires that a new chemical be subjected to a base set of tests to define its physical and chemical properties, mammalian toxicity, and ecotoxicologic effects. The base set of mammalian studies consists of the following: acute oral LD_{50}, acute dermal LD_{50}, acute inhalation LC_{50} (if applicable), skin and eye irritation, and dermal sensitization. A manufacturer or importer is required to furnish the appropriate authorities in his EEC member state with a notification containing, in part, the results of these tests with the new chemical. Such notification must be filed 45 days before marketing in the member state in which it is to occur.

The amendment to the EEC directive is the counterpart of the U.S. TSCA. There are, however, some differences, not the least of which is in the approach to toxicity testing. The EEC requires only notification of a new chemical prior to marketing whereas TSCA demands that the notification, in the case of a domestic manufacturer, be given to the EPA at least 90 days before manufacture.

The EEC has also provided guidance on the evaluation of safety and efficacy of drugs. The EEC has now adopted guidance notes for efficacy, testing of pharmacokinetics in humans and bioavailability of drugs for long-term use, as well as a number of more specific activity groups including cardiac glycosides, oral contraceptives, topical corticosteroids, nonsteroidal anti-inflammatories, antimicrobials, anticonvulsants, antianginals, and chronic peripheral arterial disease agents. Safety guidance notes also were adopted for single-dose and repeated-dose toxicities, pharmacokinetic metabolism, mutagenic potential, carcinogenic potential, and reproduction studies.[87]

As discussed earlier in this chapter, with many countries establishing their own regulations for safety assessment, it became more likely that manufacturers would have to perform several different versions of the same tests to satisfy the requirements of different countries. In an attempt to avoid unnecessary and wasteful duplication of work, the OECD, a group comprised of experts from a number of nations throughout the world, produced a set of toxicity testing guidelines that enable tests to be carried out in a similar manner in different countries.[9] The OECD package, which has been revised 10 times to implement improvements to individual study designs, includes guidelines on acute oral, dermal, and inhalation studies; eye and skin irritation and skin sensitization studies; subchronic oral, dermal, and inhalation studies; and teratogenicity, carcinogenicity, and chronic and combined chronic/carcinogenicity studies. Results from studies conducted according to OECD guidelines are generally fully acceptable to the various regulatory bodies throughout the world.

In order to facilitate more universal acceptance of data generated to support approval and use of pharmaceutical products, the ICH has developed guidance documents concerning the efficacy, quality, and safety of these drugs. ICH is made up of regulatory authorities and trade organizations from the United States, Japan, and the European Union. Instead of providing detailed instructions for study design or conduct, the ICH guidance addresses specific issues related to testing, for example,

TABLE 24.17
Internet Addresses for Regulatory Websites

Regulatory Subject	Site Address (http://)
International	
European Union	www.eurunion.org
EU General Product Safety Directive	ec.europa.eu/consumers/cons_safe/gpsd/index_en.htm
Organization for Economic Cooperation and Development	www.oecd.org
OECD Testing Guidelines	www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html
International Conference on Harmonization	www.ich.org
ICH Guidance Documents	www.ich.org/products/guidelines.html
United States	
Food and Drug Administration	www.fda.gov
FDA Center for Food Safety and Applied Nutrition	www.fda.gov/AboutFDA/CentersOffices/OrganizationCharts/ucm135675.htm
FDA Center for Drug Evaluation and Research	www.fda.gov/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDER/default.htm
FDA Center for Devices and Radiological Health	www.fda.gov/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDRH/default.htm
Environmental Protection Agency	www.epa.gov
EPA Office of Pollution Prevention and Toxics	www.epa.gov/oppt/
EPA Office of Pesticide Programs	www.epa.gov/pesticides
Toxic Substances Control Act	www.epa.gov/lawsregs/laws/tsca.html
Federal Insecticide, Fungicide, and Rodenticide Act	www.epa.gov/oecaagct/lfra.html
Federal Food, Drug, and Cosmetic Act	www.fda.gov/opacom/laws/fdcact/fdctoc.htm
Food Quality Protection Act	www.epa.gov/opp00001/regulating/laws/fqpa/
Food and Drug Administration Modernization Act of 1997	www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/SignificantAmendmentstotheFDCAct/FDAMA/default.htm
EPA Testing Guidelines	www.epa.gov/ocspp/pubs/frs/home/guidelin.htm
FDA Guidance Documents	www.fda.gov/regulatoryinformation/guidances/default.htm
FDA, EPA, and OECD GLP Comparison Chart	www.fda.gov/downloads/ICECI/enforcementactions/bioresearchmonitoring/UCM133724.pdf

the duration of chronic toxicity testing, definition of an acceptable battery of genotoxicity studies, and required elements of chemical stability testing of new drug products.[88] Because of ICH efforts, many hindrances to the approval and use of valuable pharmaceuticals around the world have been removed.

Regulatory Internet Sites

Many of the regulatory agencies and organizations worldwide that have responsibility for protection of humans from the hazardous effects of chemicals maintain Internet websites. These websites usually include information concerning the history, structure, and specific responsibilities of these organizations. The sites also generally contain or reference the statutes and guidelines under which the organizations operate. Internet addresses for a number of informative regulatory sites are listed in Table 24.17.

QUESTIONS

24.1 Five related compounds are under consideration for development by a pharmaceutical company named Nomohats Ltd. The compounds are hair regrowth agents. The intended treatment population includes males and females from 24 to 90+ years of age. All of the compounds have been thoroughly characterized chemically, and each has shown adequate efficacy following oral exposure during preliminary testing in animals. The effects of oral exposure in single-dose acute studies were similar for each of the compounds and none of the compounds were genotoxic in preliminary screens. Nomohats has budgeted funding to allow the development of one of the compounds. You are the toxicologist responsible for generating additional toxicity data to assist in selecting the compound to be carried forward in the development program. You have 2 months because a meeting with FDA has been scheduled by Nomohats 10 weeks from today. The purpose of the meeting is to review the chemical characterization and preliminary toxicity data for the compound and to suggest a toxicology program to demonstrate that there is no unreasonable risk-associated human exposure to the compound. Describe the study(ies) that you would conduct to aid in the selection of the compound for further development, remembering that these studies will also generate most of the new toxicity data to be reviewed during the meeting with FDA.

TABLE 24.18
Selected Results from the Subchronic Toxicity Study of *Fuzzypate* in Minipigs

	Compound Group: Males				Compound Group: Females			
Variable/Finding/Interval	**Control**	**Low**	**Mid**	**High**	**Control**	**Low**	**Mid**	**High**
Body weight (kg)	9.3 ± 0.7	8.6 ± 1.5	10.2 ± 0.6	7.2 ± 1.1[a]	9.9 ± 1.2	10.1 ± 0.9	9.2 ± 0.7	8.1 ± 0.6[a]
Hepatocellular hypertrophy (no. with finding/total no.)								
Week 4	0/4	1/4	0/4	2/4	1/4	0/4	1/4	1/4
Week 13	1/6	1/6	2/6	3/6	0/6	2/6	1/6	1/6
Renal tubule hyperplasia (no. with finding/total no.)								
Week 4	1/4	0/4	4/4	4/4	0/4	1/4	2/4	2/4
Week 13	1/6	1/6	2/6	6/6	0/6	0/6	3/6	4/6

[a] Statistically significantly different from controls ($p < 0.05$).

24.2 After consulting with the FDA, you have determined that the hazard assessment program for your compound, *Fuzzypate,* should include subchronic toxicity testing. In a 14-day repeated-dose study with this compound in Göettingen minipigs at 5 mg/kg/day, dermal administration resulted in renal toxicity in 40% of the animals. These animals also exhibited mildly decreased body weight gain, urinary incontinence, and slightly enlarged and discolored kidneys. In the mid-dose group, 2.5 mg/kg/day, no renal toxicity was reported, and body weight of the animals was slightly lower than the untreated control group. At 1 mg/kg/day, only a statistically significant increase in serum levels of liver enzymes was observed. Efficacy testing revealed that *Fuzzypate* is effective for its intended use at 0.2 mg/kg/day. Pharmacokinetic determinations indicate that the material is excreted almost entirely in the urine and its serum half-life is 120 min. What dose levels would you select for the subchronic toxicity study? Why? In addition to the standard parameters, what special endpoints would you include for investigation during the subchronic study?

24.3 The data in Table 24.18 above were obtained during the subchronic study with *Fuzzypate*. In what dose groups is there a compound-related effect on body weight? Are liver and kidney target organs for *Fuzzypate* toxicity? If so, in which dose group(s) is there a compound-related effect? Based on the data in the table, what is the NOAEL for this study?

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25 Carcinogenicity of Chemicals
Assessment and Human Extrapolation

Gary M. Williams, Michael J. Iatropoulos, Harald G. Enzmann, and Ulrich F. Deschl

CONTENTS

CHEMICALS WITH CARCINOGENIC ACTIVITY

Cancer is a leading cause of morbidity and mortality in the developed parts of the world and in many other regions [210,232]. The etiologies of most types of cancer are not known, but some are caused by exogenous chemicals, for example, lung cancer and cigarette smoke [210] and liver cancer and aflatoxin B_1 [210]. Accordingly, in the toxicological assessment of chemicals, testing for carcinogenic activity constitutes a critical component and is required under numerous governmental regulations or agreements (Table 25.1). A large database on the carcinogenic, or oncogenic, activities of chemicals in rodents has accrued [147,148,149,316] as a result of almost 100 years of basic research, as well as the output from national testing programs in several countries, particularly the United States and Japan, and regulatory studies conducted by the pharmaceutical, chemical, food, and cosmetic industries. In the United States, under the aegis initially of the National Cancer Institute (NCI) and subsequently the National Toxicology Program (NTP) [474], results of cancer bioassays in rodents, mainly mice and rats, have been reported for over 650 chemicals [324], and further testing is ongoing.

The definitive bioassay for carcinogenic activity in animals is the rodent cancer bioassay (RCB), which in its various forms is detailed here and in the previous version of this chapter [492], as well as elsewhere [177,199,200, 300,447,448,476]. The current method for the RCB is exemplified by that presently in use by the NTP [33]. This method was refined from basic procedures developed beginning in the 1960s largely by the pharmaceutical industry under guidance from the U.S. Food and Drug Administration (FDA) and by the NCI Bioassay Program [67,68,84,474]. The scientific basis for the procedures used has been recently reviewed [177]. Findings from RCB on a wide variety of chemicals have led to the recognition that results cannot be unquestioningly extrapolated to humans [305,476], in which, of course, the carcinogenic activity of a chemical is established only by epidemiological studies, involving the application of rigorous criteria [4,182,416]. Available mechanistic procedures discussed below (in the Systematic Approach to Testing section) assist in the assessment of RCB results for potential human hazard.

TABLE 25.1
Regulations, Agreements, or Their Latest Amendments and Recommendations for Carcinogenicity Testing

Legislation/Guidance	Agency	Agents of Concern
Commission Directive 318/EEC (1975) and further updates (2003)	EU	Registration of new human medicines in Europe
Commission Directive 414/EEC (1991)	EU	Plant protection products
Pesticide Registration Directive (1991)	EU	Pesticides
Commission Directive 67/EEC (1993)	EU	New notified substances
Commission Regulation 1488/EEC (1994)	EU	Existing substances
Dangerous Substances Directive (1967; amended 1992)	EU	Industrial chemicals
Commission Directive 27 (2004)	EU	Registration of new human medicines
Commission Directive 28 (2008)	EU	Registration of veterinary medicines
Commission Directive 24 (2004)	EU	Registration of herbal medicines
Federal Hazardous Substances Act (1960; amended 1988)	U.S. CPSC	Household products
Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (1948; amended 1978)	U.S. EPA	Pesticides
Toxic Substances Control Act (TSCA) (1976; amended 1992)	U.S. EPA	Hazardous chemicals not covered by other laws, includes premarket review
Food, Drug, and Cosmetics Act (1906, 1938, amended 1992)/FDA Redbook II (1993) [447]	U.S. FDA	Food, medicines, cosmetics, food additives, color additives, new drugs, animal and feed additives, medical devices
Guidance on Toxicology Study Data for Application of Agriculture Chemical Registration (1985)	MAFF (Japan)	Agricultural chemicals
Guideline for Toxicity Testing of Chemicals (1990)	MHW (Japan)	Chemicals
Pharmaceutical Affairs Law (1980)/Guidelines for Toxicity Studies of Drugs Manual (1990)	MHW (Japan)	Medicines
Guidelines for Toxicity Studies of New Animal Drugs (1988)	MHW (Japan)	Animal medicines
Technical Requirements for the Registration of Pharmaceuticals for Human Use (1995)	ICH	Medicines
S1A. Need for Carcinogenicity Studies of Pharmaceuticals (1995)		
S1C (R2). Dose Selection for Carcinogenicity Studies of Pharmaceuticals (2008)		

Note: EEC, European Economic Communities; EU, European Union; ICH, International Conference on Harmonization; MAFF, Ministry of Agriculture, Forestry, and Fisheries; MHW, Ministry of Health and Welfare; U.S. CPSC, United States Consumer Product Safety Commission; U.S. EPA, United States Environmental Protection Agency; U.S. FDA, United States Food and Drug Administration.

Chemical Carcinogenic Activity

Carcinogenicity is not an intrinsic property of a chemical; rather, it is an activity of the chemical under specific conditions. Carcinogenic activity has been defined in various ways. A committee of the International Federation of Societies of Toxicologic Pathologists (IFSTP) adopted the definition of a chemical carcinogen as a "substance that causes a cell or group of normal cells, which would not otherwise have shown this property, to change its biological behavior and demonstrate progressive growth of a malignant character" [118]. In an RCB, the carcinogenic activity of a test substance (TS) is best documented by the finding of unequivocal evidence in either sex of the experimental animal of induction of a type(s) of malignant neoplasm not seen in contemporary controls [492,500,513], and above that recorded in databases of background neoplasms (see Table 25.7), thereby indicating *ab initio* induction of neoplasia. A marginal increase in a very rare malignancy also can incriminate a TS. Another generally used criterion is an increase in the incidence of a type of malignant neoplasm present in controls, especially if the incidence(s) in dosed animals exceeds the range of historical controls. The malignant neoplasm can be of any histological type, epithelial or mesenchymal, and, although a clear increase in the incidence of a malignant neoplasm is most persuasive, an increase in the combined incidence of benign and malignant neoplasms of the same cell type of origin, where the latter is not significantly increased, is generally accepted as reflecting carcinogenic activity [198,290]. The evidence of malignancy is best established by the presence of invasion or metastasis. For most rodent neoplasms, however, the diagnosis of malignancy is typically made histologically on evidence of cellular atypia. This can be problematic since some diagnoses are controversial, as discussed in the following sections on anatomic pathology and cancer hazard evaluation. The finding of an increase in only benign neoplasms, especially if the type of neoplasm is not established to be premalignant, does not constitute sufficient evidence for carcinogenicity, but does provide some limited evidence. In addition to these criteria, an increase in the multiplicity of neoplasms above that in controls or a reduction in the latency period for the development of neoplasms has also been considered [513]. Although these latter findings can indicate an influence of the chemical or stress/toxicity produced by it on the development of neoplasia, they can reflect modulation of host control of background neoplasia and are less definitive evidence of carcinogenic activity. These criteria apply to findings in any species, strain, or sex. An effect in more than one sex or species, in more than one dose group or in independent experiments, of course, strengthens the evidence. For regulatory RCB, assessment of results is ultimately made by the responsible expert body using criteria deemed appropriate (see Assessment and Classification of Evidence of Carcinogenicity section), a process often influenced by a high level of concern for hazard identification in the interests of public health protection. It must be kept in mind that carcinogenicity in a rodent study is not proof of a cancer hazard to humans, as discussed below. Ultimately, in assessing human hazard (which is the reason why animal tests are done), scientific judgment is used in evaluating all the information available, including, importantly, mechanism (or mode) of action, including any known pharmacologic activity.

Types of Carcinogens

A wide variety of organic and inorganic chemicals has exhibited carcinogenic activity in rodents [147,148,149,201,206,207]. This diversity reflects the fact that the multistep process of

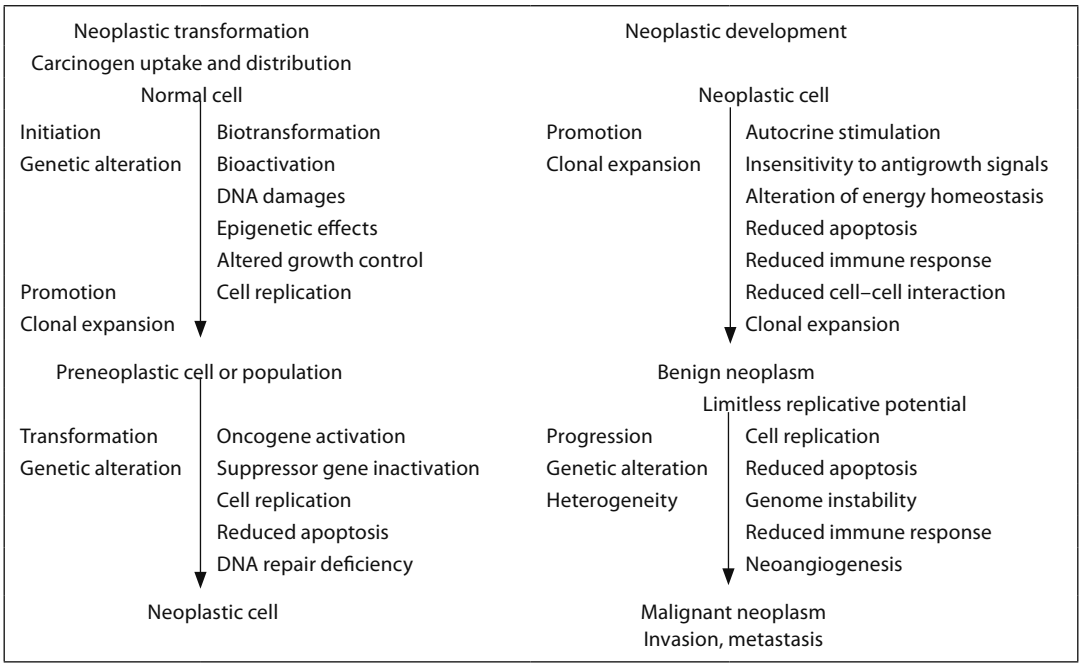

FIGURE 25.1 Sequence of oncogenesis.

oncogenesis (Figure 25.1) can be influenced by chemicals in various ways, mainly involving either chemical reactivity and genotoxicity as a key event in effecting neoplastic transformation (initiation [IN]) or epigenetic effects leading to either neoplastic transformation or enhancement of cell growth facilitating neoplastic development (promotion [PM]). Thus, chemicals can give rise to increases in neoplasms through a variety of modes of action (MOAs) that have been broadly characterized as either DNA reactive or epigenetic (Tables 25.2 and 25.3) [488,492,497]. A DNA-reactive carcinogen can be defined as an agent that reacts with DNA in the target somatic cell of carcinogenicity under conditions of carcinogenicity to produce mutations that lead to neoplastic transformation and IN of neoplasia [364]. The critical genes affected are known as oncogenes and tumor suppressor genes [43]. An epigenetic carcinogen can be defined as an agent that under conditions of its carcinogenicity produces a non-DNA-reactive cellular effect in the target tissue of carcinogenicity, which either indirectly results in DNA alteration (e.g., DNA oxidation, aberrant methylation) leading to a procarcinogenic mutation or facilitates the

TABLE 25.2
Classification of Substances with Experimental Carcinogenic Activity

A.	DNA-reactive chemicals	
	1. Activation independent	Alkylating agents: nitrogen mustards, ethylnitrosourea, cyclophosphamide
		Epoxides: ethylene oxide
	2. Activation dependent	Aliphatic halides: vinyl chloride
		Aromatic amines, aminoazo dyes, and nitro-aromatic compounds: *o*-toluidine, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP), polycyclic 4 aminobiphenyl, benzidine, dimethylaminoazobenzene, 1-nitropropane (nitroalkane)
		Polycyclic aromatic hydrocarbons: benzo(*a*)pyrene
		N-nitroso compounds: dimethylnitrosamine, *N*-nitrosonornicotine
		Hydrazine derivatives: 1,2-dimethylhydrazine, azoxymethane, methyl-azoxymethanol
		Mycotoxins: aflatoxin B1, pyrrolizidine alkaloids, ochratoxin
		Pharmaceuticals: chlorambucil, tamoxifen
		Triazines (diazoamino compounds): 3,3-dimethyl-1-phenyltriazene
B.	Epigenetic chemicals	
	1. Promoter	Liver enzyme-inducer-type hepatocarcinogens: chlordane, DDT, pentachlorophenol, phenobarbital, polybrominated biphenyls, polychlorinated biphenyls
		Urothelial cell proliferation enhancers: saccharin
	2. Endocrine-modifier	Estrogenic hormones: estrogens, diethylstilbestrol, and hormone modifiers atrazine and chloro-*S*-triazines
		β_2-Adrenoreceptor agonists in female rats: soterenol, salbutamol
		Antiandrogens: finasteride, vinclozolin
		Antithyroid thyroid tumor enhancers: thyroperoxidase inhibitors (amitrole, sulfamethazine); thyroid hormone conjugation enhancers (phenobarbital, spironolactone)
		Gastrin-elevating inducers of gastric neuroendocrine tumors: omeprazole, lansoprazole, pantoprazole, alachlor, butachlor
		Neuroleptics (dopamine inhibitors), gonadotropin-releasing-hormone-like drugs (goseline)
	3. Immunomodulator	Purine analogues: azathioprine
		Cyclosporine
	4. Cytotoxin	Forestomach toxicants: butylated hydroxyanisole, propionic acid, diallyl phthalate, ethyl acrylate
		Nasal toxicants: chloracetanilide herbicides (alachlor, butachlor)
		Renal toxicants: potassium bromate, nitrilotriacetic acid
		Male rat $\alpha_{2\mu}$-globulin nephropathy inducers: D-limonene, *p*-dichlorobenzene
	5. Peroxisome proliferator-activated-receptor α/γ agonist	Hypolipidemic fibrates: ciprofibrate, clofibrate, gemfibrozil
		Phthalates: di(2-ethylhexyl) phthalate (DEHP), di(isononyl) phthalate (DINP)
		Miscellaneous: lactofen
	6. Inducer of urine pH extremes	Melamine, dietary phosphates, carbonic anhydrase inhibitors
C.	Inorganic compounds[a]	
	1. Metal or metal salt	Beryllium, cadmium, chromium, nickel, silica
	2. Fiber	Asbestos
D.	Unclassified	Acrylamide, acrylonitrile, benzene, dioxane, dioxin, furan, methapyrilene, nucleoside analogues (entecavir, zidoriudine, zalcitabine)

[a] Some are categorized as genotoxic because of evidence for damage of DNA; others may operate through epigenetic mechanisms such as alterations in fidelity of DNA polymerases.

TABLE 25.3
Classification of Chemicals and Mixtures Considered Carcinogenic to Humans by the International Agency for Research on Cancer

Aflatoxins	**Melphalan**
DNA reactive	
4-Aminobiphenyl	MOPP (nitrogen mustard, vincristine, procarbazine, and prednisone)
5-Azacytidine	2-Napththyamine
Benzidine	Nickel and nickel compounds
Betel quid with tobacco	Phenacetin-containing analgesic mixtures
bis(Chloromethyl)ether	Soot
1,4-Butanediol dimethanesulfonate (myleran)	Sulfur mustard
Chlorambucil	Tobacco smoke and products *o*-Toluidine
1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (methyl-CCNU)	
Chromium compounds, hexavalent	Triethylethiophosphoramide (thiotepa)
Coal tars	Vinyl chloride
Cyclophosphamide	
Diesel engine exhaust	
Ethylene oxide[a]	
Epigenetic	
Azathioprine	Oral contraceptives
Cyclosporine	Tamoxifen
Diethylstilbestrol	2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin (TCDD)[a,b]
Estrogens, steroidal, conjugated	
Unclassified	
Alcoholic beverages	Mineral oils
Arsenic and arsenic compounds; gallium arsenide	Shale oils
Benzene	

Source: IARC, *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, International Agency for Research on Cancer, Lyon, France, 1997.

[a] Based on evidence for a relevant mechanism in humans.

[b] Based on overall increase in neoplasia.

development of a normal or preexisting preneoplastic cell into a neoplasm. A DNA-reactive carcinogen can also produce epigenetic effects, usually at higher doses than required for DNA reactivity, which facilitate neoplastic development.

The types of organic chemicals that can be assigned to these two categories of carcinogens, as well as inorganic carcinogens, are given in Table 25.2 and have been discussed in detail elsewhere [477,497]. Carcinogens are both naturally occurring (e.g., aflatoxins) and synthetic (e.g., vinyl chloride).

Of the many chemicals with carcinogenic activity in RCB, few are associated with cancer in humans, as assessed by the World Health Organization (WHO) and International Agency for Research on Cancer (IARC) (Table 25.3). Thus, the specificity of the RCB is obviously low, detecting many *rodent-only* carcinogens, whereas its sensitivity is high in that it can detect all substances that really are established to be carcinogenic in humans. Most of the known human carcinogens are of the DNA-reactive type [500], indicating the importance of this MOA in human hazard. As a further indication of the significance of this MOA, several DNA-reactive carcinogens have been carcinogenic transplacentally in rodents [373] and in primates [418]. Human contacts with, or intake of, DNA-reactive carcinogens occur mainly with tobacco use and as contaminants in food, and also in the workplace or as therapeutic interventions [477,515]. Human contacts with these types of carcinogens are usually substantial compared to those resulting from trace levels of environmental chemicals (e.g., pesticides). A variety of neoplasms is induced in humans by such chemicals, including notably lung (tobacco smoke, bischloromethyl ether), urinary bladder (benzidine, tobacco smoke), hematopoietic system (benzene, chlorambucil), and liver (aflatoxin B_1, vinyl chloride). The few human epigenetic carcinogens [500] are mainly pharmaceuticals, and these are associated with cancer increases only at therapeutic doses that produce the cellular effect that underlies their carcinogenicity in rodents, mainly hormonal perturbation or immunosuppression. With hormonal agents, responsive target tissues include endometrium (e.g., tamoxifen), while with immunosuppression, risk of lymphatic tissue neoplasia (e.g., cyclosporine) is greatly increased. Thus, a

primary objective of cancer hazard assessment is to identify chemicals with DNA reactivity and those with epigenetic effects known to be relevant to human hazard.

Because of the significance of DNA reactivity and the reliability with which it can be identified, agents of this type are generally not considered for uses in which there is any intentional human contact, apart from chemotherapeutic alkylating agents. In contrast, agents that elicit rodent neoplasm increases by epigenetic MOA are widely used; for example, as of 1995, more than 80 approved medicines were oncogenic in RCB [82], and the number has increased [18]. Generally, epigenetic carcinogens have not been carcinogenic in primates [398,418]. An important aspect of safety assessment is to elucidate any MOA that might underlie an increase in neoplasia in an RCB to guide mechanistic research for informed hazard evaluation [505].

Potency

The magnitude of carcinogenic activities in rodents of chemicals with respect to dose varies more than 10 million-fold [147]. The most extensive compilation of quantitative indices of carcinogenic potency is the Carcinogenic Potency Database [147,148], which uses TD_{50} values as its metric, defined as the daily dose rate required to halve the probability of an experimental animal remaining tumor free at the end of its standard life span [354]. A simplified method of expressing potency proposed for use in the regulatory setting in Europe is the TD_{25}, defined as the chronic dose rate in mg per kg body weight per day, which will cause neoplasia in 25% of the animals at a specific site, after correction for the spontaneous incidence, within the standard lifetime of that species [97]. Potency can also be reflected in reduced latency or increased multiplicity of neoplasms compared to controls. Specifically, for DNA-reactive carcinogens, a general, but not exact, relationship exists between carcinogenicity and DNA binding, which differs over several orders of magnitude [343,346] reflecting the fact that not all DNA binding is necessarily mutagenic [462] or carcinogenic [326].

REQUIREMENTS FOR TESTING

Testing of chemicals in experimental animals for carcinogenic activity is done to assess potential human cancer hazard under conditions in which humans might have contact with the chemical. The U.S. federal government has enacted numerous laws that establish requirements for carcinogenicity testing of substances for which human contact or environmental release occurs (Table 25.1). Other countries have similar provisions (Table 25.1). The requirements for testing of industrial chemicals in the United States and the European Union (EU) have been undergoing revision.

Food substances generally require technical evidence of safety [461]. The procedures have been detailed by the U.S. FDA [447]. Food substances, such as flavors, many of which are naturally occurring and which are used at extremely low levels, if evaluated by a group of qualified experts can be deemed "generally recognized as safe" and do not require premarket approval [461].

Cosmetics usually do not require carcinogenicity testing. Moreover, under the seventh amendment to the EU cosmetics directive [113], since 2009, the use of animals in the safety assessment of cosmetic ingredients is not permitted [165].

For pharmaceuticals, circumstances requiring carcinogenicity studies have been agreed upon by the International Conference on Harmonization (ICH), a tripartite body comprised of regulatory scientists from North America, Europe, and Japan. The requirements are available on the Center for Drug Evaluation and Research (CDER) website (http://www.fda.gov/cder). In general, candidate medicines with positive genotoxicity findings, or which are intended for chronic use (>6 months), usually require carcinogenicity testing.

The requirements for the testing of biopharmaceuticals present specific issues [395] for which the FDA has provided guidance (http://www.fda.gov/cber). Several nucleoside analogues have been found to be carcinogenic in rodents [515], possibly through perturbation of nucleotide pools leading to errors in DNA synthesis [357]. Growth factors and immunosuppressive antibodies are noted as raising concern for carcinogenic potential. Normal hormones or growth factors that are intended to correct deficiency states (e.g., insulin) may not need to be tested, unless they are structurally modified, are administered at doses exceeding the physiological levels, or are given by a route that results in substantially increased exposure in some tissues. Modified proteins with new biologic properties require testing, because, for example, it was found that a modified insulin [408] with an increased affinity for the insulin-like growth factor 1 (IGF-1) receptor produced mammary tumors in rats [92]. Also, a portion of parathyroid hormone with bone trophic activity produced bone neoplasms in rats [356]. Certain modifications of proteins to improve bioavailability, such as with conjugation to polyethylene glycol, have not raised carcinogenicity issues.

An aspect of regulatory concern is potential photochemical carcinogenicity [188], discussed below in the section Photochemical Carcinogenesis. Indications for the possible need for photochemical carcinogenicity testing include (1) long-term exposure of the skin to a chemical that can undergo photoactivation, (2) alteration of the structure of the epidermis, (3) sensitization of the skin to ultraviolet radiation (UVR), and (4) exacerbation of suspected UVR-induced carcinogenesis [454].

Nanosized particles (NSPs; <100 nm) are increasingly being used in medical and other applications [21,27,115,286,301,460]. NSP can have inflammatory, prooxidant, and antioxidant activities [286,333], which raise concern. Several regulations require the toxicity assessment of NSP products [115,460].

Thus, for the uses of many types of chemicals, RCBs are required, usually in both rats and mice. For chemicals with negligible human contact, similar to food contact materials [340], application of the threshold of toxicological concern (TTC) (see Risk Assessment) can be utilized [25,136,264,314,451]. Also, expedited approaches have been proposed to provide the assessment of potential carcinogenic activity rapidly and economically, with minimal use of

experimental animals, in order to delineate potential hazard before extensive development or significant contacts with humans takes place. These are discussed in the next section Systematic Approach to Testing.

SYSTEMATIC APPROACH TO TESTING

The goal of a systematic approach to testing is to obtain reliable data enabling the evaluation of the TS at the earliest possible stage for potential human hazard. An approach that incorporates the mechanistic concepts described earlier is the decision point approach (DPA) [475,476], which is presented here as a general framework for the concept (Table 25.4). Such an expedited approach to testing can assist in prioritizing from the large reservoir of existing untested chemicals and in selecting for progression new molecular (or chemical) entities, both small and large (e.g., protein) molecules.

The endpoints detailed later provide guidance in identifying a potentially carcinogenic TS, but negative results do not preclude carcinogenicity, and, in any event, carcinogenicity testing ultimately may be required. Recently, an analysis of data on 182 pharmaceuticals reported that a positive finding for either genotoxicity, histopathological changes indicative of preneoplasia, or hormonal perturbation could be highly predictive of carcinogenic activity [400], supporting the value of critical data prior to carcinogenicity testing. A review of the FDA/CDER database of pharmaceuticals, however, revealed that short-term toxicity studies, including in transgenic mice, do not accurately and reliably predict neoplastic findings in long-term assays [225].

Implications of Chemical Structure (Stage A)

From the classes of DNA-reactive organic carcinogens given in Table 25.2, the types of electrophiles that are involved in chemical reactivity and hence DNA binding are well known (Figure 25.2). Such molecular features also have been referred to as structural alerts [12]. In general, a relationship exists between DNA binding and carcinogenicity [455,462], although not all DNA binding is necessarily mutagenic [462] or carcinogenic [326], since adducts can be unstable or involve sites on bases that are not involved in base pairing. Among both DNA-reactive and epigenetic carcinogens, numerous classes have common structural features within the class. The presence of one of these features in a new molecular entity of unknown carcinogenicity suggests potential activity. The FDA Center for Food Safety and Nutrition (CFSAN) has grouped food substances into classes by chemical structure, estimating their potential toxicity [447,450], similar to the Cranmer classes [75]. These structural classes are used for assignment to levels of concern. Substances with functional groups of high probable toxicity are assigned to Category C; substances of intermediate or unknown toxicity are assigned to Category B; and substances of low probable toxicity are assigned to Category A. The recognition of potential toxicity can provide a guide to potential epigenetic carcinogenicity. Several artificial intelligence systems (in silico) for assessing potential toxicities related to structures are available [43,69,257].

TABLE 25.4
Decision Point Approach in Carcinogen Testing

Stage A. Structure of chemical
1. Possible electrophiles
2. Relation to known carcinogens

Stage B. Short-term genotoxicity assays
1. In vitro: Bacterial mutagenesis; hepatocyte DNA repair test
2. In vivo*:* Micronucleus test

Decision Point 1: Evaluation of findings in stages A and B

Stage C. Assays for epigenetic effects
1. Cultured cells
   - Mitogenesis
   - Induction of cytochrome P450 (CYP)
   - Peroxisome proliferation
   - Gap junction protein downregulation
   - Inhibition of cell–cell communication
   - Hormone modulating effect
   - Altered gene expression
2. In vivo
   - Increased cell proliferation
   - Reduced cell apoptosis
   - Induction of cytochrome P450 (CYP)
   - Peroxisome proliferation
   - Hormone homeostasis disruption
   - Gap junction protein reduction
   - Enhancement of preneoplastic lesions
   - Immunosuppression
   - Altered gene expression

Decision Point 2: Evaluation of results from stages A through C

Stage D. In vivo assays
1. DNA reactivity
2. Limited bioassays
3. Preneoplastic lesions (rat liver, mouse skin, mouse lung, rat breast)
4. Transgenic mice

Decision Point 3: Evaluation of results from stages A to C and selected tests in stage D

Stage E. Carcinogenicity bioassays
1. Accelerated bioassays
2. Long-term bioassays

Decision Point 4: Final evaluation of all results and cancer hazard assessment

Short-Term Assays (Stage B)

In Vitro

A large number of short-term assays for various genetic endpoints is available [43,177,485,253], and all regulatory agencies have specific testing recommendations or requirements, which may extend beyond the intent to predict potential carcinogenicity. For pharmaceuticals, a core battery

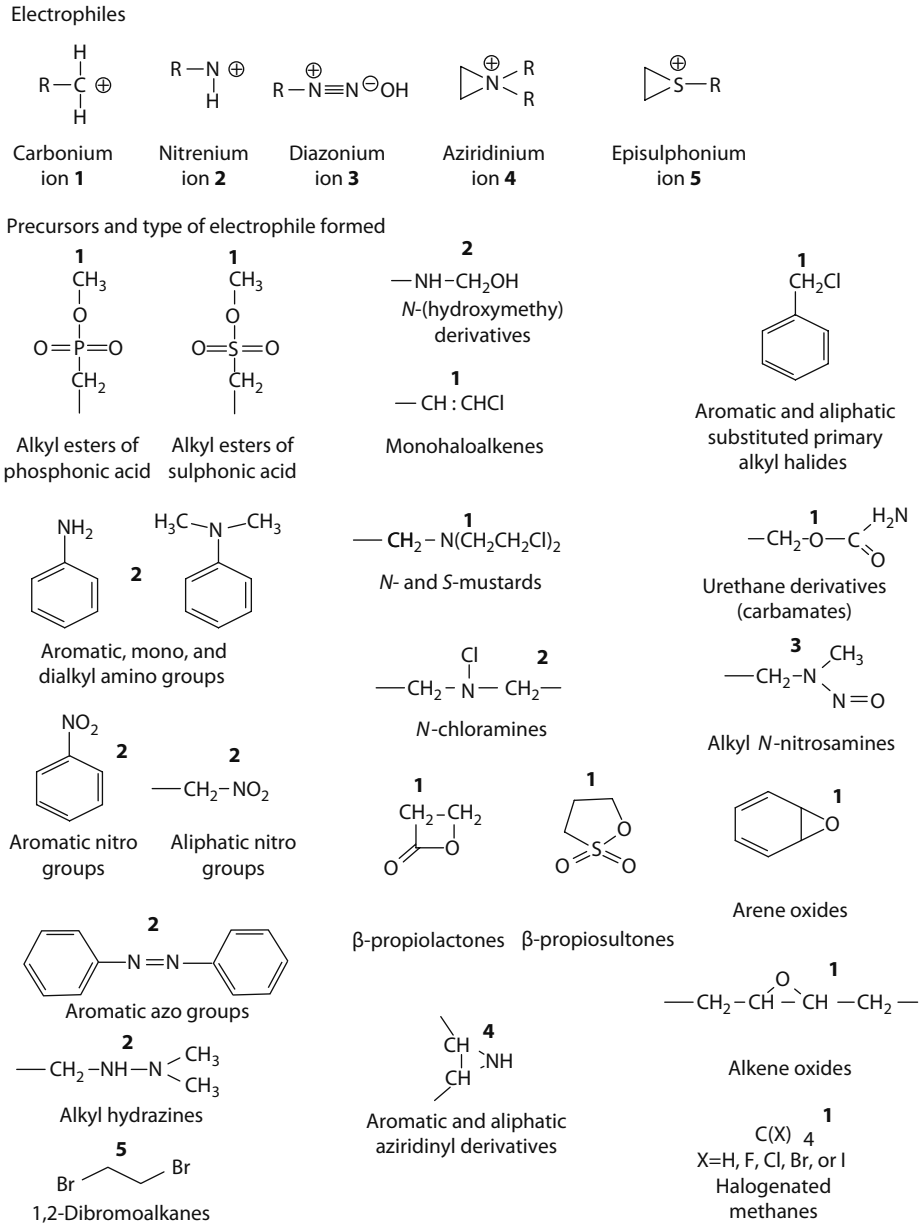

FIGURE 25.2 Structure of reactive electrophiles and precursors.

including both in vitro and in vivo assays has been agreed upon by the ICH [79,216]. For cosmetics, more extensive testing has been proposed in the EU [391], because animal testing of cosmetics was eliminated in 2009 [113]. Nevertheless, an expedited approach may still be possible [254]. These batteries generally include a bacterial mutagenicity assay (Ames), a mammalian cell mutagenicity/chromosome aberration assay, and an in vivo mutagenicity/chromosome aberration assay [252,43,216]. Each of these tests can yield false-positive and false-negative results in relation to carcinogenicity [253].

The predictivity of most genotoxicity assays (i.e., the percentage of positive chemicals that prove to be carcinogens) is limited largely to DNA-reactive carcinogens as a consequence of the fact that DNA alteration is the MOA of this class of carcinogen. Nevertheless, some assays, such as mammalian cell transformation, appear to respond to both DNA-reactive and epigenetic agents [269].

A bacterial mutagenicity assay [147] is required in all testing batteries and has reasonably high predictivity for carcinogenicity [43,519]. A substantial number of bacterial mutagens, however, are noncarcinogenic [251], for example, quercetin. Also, the NTP has found that certain in vivo effects, such as increased liver weight and hepatocellular hypertrophy (for both mice and rats) and hepatocellular necrosis (for mice), are better predictors for liver carcinogenesis than bacterial mutagenicity [9]. The ability of *Salmonella* mutagenicity to differentiate carcinogens is not increased by certain other standard in vitro assays, such as mammalian cell mutagenicity and chromosome aberration assays [137,519]. To reinforce

assurance of the predictiveness of a positive *Salmonella* mutagenicity finding in the DPA (Table 25.4, Stage B), another well-established assay, the hepatocyte/DNA repair synthesis assay [504], can be used. Positive results in the two assays in one substantial data set provided essentially perfect predictivity for the detection of DNA-reactive carcinogens [503]. Other assays for DNA damage include binding of radiolabeled TS, the alkaline single-cell gel electrophoresis (comet) assay [119], the nucleotide postlabeling (NPL) assay [366], and increases in the expression of DNA damage response genes [23]. The features of these assays have been reviewed [184]. The utility of hepatocytes is that they provide intrinsic bioactivation for both phase I and II metabolism. Also, hepatocytes from various species [295], including humans [296], can be used. Clear positive results in both of these assays, therefore, raise serious concerns for potential carcinogenicity. Other combinations of tests comprising the Ames and in vitro micronucleus tests have been found to be sufficient to detect rodent carcinogens and in vivo genotoxins [251].

Genetic toxicity studies unfortunately are not particularly informative for biopharmaceuticals because the large molecules are unlikely to enter the cells used in assays, particularly bacteria. Moreover, it is exceedingly improbable that such macromolecules would produce genetic effects. An issue with impurities is unlikely because chemical synthesis is not involved in most cases.

The FDA/CDER has issued a guidance for the integration of results from genetic toxicology studies [457]. If testing at this level, however, yields equivocal findings, in vivo assays for DNA reactivity can be used (see below).

In Vivo

In vivo genotoxicity assays are undertaken, as indicated in Table 25.4, at Stage D, if a suspicion of potential DNA reactivity for the TS has not been resolved by in vitro assays. Where possible, it is desirable to conduct these assays in rats, as most toxicity and pharmacokinetic studies will be performed in this species. Some assays included in recommended batteries, such as the bone marrow micronucleus assay [390], are not specific for DNA reactivity and assess only a single tissue, which in the case of bone marrow is of low chemical biotransformation capability. Moreover, systemic effects, such as hypothermia, hypoxia, and increased erythropoiesis, can yield positive findings [11,39,152,431]. DNA binding can be assessed if radiolabeled TS is available [278]; otherwise, assays for DNA damage, as mentioned earlier, that can be applied include the comet assay for DNA breakage [387], which is gaining acceptance [38], nucleotide postlabeling for adduct formation [355], and the in vivo/in vitro hepatocyte DNA repair assay [47]. Positive results in these DNA damage assays, if considered insufficient, direct the need for a radiolabeled chemical binding assay, which should include the demonstration of any adducted DNA base [387].

A model under development for in vivo detection of DNA damage uses avian eggs [352,511], which can also be used for the assessment of carcinogenic activity (see the following). An advantage of the in ovo model is that it employs an intact organism that is not a live animal and hence can be used for situations where use of animals is undesirable or precluded.

In addition to conventional in vivo mutagenicity assays, such as the bone marrow micronucleus assay and the bone marrow chromosome aberration assay, other models for in vivo mutagenicity include transgenic animals such as the Muta™ Mouse and Big Blue® transgenic constructs [43,309,514]. These latter assays allow for the detection of mutations in various tissues and can provide information on the molecular nature of induced mutations.

Assays for Epigenetic Effects (Stage C)

Histopathological findings in standard toxicity assays can indicate potential carcinogenicity. For example, liver enlargement is associated with a probability of liver carcinogenicity [9,54]. Specific assays for epigenetic effects (Table 25.4) are applied selectively depending on the properties of the chemical (i.e., structure, biologic/pharmacologic action, and toxicity). These assays provide evidence for an epigenetic mechanism that could result in an increase in neoplasms in rodents with long-term dosing. Many can be conducted in cultured cells, particularly hepatocytes, which provide intrinsic bioactivation. Even when applied in vivo, the experiments are of short duration, except for assays for promoting activity, the detection of which is described further in the Limited Carcinogenicity Bioassays section. Positive results indicate potential oncogenicity, in which case the potency relative to that of established carcinogens with a similar mechanism and organotropism provides a guide to hazard assessment. A particularly valuable endpoint to monitor is cell proliferation, because many nonspecific alterations in proliferation can lead to an enhancement of neoplasia [48,62].

Technologies are available for screening for effects on gene expression and function, including assays for RNA (genomics) and protein (proteomics) levels [1,18,43,91,248]. The increased expression of specific genes, such as hepatic acyl-CoA oxidase (increased by peroxisome proliferator–activated receptor-alpha (PPARα) liver carcinogens) or hepatic cytochrome P450s (increased by a variety of liver neoplasm promoters), is linked to epigenetic carcinogenesis, and thus these methods have utility for screening.

Limited Carcinogenicity Bioassays (Stage D)

Limited carcinogenicity bioassays (LCBs) are based on either neoplasms or established preneoplastic lesions as their endpoint [104,106,490]. These can be applied as IN assays, in which the TS is tested for its ability to induce the endpoint lesion, or as PM assays, in which the TS is administered after an agent that induces the endpoint lesion to determine the ability of the TS to enhance development of the lesion [423].

Neoplastic Initiation (IN)/Promotion (PM)

In early experimental studies of IN of skin carcinogenesis, IN was achieved with a single administration. Although this is possible with potent DNA-reactive agents, repeated exposure is

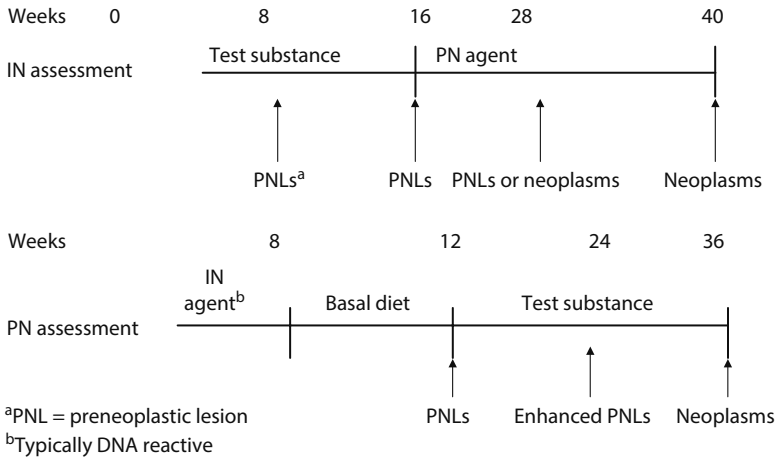

FIGURE 25.3 Limited bioassays for initiation and promotion in rodent liver.

required for an adequate assessment of IN by a TS. Because PM requires an even longer time for expression, more extensive dosing, up to 6 months, is required for an adequate test. Essentially, an assay for IN activity is directed largely toward assessing potential in vivo genotoxicity of the TS, whereas the assay for PM activity assesses an epigenetic MOA. Accordingly, assays for PM activity can also be deployed in the DPA at Stage C in vivo assays for epigenetic effects (Table 25.4).

An outline for IN and PM assays is shown in Figure 25.3. The most extensively validated and used model for an LCB is the rat liver hepatocellular focus assay [95,104,106,397]. This assay in the liver takes advantage of the extensive capability of chemical biotransformation in this organ and the availability of sensitive and reliable markers for preneoplastic lesions. Other commonly used LCBs are the mouse skin papilloma/carcinoma, the mouse lung adenoma/carcinoma, and the rat mammary gland adenoma/carcinoma assays [104,106,476]. These have advantages for specific types of chemicals, for example, mouse skin is very responsive to polycyclic aromatic hydrocarbons and is appropriate for the assessment of topical products.

Positive findings for IN are highly indicative of potential carcinogenic activity [208]. In fact, it has been calculated that an IN/PM assay (of 40-week duration) can be as sensitive as the 2-year chronic bioassay in detecting carcinogenic activity [510]. PM activity also suggests a likelihood of carcinogenic activity [208]. In either case, it is possible to establish dose–effect data and no-effect levels (NELs) for the design of chronic bioassays or risk assessment.

Transgenic Mice

Another type of LCB, increasingly in use, employs transgenic mice [114,279] in which the principal genetic targets in the transgene are specific oncogenes (H-ras model), tumor suppressor genes (p53 model), or the entire genome in DNA-repair deficient animals ($XPA^{-/-}$ deficient model). Four models that have received the greatest attention are the p53 heterozygous mouse ($p53^{+/-}$), the Tg.AC mouse, the CB6F1-Tg ras H2 mouse, and the $XPA^{-/-}$ mouse. Other models, such as Min, p53/XPC double mutant, Eμ-pim-1, and ARF-deficient, have shown responsiveness to carcinogens but have not been adopted for regulatory studies [114]. The studied transgenic models have responded appropriately to a number of carcinogenic and noncarcinogenic agents [219]; they have been introduced as alternatives to the RCB in mice and accepted as providing evidence of carcinogenicity [208,279].

Three of the commonly used models are based on alterations in genes that are relevant to gene changes in many human and rodent neoplasms (e.g., the p53 tumor suppressor gene and the H-ras oncogene), while the $XPA^{-/-}$ model provides an enhanced response to DNA damage as a consequence of the elimination of nucleotide excision repair. Each model has specific features. Mice heterozygous for p53 differ in response depending on the strains used as parents; for example, the C57BL mouse, which is the most widely used [94], has a low incidence of background liver cell neoplasms, whereas liver neoplasms in unexposed controls are more frequent in C3H mice. The Tg.AC model carries a v-Ha-ras oncogene fused to the promoter of ζ-globin gene in the FVB/N mouse strain [270], a strain not commonly used in toxicology and which is susceptible to audiogenic seizure. The Tg ras H2 mouse carries five to six copies of human c-H-ras gene integrated in tandem array in the genome of F1 mice of transgenic male C57BL/6J mice and normal female BALB/cByJ mice [381]. The C57BL/6J is not very susceptible to hepatocarcinogenesis or lung carcinogenesis, whereas the BALB/c is susceptible to lung carcinogenesis [345]. Currently, the $p53^{+/-}$ and Tg.AC mice are widely available, but the Tg ras H2 is less accessible outside Japan, where it was developed.

Although any route of exposure can be used with these models, most data have been obtained by the oral route for the $p53^{+/-}$ and Tg ras H2 assays, whereas topical application is the preferred route for the Tg.AC. In these models, neoplasms can be elicited within 6 months with few or no neoplasms in controls. Beyond 6 months, these animals begin to develop a high incidence of genetically determined neoplasms as follows: the $p53^{+/-}$ with a C57BL parent develops lymphomas and sarcomas [282]; the Tg.AC develops odontogenic tumors, erythrocytic leukemia, and salivary gland and ovarian neoplasms [281,282]; and the H-ras2

develops benign and malignant lung neoplasms, splenic hemangiosarcomas, and forestomach papillomas [381]. The evaluation of increases in any of these neoplasm types must consider whether the increase is attributable to induction of neoplasia as opposed to acceleration of neoplastic development. The XPA-deficient mice do not show appreciably increased incidences of background neoplasms (only some liver neoplasms in the C3H-derived strain) [90,466]. All of these models respond primarily to DNA-reactive carcinogens, although the Tg.AC model has the potential to identify epigenetic skin carcinogens [93,279,407], and the Tg ras H2 has also responded to epigenetic carcinogens [279,518].

The $p53^{+/-}$ mouse, in the studies available so far, clearly responds with accelerated development of thymic lymphomas (e.g., phenolphthalein, cyclophosphamide, and melphalan [219]), but compared to the wild-type background, it has not exhibited an accelerated response to DNA-reactive carcinogens targeting liver (diethylnitrosamine) [244], mammary gland (9,12-dimethylbenz(*a*) anthracene), colon (1,2-dimethylhydrazine), or lung (urethane) [244,345]. This may reflect the fact that p53 mutation is not an early event in murine carcinogenesis for some tissues. Also, this model has, on occasion, failed to respond to the positive control *p*-cresidine [219,279,409]. Among candidate pharmaceuticals for which the FDA/CDER has received bioassay data, 0 of 23 yielded positive results, although some were oncogenic in rats [225]; thus, the usefulness of the p53 model for chemical screening seems questionable. In the Tg.AC model, the positive control generally used is the skin tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate [219,279]. The fact that this chemical by itself elicits skin tumors in the Tg.AC mouse demonstrates the hypersensitivity of this model. A number of innocuous materials have been positive in the Tg.AC, strongly indicating that it should be avoided where possible. For all of these models, evaluation may be enhanced by the measurement of cell proliferation in critical target organs. In the case of Tg.AC, stimulated cell proliferation can be the basis for skin neoplasm enhancement.

Currently, the Tg ras H2 model seems the most promising model [310,324] and is favored by regulatory guidelines [129]. A critical aspect of application is the demonstration of assay sensitivity. An appropriate selection of the positive control is paramount, and it may be advantageous for a convincing demonstration of a relevant sensitivity to consider the use of a nongenotoxic positive control, for example, a $PPAR_{\alpha}$ agonist. A sufficiently high exposure of the animals should be demonstrated. For pharmaceuticals, the 25-fold AUC is a well-defined and accepted upper limit for the dose selection (see RCB dose selection). A positive result in any transgenic model will be usually accepted as evidence for a carcinogenic potential. Important is that a negative Tg ras H2 has been accepted by the EMA as a substitute for the long-term mouse carcinogenicity assay. Experience so far has been limited as only a minority of applications for marketing authorization has included a transgenic assay in the last few years.

Other Models

The newborn mouse is also used in LCB [122]. In this model, newborn mice of any strain are administered the TS by intraperitoneal injection or oral intubation at days 8 and 15 after birth and then held for observation for up to 1 year of age. The model exhibits high sensitivity to DNA-reactive carcinogens but is unlikely to respond to epigenetic agents because of the limited exposure that is provided.

Another model under development is the In Ovo Carcinogenicity Assessing Assay (IOCA) using turkey or chicken eggs in which effects in the embryo–fetus are assessed [41,105–109,493,511]. TS is injected into the egg, either single or repeated, and the fetuses are harvested prior to hatching for histopathological examination of the liver for preneoplastic/neoplastic-like lesions. The key histopathological features are the foci and nodules of altered hepatocytes, which have the phenotype of carcinogen-induced lesions in rodent liver [107,108,493]. An interlaboratory validation of this assay has been completed [105]. This assay is very effective in monitoring the processes of proliferation, differentiation, apoptosis, DNA damage assessment [352,511], and developmental effects [493]. It also has the advantage of being defined as a nonanimal method for carcinogenicity testing. In addition, one of the added features of IOCA is that the organisms are bacteria-free, thus excluding the factor of bacterial flora, which is present in all organs of an adult organism and which is different and more numerous in rats and mice compared to humans.

Accelerated Cancer Bioassay (Stage E)

The accelerated cancer bioassay (ACB) model can be used to develop data on carcinogenicity when there is not a requirement for an RCB or there is an urgent need to obtain data [80,492]. It also can be used as an alternative RCB for one species. The ACB is essentially a composite of six or more IN/PM LCBs for rodent organs in which carcinogenicity has been found for known human carcinogens (i.e., liver, lung, kidney, urinary bladder, stomach, hematolymphoreticular tissue (HLR), and mammary gland). The protocol involves two segments: one in which the TS is administered at an appropriate high dose (see Dose Selection Studies for Bioassay section) for 16 weeks in an IN segment followed by promoters for the relevant target organs, and a second part in which the TS is administered in a PM segment for 24 weeks after an administration of initiating agents for the target organs (Table 25.5). The TS is also given alone for 40 weeks to assess carcinogenicity.

A rat model involving partial hepatectomy and administration of the liver carcinogen 2-acetylaminofluorene to enhance liver carcinogenicity has also been developed [224]. The use of these adjuncts, however, requires extra effort, limits the doses that can be tested, and is potentially confounding.

The ACB has a number of advantages: (1) it takes less time than the RCB, as the name implies; (2) it provides mechanistic data on IN/PM; and (3) the animals exhibit much less

TABLE 25.5
Accelerated Carcinogenicity Bioassay

Initiation Segment (IN)			Promotion Segment (PM)		
Control	16 weeks		Control	24 weeks	
TS	16 weeks		Promoter	24 weeks	
				Liver	PB
				Kidney	NTA
				Bladder	NTA
				Stomach	BHA
				Lung	BHT
				Breast	DES
				HLR	AZT
Initiator	10 weeks		TS	24 weeks	
	Liver	DEN			
	Kidney	EHEN			
	Bladder	BHBN			
	Stomach	MNU			
	Lung	DMN			
	Breast	DMBA			
	HLR	MNU			
TS	16 weeks		TS	24 weeks	

Note: AZT, azathioprine; BHA, butylated hydroxyanisole; BHBN, *N*-butyl-*N*(4-hydroxybutyl)nitrosamine; BHT, butylated hydroxytoluene; DEN, diethyl-nitrosamine; DES, diethylstilbestrol; DMBA, 7,12-dimethylbenz(*a*)anthracene; DMN, dimethylnitrosamine; EHEN, *N*-ethylhydroxy-ethylnitrosamine; HLR, hematolymphoreticular tissue; MNU, methylnitrosourea; NTA, nitrilotriacetic acid; PB, phenobarbital; TS, test substance.

age-related pathology at termination since they are less than 1 year of age at the end of the study and background rodent neoplasms occur predominately after 50 weeks (Table 25.6) [404]. The chief limitation is that the ACB is not as comprehensive as the RCB, although it has been calculated that, because of the PM stimulus of tumor development, the IN/PM model is as sensitive as a chronic bioassay [496,510]. Moreover, the ACB, with the addition of bone marrow, interrogates all target tissues affected in rodents by human DNA-reactive carcinogens (Table 25.5).

RODENT CANCER BIOASSAY (STAGE E)

Usually, rats and mice are required, although other species may be used (as discussed in the Animals and Their Environment section). For pharmaceuticals, the value of a mouse RCB has been questioned [465]; nevertheless, a mouse RCB would be indicated when (1) the biological effect of the chemical is expressed in mice, but not in rats, (2) the chemical has a pharmacologic effect on the gall bladder (absent in rats), or (3) mice are more representative of human toxicokinetics [225]. RCB are discussed in the Limited Carcinogenicity Bioassays section above.

In the EU, a comprehensive evaluation of carcinogenicity studies of medicinal products has been compiled for the years 1995–2009 [129]. Due to the high number of rodent tumor findings with unlikely relevance for humans, the value of the currently used testing strategy for carcinogenicity assessment appears questionable. Reconsideration of the carcinogenicity testing paradigm is warranted.

TABLE 25.6
Profiles of Percent Incidences of Common Spontaneous Neoplasms Expressed by Time of Death in Rats and Mice

	Males			Females		
Species, Sites/Neoplasms	**Rank**	**<50 Weeks[a] (%)**	**>50 Weeks[a] (%)**	**Rank**	**<50 Weeks[a] (%)**	**>50 Weeks[a] (%)**
SD rats						
Pituitary gland adenoma	1	0	100	1	8	92
Mammary gland fibroadenoma	2	9	91	2	7	93
Skin fibroma	3	8	82	NA[b]	NA[b]	NA[b]
Lymphoma, multicentric	4	60	40	5	40	60
Skin fibrosarcoma	5	11	89	NA[b]	NA[b]	NA[b]
Mammary gland adenocarcinoma	NA[b]	NA[b]	NA[b]	3	27	73
Pituitary gland carcinoma	NA[b]	NA[b]	NA[b]	4	0	100
CD-1 mice						
Lymphoma, multicentric	1	39	61	1	24	76
Bronchiolo-alveolar adenoma	2	14	86	2	7	93
Hepatocellular adenoma	3	13	87	NA[b]	NA[b]	NA[b]
Bronchiolo-alveolar carcinoma	4	5	95	NA[b]	NA[b]	NA[b]
Hepatocellular carcinoma	5	14	86	NA[b]	NA[b]	NA[b]
Histiocytic sarcoma	NA[b]	NA[b]	NA[b]	3	14	86
Mammary gland adenocarcinoma	NA[b]	NA[b]	NA[b]	4	12	88
Myeloid leukemia	NA[b]	NA[b]	NA[b]	5	25	75

Source: Adapted from Son, W.C. and Gopinath, C., *Toxicol. Pathol.*, 32, 371, 2004.

[a] Values reflect percent of total at the two arbitrary intervals (i.e., before and after 50 weeks).

[b] NA, not analyzed either because inappropriate or not common neoplasms.

The RCB was developed for the testing of small molecules, but is also being used to test large biological molecules. Such biomolecules were introduced into medicine beginning with vaccines and now also include hormones, oligodeoxynucleotides, genes, recombinant human proteins, humanized monoclonal antibodies, blood products, and cellular therapies. Each of these molecules presents specific issues in the conduct of an RCB. The ICH has produced a framework for preclinical safety evaluation of biotechnology-derived pharmaceuticals [217,395,452]. The limitations of genetic toxicity testing are discussed above.

Customized approaches are usually required for chronic preclinical safety assessment of biotechnology-derived pharmaceuticals and genetically engineered food products [452]. Some of these products are intended for intravenous administration, which is a challenging route of administration for an RCB. Immunogenicity presents an additional complication with proteins, because the development of neutralizing antibodies can abolish the biological activity of the protein, and the chronic antigenic stimulation can compromise carcinogenicity testing. Guidances concerning the design of carcinogenicity studies are generally based on the clinical indication or in-use exposure. Exceptionally, with these products, the possibility arises of using relevant but nontraditional species or using an animal model of the target disease [395]. Any testing should, of course, be done in a species in which the molecule has biological activity. For an immunogenic protein, one solution to this problem may be to test a homologous rodent molecule in the corresponding tolerant species.

Design

Most RCBs are performed to meet regulatory requirements, as listed in Table 25.1; otherwise, there are more efficient approaches to carcinogen identification (as discussed in the earlier Systematic Approach to Testing section). An RCB performed for regulatory requirements must follow prescribed guidelines [111,118,214,448,452] and, in particular, the regulations for good laboratory practices (GLPs; described below). Detailed descriptions of standard procedures for an RCB with chemicals have been published [298,476,495]. Aspects of the design, conduct, analysis, evaluation, reporting, and interpretation are given later.

Lifestage and Duration

Experimental transplacental carcinogenesis has been extensively studied, mainly in rats and mice [421] but also in primates [373,418]. The design of experiments ranges from dosing during embryogenesis or fetal or neonatal development [6,421] to multigenerational, involving exposure of germ cells of one or both parents and subsequently the progeny. These approaches are based on the well-established fact that younger animals because of greater rates of cell proliferation can be more sensitive to carcinogens [292,372]. It has frequently been considered whether exposures during pregnancy and lactation should be included in a routine RCB [403,421], but so far it is accepted that a conventional RCB can identify carcinogens that might have activity in developmental stages [45,177]. Thus, the approximate start of the RCB is usually around 6 weeks of age [176].

The anticipated life span for commonly used strains of rats is 24–30 months and 18–24 months for mice. The usual duration for both rat and mouse RCB is 24 months, although for a mouse RCB, 18 months can be acceptable if the strain does not have a sufficiently high survival for 24 months [67]. Some studies have been conducted with lifetime administration in which animals are allowed to die spontaneously [403]. Such a procedure, however, presents problems such as imbalances in the survival of groups and potential impairment of the quality of pathology due to postmortem autolysis.

Dosing should start shortly after weaning at about 4–6 weeks of age and should be daily. Test groups are not allowed to live longer than control groups; otherwise, late-developing background neoplasms in these groups could appear to be induced. If a high-dose group experiences high mortality (greater than 50%) due to TS administration, it should be terminated; the other test groups and controls should continue until 24 months are completed. In general, survival should not be less than 50% for mice at 15 months and rats at 18 months or 25% for mice at 18 months and rats at 24 months [67].

Feeding Procedures

During the past 20 years, increased variability in body weights, survival, and incidences of background neoplasms in Sprague–Dawley, Wistar, and F344 strain rats and CD-1 and $B6C3F_1$ mice have been observed [5,28,59,100,242,360, 369,413,414,430,470]. The changes in these parameters can confound and even jeopardize the interpretation of an RCB [28,100,240,242,322,367,394,413,414,419].

Most RCB are conducted using ad libitum (AL) feeding. To overcome the problem of overeating with AL feeding, two solutions have been proposed [59,242,322,419]. One is referred to as the caloric optimization diet (COD), which consists of limiting caloric intakes to 50%–80% of AL consumption [59,163]. The other is the diet-restricted (DR) model in which animals are fed diets limited in the offered quantity of feed sufficient to produce a 15% reduction in body weight compared to the AL controls [2,164,322]. In addition to these procedures, the use of weight-matched groups that are fed in such a way that their mean body weight is matched with that of the high-exposure AL group has been evaluated [2,322] but is not currently being used. The COD and DR procedures clearly improve the health and survival of animals, as they reduce the occurrence of age-related pathology, such as chronic progressive nephropathy and myocardiopathy in rats [175,197], and of certain background neoplasms [19,175], such as uterine polyps, pituitary gland neoplasms, and mononuclear cell leukemia (MCL; see Species and Strain section) [40,175].

In the past, dietary control was routinely used in the testing of oral contraceptives [234]. Proposals have been made for the use of dietary control for all medicinal and chemical products [5]. Differences in the incidences of neoplasias

between AL-fed and DR rodents are available for the two commonly used rat strains, Sprague–Dawley and F344, and the B6C3F$_1$ hybrid mouse [34,40,59,174,175,293,322,411,430]. In both rats and mice, especially the latter, hepatocellular neoplasia in both sexes is reduced in DR groups. Similarly, reductions in pancreatic (both acinar and islet) neoplasia (especially in male rats), pituitary neoplasia (especially in female rats), and adrenomedullary neoplasia (especially in male Sprague–Dawley rats) were achieved in both species. In general, these decreases in DR groups are due to changes in the metabolism and hormonal homeostasis. In addition, certain tumor decreases in the DR groups are present only in one species (skin fibroma in rats), one strain (thyroid C-cell, mammary gland, ovarian, and hematopoietic neoplasms in F344 rats), or one sex (pulmonary neoplasia in male mice). Also in female F344 rats, uterine polyps and pituitary gland neoplasia have been significantly reduced in feed studies with NTP-2000 diet [175], which was developed to reduce the background pathology, particularly renal, in rats. The mechanism of these decreases is not fully understood. Available information, however, on the impact of either COD or DR on response to well-studied carcinogens generally reveals a reduced response. Accordingly, detection of carcinogenic effects may be masked at sites sensitive to tumor reduction by body weight gain inhibition. Analyzing tumor incidences within body weight strata can reduce bias introduced by weight differences (see Statistical Analyses section).

Groups and Identification

After acclimatization in the test facility, animals should be assigned to groups using randomization procedures immediately prior to study initiation. Randomization eliminates bias, but if there is another source of variation, such as sex, cage position, or order of euthanasia at termination, then a stratified randomization is more appropriate. This involves separate randomization within each level of stratifying variable, such as body weight or cage position [271].

The use of two independent control groups helps to assess biological variability in incidences of commonly occurring background neoplasms [14,170]. The minimum group size is 50, which permits detection of neoplasms with incidences in the range of 5%–10%. Larger groups, if indicated by 3 month results, can be useful in allowing interim (12- or 16-month) terminations. Such information is not otherwise available when no unscheduled deaths occur due to neoplasms [135,271,404] (Table 25.6). To monitor for intercurrent diseases (see Animals and Their Environment section), a satellite group of six or nine can be used.

The groups should consist of a high-dose group (see Dose Selection Studies for Bioassay section) and at least two lower doses. Many testing laboratories, including the NTP, use only two groups with the low-dose group at half the high dose to provide sufficiently dosed animals in the event that the high dose impairs adequate long-term survival. Others space doses by one-third or one-fourth. The factor between doses should not exceed 5. Because mainly TS that are not DNA-reactive advance to an RCB, a valuable third group is one at the NEL for any epigenetic effect identified at higher doses that may lead to oncogenesis. This NEL should yield a cancer NEL, which is valuable for risk assessment. If quantitative risk assessment is envisioned, four or more dose levels will be needed to establish the shape of the dose–effect curve.

Cages must be identified with study information including study number and animal numbers. Animals must be individually identified. This can be done by tattoo or by implantation of an electronic transponder. The latter is not advisable for transgenic mice, as subcutaneous sarcomas have been induced [26].

GOOD LABORATORY PRACTICE

As regards the RCB, the main intent of GLPs is maintaining the integrity of a complex system of data management. This requires that the study be designed, conducted, evaluated, and reported according to standard operating procedures (SOPs) and that records are maintained in a manner that ensures a comprehensive and independent review. GLP must be conducted in such a way that all data can be validated. GLP is a global process that has been implemented by several national bodies [214,341,438,446]. The elements were detailed in the previous version of this chapter [126] and have been recently addressed [177].

In a GLP study, the study director plays the critical roles of moderator, catalyst, and gatekeeper and is responsible for the integrity of study data. For more details, see Williams et al., 2008 [126].

All raw data must be properly identified and stored, eventually in study notebooks that ensure the integrity of the data. All data must be entered in a permanent and legible manner. Any changes to data must be dated and identified as changes, with reasons noted for any change, as well as the identification of the scientist making the change. Further, the professionals responsible for data entry, verification, and review should also be identified. Finally, documentation of the data so described must be maintained at all times, and secure audit trails must be created for authorized changes in the database and also in the study notebook [438]. In histopathology, the most important study materials are the tissues on glass slides and their respective blocks, which require a specific trail leading from sampling records during necropsy and trimming records after necropsy [32,76,446].

To obtain proper material for pathology, tissues are prepared according to standardized procedures with respect to location, type of section (e.g., cross), and orientation on the slide. In addition, slides of lesions should include both lesion and surrounding normal tissue. Any failure to adhere to this regimen is a form of censorship and results in noncompliance. It is of paramount importance to integrate clinical (cage-side observation), structural (macroscopic and microscopic), and functional (cellular and biochemical) data. Failure to integrate these three types of data will result in compromise of data and loss of data integrity. The pathologist must keep the

study director apprised of events as they occur. The responsibilities of the study pathologist include keeping account of all the lesions reported at necropsy and performing microscopic evaluation of the normal and abnormal tissue changes. In this way, the pathologist ensures that appropriate tissues are collected, processed, and evaluated in a manner that satisfies the objective of the study. Finally, the pathologist ensures consistency in diagnosis, integration of data, and grading of pertinent lesions, avoiding diagnostic drifts and censorship. For a facility to be GLP compliant, specified environmental conditions (see the Animals and Their Environment section) must be maintained and monitored by a program applied at appropriate intervals.

The final regulatory end product for every study is the compliance statement, which is provided in the final report and signed by the study director. In this section of the report, all modifications, deviations, and amendments to the protocol are listed. A second page with a quality assurance statement is signed by the quality assurance auditor. During the last 33 years of GLP implementation, several common findings of importance (i.e., deficiencies listed in form FD-483) have been compiled by various agencies. The deficiencies noted were in all subparts of the FDA regulations (subparts A–J, 58.10–58.190). Those pertaining to pathology are detailed in the Anatomic Pathology section.

HEALTH AND SAFETY PROCEDURES

A comprehensive, rigorously followed health and safety plan is necessary for the proper conduct of an RCB. The fact that a substance is being tested for carcinogenic activity makes it, in effect, a suspect carcinogen, although information in the Material Safety Data Sheet, such as genotoxicity or reproductive toxicity, influences the stringency of the handling procedures instituted, from receipt of the chemical through disposal of animal waste and processing of tissues for histopathological examination. All measures are subject to quality control.

The safety plan [317,337] must address the responsibility within management for the development and adherence of the plan; medical surveillance for employees; employee training; safe handling practices for the chemical; animal handling; general laboratory safety; safe personnel practices; safe work area practices (e.g., spill control and decontamination); handling of air, liquid, and solid wastes; monitoring of workers and physical equipment; emergency control; recordkeeping; design of facilities; and pollution potential. Applicable regulations of the U.S. Occupational Safety and Health Administration (OSHA) provide only a minimum structure from which to work, and lessening of the hazard within the particular facility must be addressed individually and with ingenuity. Laboratory directors must appreciate that chemicals, especially highly volatile ones, may penetrate protective clothing and travel a considerable distance from their point of use [336,383–385,441,443]. Indeed, the finding of TS in the blood of control animals [329] testifies to migration.

No safety measure is unique to an RCB; it is the degree of adherence to such procedures that distinguishes the conduct of these studies from all others. It is beyond the scope of this section to address each individually. A few examples of aspects that are often inadequately addressed include the following: (1) use of a properly ventilated cage dumping area or an enclosed animal bedding disposal cabinet to prevent the inhalation of contaminated dust and aerosols by employees; (2) an air-handling system that provides decreasing gradations of air pressure from clean corridor to the animal rooms to the dirty corridor and that is periodically tested under such stress as several doors being opened at one time or with all possible chemical hoods in operation; (3) maintenance personnel, as well as scientific supervisors, following the same rules as technicians for personal protection; (4) storage facilities that protect the integrity of the TS over extended periods of time during which unused material may be held and the immediate containers checked for deterioration; (5) a breathable air line available for use with an air-supplied respirator in the TS preparation areas; and (6) workers, including weekend staff, who are familiar with emergency safety instructions within the laboratory and know whom to notify in the event of various types of potential emergency situations.

ANIMALS AND THEIR ENVIRONMENT

The use of animals in research is subject to national regulations. In the United States, the use of rats and mice is not regulated, but usually follows the guidelines for other animal use that have been provided by the U.S. Department of Health and Welfare for Care and Use of Laboratory Animals [317,318,436], the latest amendment of the U.S. Congress Animal Welfare Act [432], the U.S. Public Health Service Policy of Humane Care and Use of Laboratory Animals [318], the U.S. Department of Agriculture Animal Welfare Rules [435], the Animal Welfare Act of the National Research Council [321], and the U.S. EPA Health Effects Test Guidelines [444]. Institutional responsibilities include making available all protocols for review by a committee and providing veterinary care. More extensive coverage is provided in Chapter 20.

Species and Strain (Genotype)

In the past, large animals have been used in carcinogenicity studies for specific purposes; for example, dogs were used for the testing of aromatic amines, whose effects in the dog bladder have been shown to be related to frequency of urination [235]. Hamsters have also been used as a second rodent species when either rat or mouse exhibits high nonrelevant effects, for example, nasal toxicity [231].

For the rat RCB, several strains have been widely used. The NTP generally uses the inbred F344, although this is under evaluation [250], while the pharmaceutical and chemical industries have favored the outbred Sprague–Dawley or Wistar to obtain genetic diversity. For the mouse RCB, industry generally favors the CD-1 strain, whereas the NTP uses

the $B6C3F_1$ hybrid, which is a first-generation cross between male C3H and female C57BL/6 strains, for the purpose of reducing the high spontaneous liver neoplasm development of the C3H.

Both rat and mouse strains, including hybrids, differ substantially in their background of neoplasms and their susceptibility to the induction of tumors [60], for example, the F344 has very high incidences of several neoplasms, notably testicular (Table 25.7). Among mouse strains, those derived from the C3H (i.e., $B6C3F_1$) have high incidences of liver tumors. The A/J mouse has a high lung tumor incidence, which is taken advantage of in limited cancer bioassays. The comparative percent incidences of the principal spontaneous neoplasms from five different strains of rats and mice are given in Table 25.7.

Several factors that influence response to carcinogens differ among genotypes. Notably, biotransformation activities for certain chemicals differ considerably. In addition, sex-dependent differences in xenobiotic biotransformation are most pronounced in rats. The differences involve mainly cytochromes P450 (CYP), sulfotransferases, glutathione transferases, and glucuronyltransferases [311,312,325]. Thus, it is critical that strains used are obtained from providers that follow procedures designed to minimize genetic drift in the founding colony [177].

A troublesome feature of the F344 rat and $B6C3F_1$ mouse is that their average survival has progressively decreased, and increases have occurred in the incidences of liver neoplasms in female and male mice; pituitary neoplasms in female mice; thyroid neoplasms, adrenal pheochromocytomas, and leukemias in male rats; and mammary neoplasms in female rats [368,428]. Some of these neoplasm increases are positively correlated with excessive body weight [428] resulting from overeating (a detailed discussion of this issue was presented earlier in the Feeding Procedures section). Likewise, in Charles River Sprague–Dawley rats, decreases in survival have been reported [241,242]. These changes may be due to breeding practices. Currently, the Wistar strain does not appear to present this problem [360].

The species and strains selected in combination should achieve exposure to all known human metabolites.

Feed and Water

Four major types of diets are available: (1) natural-product, unrefined, largely cereal-based formulations usually referred to as *chow-type* diets, such as NIH-07 (Purina 5018 or Purina 5001); (2) semipurified diets, formulated from refined nutrient ingredients, such as AIN76A (with sucrose) or modified AIN76A (with dextrose); (3) open-formula diets, such as NIH-31 (Purina 7017), which are formulated to contain researcher-specified quantities of nonproprietary ingredients; and (4) chemical-defined diets, which are individually specialized [258,321]. Industry generally favors the open-formula diet. In 2000, the NTP introduced the NTP-2000 diet, which was developed to reduce the background tumor burden [175]. Interestingly, the natural unrefined diet is reported to protect against the effects of several chemical carcinogens [328]. Details about various regimens of feed availability were given earlier in the Feeding Procedures section. Finally, the drinking water, which is usually municipal tap water, should be periodically monitored for microbiological and chemical contaminants.

Caging and Stratification

During the planning stage, the possibility of cross contamination of control groups [329], especially when the administered chemicals are volatile, should be anticipated. Once established in preliminary pilot toxicokinetic (TK) studies, then steps can be taken to address the issue as indicated later.

Following randomization, animals can be caged individually or with more than one animal in each cage; each approach has advantages and disadvantages. Individually caged animals tend to overeat. Multiple animal caging leads to a conflict for hierarchy in the cage and consequent cage differences. Group-caged female mice develop pseudopregnancy, which results in uterine decidual reactions, whereas wounding as a result of fighting is common in group-housed male mice. Also, group caging of mice produces almost a doubling of the lymphoma incidence in both males and females [417]. In general, group-caged rodents demonstrate higher survival rates and lower background pathology [376]. For inhalation and dermal administration studies, single housing is required. Cages used can be either metal (stainless or galvanized steel) or plastic (polycarbonate, polyethylene, or polypropylene), with a minimum stipulated cage size [436]. The floor of the cages can either be solid or wire bottom for a suspension rack. Solid-floor cages require bedding that does not have the enzyme-induction properties of, for example, hardwood bedding [468]. A problem with wire-bottom cages is foot injury. The tops of cages should be protected from airborne contaminants [479] at least by providing a microisolator filter cover. Cages and racks should be rotated periodically to balance known confounding sources of variability such as proximity to fluorescent lights [266,371]. Consequently, rodents within groups should be distributed in cage racks in such a way as to be present equally at all vertical levels of cage placement. A thorough documentation of such cage and rack rotation is mandated.

All groups should be housed in the same room to avoid nonuniform environmental influences [479].

Environment and Emergency Power

Environmental stress experienced by test animals must be minimized, particularly with mice, which are easily stressed even when maintained under conventional housing conditions and handled in the usual manner. The incidence of tumors in mice infected with viruses can be increased by chronic stress [375,393]. Standards for care are detailed in the *Guide for the Care and Use of Laboratory Animals* [321]. These include (1) 10–15 fresh air changes per hour occur in each animal room; (2) air pressure maintenance so the animal rooms are slightly

TABLE 25.7
Comparative Percent Incidence of Pertinent Neoplasia in Strains of Rats and Mice (104-Weeks Old) Used in Bioassays

	Sprague–Dawley Rats[a] (%)		Wistar Rats[b] (%)		F 344 Rats[c] (%)		1CRCr:CD-1 Mice[d] (%)		B6C3F1 Mice[e,f] (%)	
Types of Neoplasia	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Hepatocellular neoplasia	5	3	1	2	3	1	18	3	22	17
Pancreas islet neoplasia	7	3	4	2	6	1	1	1	1	0
Pancreas acinar neoplasia	1	<1	13	1	6	0	<1	0	2	0
Pheochromocytoma	11	4	8	2	21	4	<1	<1	0	2
Adrenocortical neoplasia	2	3	6	6	1	2	1	<1	<1	0
Pituitary adenoma	46	78	16	29	25	42	0	5	2	8
Thyroid C-cell neoplasia	5	5	6	8	12	8	0	0	0	0
Thyroid follicular neoplasia	3	2	2	1	2	1	1	<1	2	6
Mammary gland fibroadenoma	1	18	3	9	4	57	<1	1	0	0
Mammary gland carcinoma	<1	40	1	27	0	25	0	6	0	0
Skin fibroma	5	<1	5	1	10	2	<1	<1	1	2
Skin papilloma	2	1	2	<1	5	<1	<1	0	0	0
Pulmonary neoplasia	<1	<1	<1	0	4	4	15	15	22	6
Preputial gland neoplasia	1	NA	<1	NA	10	NA	<1	NA	<1	NA
Leydig cell neoplasia	4	NA	6	NA	84	NA	1	NA	0	NA
Clitoral gland neoplasia	NA	<1	NA	<1	NA	14	NA	0	NA	<1
Uterine polyps	NA	4	NA	12	NA	14	NA	<1	NA	1
Ovarian neoplasia	NA	1	NA	5	NA	6	NA	1	NA	6
Mononuclear cell leukemia	<1	<1	<1	<1	47	22	2	2	0	0
Lymphoma	2	2	3	2	<1	<1	8	22	14	24
Forestomach neoplasia	<1	<1	0	<1	0	2	<1	<1	5	2
Scrotal mesothelioma	1	NA	2	NA	5	NA	0	NA	0	NA

Note: NA, not applicable.

[a] Data from Brix et al. [40], Christian et al. [59], Giknis and Clifford [146], and McMartin et al. [293].

[b] Data from Bomhard and Rinke [28], Eiben and Bomhard [99], Giknis and Clifford [145], Poteracki and Walsh [360], Tennekes et al. [413,414], and Walsh and Poteracki [470].

[c] Data from Christian et al. [59], Haseman et al. [174], and NTP [322].

[d] Data from Giknis and Clifford [144] and Maita et al. [283].

[e] B6C3F1 mice = (C57BL/6N + C3H/HeN)F1.

[f] Data from NTP [322], Tamano et al. [411], and Turusov et al. [430].

positive to the *dirty* corridor and negative to the *clean* one, with minimal crossovers between the corridors; (3) adequate air filtration before it enters the animal facility and dilution or filtration after it leaves to prevent possibly toxic concentrations of the TS from entering the outside air (a process that is particularly important with inhalation studies because of the large amounts of chemical used); (4) temperature and humidity control within those ranges reported to be optimal (i.e., 23.3°C ± 1.1°C or 74°F ± 2°F) and a relative humidity of 40% ± 5% in rat and mouse rooms; and (5) automatic control systems to record both temperature and humidity at least three or four times per day. Control of lighting is essential. Usually, a 12 h continuous light interval per day is used for both rats and mice (14 h light day for hamsters). Moreover, 4 h of high-intensity light (only during cage-side observation) and 20 h of low-intensity fluorescent light are recommended to avoid blindness caused by high-intensity light. In addition, because the light and temperature gradients occur vertically, a cage-rack rotation is mandated [321]. In specific situations such as with dietary administration of TS, consideration should be given to a reversed light cycle, when blood for achieved blood levels needs to be obtained during the dark cycle when animals are awake and feeding.

An emergency power source is essential to maintain the operation of storage freezers and refrigerators, lighting, autotechnicons, and some degree of air conditioning, as well as air handling, during power failure or when personnel are unable to reach the facility. The emergency power and alarm systems should be tested on a regular schedule. In a GLP-compliant facility, all these conditions must be monitored.

Also important is control of pests by adequate facility design and sanitary procedures. Pesticides must not be allowed to contaminate the animal rooms, feed rooms, or cage washing areas and accordingly should be dispensed only in closed traps in limited areas. Detergents and cleaning agents for use on floors, cage washers, and other equipment must be nonvolatile and must not leave a residue.

The environment and welfare of animals can be enriched by providing wooden chew blocks.

DOSE SELECTION STUDIES FOR BIOASSAY

Dose Selection: The Maximum Tolerated Dose

Setting of the high dose can be based on a number of endpoints, including toxicity, toxicokinetics, saturation of absorption, and maximum feasible dose [125,214,215]. The various methods for the selection of the high-dose level have been reviewed by a working group on dose selection convened by the International Life Sciences Institute [125]. Generally, it is expected that the high-dose level in an RCB is a toxicity-based dose—the maximum tolerated dose (MTD) [406]—which is also referred to as the minimum toxic dose (or the minimally toxic dose by the NTP) [172]. Testing at the MTD ensures that potential carcinogenicity has been fully evaluated; nevertheless, it creates issues in the interpretation of positive findings, because of high-dose effects, as discussed below.

The first widely used definition of the MTD was formulated by the NCI [406] as follows: "The MTD is defined as the highest dose of the test agent during the chronic study that can be predicted not to alter the animals' normal longevity from effects other than carcinogenicity." This definition did not stipulate that any toxicity needed to be produced; hence, a slight, but significant, reformulation was introduced by the U.S. Interagency Staff Group on Carcinogens [320,449]: The MTD is "the highest dose which when given for the duration of the chronic study is just high enough to elicit signs of minimal toxicity without significantly altering animals' normal life span due to effects other than carcinogenicity." The MTD so defined is a dose used in the chronic study [10], which, of necessity, is selected from subchronic studies (normally 90-day studies). The ICH defines the MTD as the dose predicted from a range-finding study to produce minimum toxicity over the course of the carcinogenicity study [215,449]. Such a condition can be produced by alterations in physiological function that would be expected to alter survival, target organ toxicity, significant alterations in clinical pathological parameters, or no more than 10% suppression of weight gain relative to controls, calculated as the difference between the starting weights and those at the end of the study [320,449]. A fortuitously selected target MTD in the RCB should suppress weight gain by 10% or slightly greater and produce only minimal other toxicities. The ideal dose selection, however, infrequently happens, and issues arise when the high dose is either below or above what is considered by a regulatory agency to be an MTD.

TK endpoints have gained acceptance for dose setting for pharmaceuticals [128]. A dose that produces saturation of absorption is considered an MTD, and a dose that produces a plasma concentration that is 25 times greater than human exposure (a 25 to 1 exposure ratio) is considered pragmatic for TS with no genotoxicity signal [212,215,459]. When using this parameter, it needs to be determined whether among humans there are poor metabolizers who would achieve higher blood levels than the general population.

Toxicodynamic endpoints can be used to establish a high dose that will produce a cellular or tissue effect, beyond which the validity of the study would be compromised. An example is renal toxicity, which can become more severe with aging in rats as they develop chronic progressive nephropathy.

To identify the target MTD or high dose, subchronic studies are performed, as described below. If the target MTD (or the high dose) cannot be established using the criteria discussed, it is usually recommended that the high-dose level or limit dose not exceed 5% in the feed [199,299,340], which translates into approximately 3–4 g/kg/day for rats and 7–8 g/kg/day for mice. This limit is based in part on concern for the nutritional impact of high proportions of TS in the diet. Certainly, some TSs at high concentrations interfere with nutritional elements, such as impairment of vitamin K function by butylated hydroxytoluene [71,220], but such problems can be overcome by appropriate nutritional supplementation. Nevertheless, reduced food intake and, hence, caloric intake will affect the outcome of the RCB, usually reducing neoplasm incidence [2,59,255,322].

In the United States, the dose selection for pharmaceuticals should be submitted to the FDA/CDER and its Carcinogenicity Assessment Committee (CAC), which strongly favors a toxicity-based endpoint. Such consultation is not yet practiced in Europe, but the EMA, as well as several national authorities, offers various forms of scientific advice procedures. The selection of other dose levels is discussed in the Rodent Cancer Bioassay section above.

Subchronic Study

The MTD is usually identified from results of a 90-day study conducted in the species and strain selected for the RCB using the intended route of administration. It is unusual that the tested doses would exactly identify an MTD, so interpolation is usually made. When the two sexes show different toxicities, which is frequent, different sets of doses need to be administered in the RCB.

Chemical Disposition Studies

Before starting an RCB, it is helpful and, in the case of medicinal products, a requirement [128,212] to determine the time course of internal exposure to parent compound and metabolites and eventually the relationship between exposure and observed effects. The species/strain used for an RCB must provide coverage of known human metabolites. For this purpose, multiple-dose TK studies measuring both parent compound and metabolites are conducted [50,212]. Plasma protein binding of the TS in rodents and humans should be determined before the initiation of carcinogenicity studies. Should binding exceed 80%, it is advisable to express exposure (plasma TS concentration) in terms of the free fraction [56,347]. In addition, tissue distribution and accumulation data can be valuable [55,362], especially because species differ in their proportion and composition of certain tissues. For example, the liver represents 4.8% of the body weight of a rat, whereas it is only 2.8% of the body weight of a human [191]. Conversely, adipose tissue is only 11% of the body weight of a nonobese rat [143], whereas it can be over 20% of the body weight of a human [191]. Furthermore, appropriate and useful extrapolation from rodents to humans involves understanding of physiologic (Table 25.8), histokinetic, and xenodynamic considerations [57], for example, smaller species (e.g., rodents) have higher rates of xenobiotic biotransformation

TABLE 25.8
Species Physiologic Differences Important in Carcinogenicity

	Species		
Parameter	**Rat**	**Mouse**	**Human**
Life span (years)	2.5	2	70
Body weight (kg)	0.40	0.02	60
Body surface (cm^2)	325	46	1600
Food consumption (g/kg/day)	50	150	2.5
Human equivalents in months[a]	34	38	1
Basal metabolic rate (kcal/kg/day)	109	188	26
Heart rate (beats/min)	350	600	75
Respiratory rate (beats/min)	97	120	12
Stomach pH	4	3	2
Bacterial flora	Numerous	Numerous	Few
Reproductive cycle	Estrus	Estrus	Menstrual
Placental barrier	HE	HE	HC
Tissue volume ratio direction compared to humans (increased, decreased, or same)[b]			
Liver	2.4 × decreased	1.3 × increased	NA
Kidney	1.5 × increased	Same	NA
Lung	6 × increased	204 × increased	NA
Adipose tissue	5 × increased	2 × decreased	NA
Intestine	4.3 × increased	3.3 × increased	NA
Heart	2.8 × increased	6 × increased	NA
Muscle	9 × increased	5 × increased	NA

Sources: Data from Iatropoulos, M.J., Comparative histokinetic and xenodynamic considerations in toxicity, in: *Drug Toxicokinetics*, Marcel Dekker, New York, 1993; Iatropoulos, M.J. et al., *Scand. J. Lab. Anim. Sci*., 13, 339, 1996; Monro, A. *Exp. Toxicol. Pathol.*, 48, 155, 1996; Williams, G.M. and Iatropoulos, M.J., *Toxicol. Pathol*., 30, 41, 2002.

Note: HC, hemochorial consisting of fetal trophoblast, fetal connective tissue and fetal endothelium; HE, hemoendothelial consisting of fetal endothelium; NA, not applicable.

[a] Human xenobiotic exposure of 1 month (or 0.1% of the life span) is 4.1% of the life span of rats and 5.5% of the life span of mice.

[b] Calculated ratio based on organ volume (mL) and organ plasma flow rate (mL/min).

per unit of body weight, faster rates of tissue distribution, and shorter tissue half-lives [191,194]. Tissue volume directly affects the volume of distribution of xenobiotics, bioavailability, half-lives, and systemic clearance [24,228,249]. In general, with increasing age, as occurs over the course of a carcinogenicity study, body weight increases, as does the proportion of an adipose tissue, which has a lower metabolic rate than skeletal muscle mass (Tables 25.8 through 25.10) [138]; thus, the volume of distribution decreases with age, resulting in an increased blood concentration of hydrophilic TS during distribution. In addition, the increased adipose tissue in rats, but not in mice (Tables 25.9 and 25.10), and the decreased water content in tissues can affect energy homeostasis, resulting in a deficiency in energy availability in rats compared to humans. Both influence the outcome of kinetic studies. In dose-proportional linear kinetics, half-life and clearance are independent of xenobiotic concentration. In contrast, in nonlinear kinetics, these parameters are dependent, because the various processes (absorption, distribution, metabolism, and excretion) can become saturated (Tables 25.8 and 25.9) [274].

The TK component of RCB often is carried out using satellite groups maintained in parallel and consists of at least three rodents per group, sex, and time interval, with interim sampling at least every 6 months. The time-course factor is very important in understanding how the various concentrations accumulate and result in different exposures [128]. The animals are maintained and dosed under identical conditions with the main study. Approximately four blood samples are taken from each animal over the duration of the RCB, with the blood removed not exceeding 15% of the total blood volume. If the route of administration is feed admixture, it is essential that sampling be done during the feeding (dark) phase; otherwise, unrepresentatively low values will be obtained. The parameters examined may include the maximum achieved concentration (C_{max}), the minimum concentration (C_{min}), the time to C_{max} (T_{max}), and the area under the plasma concentration time curve (AUC) [191], although only the AUC is required to establish exposure [212]. In an RCB, together with measuring the systemic concentration of exposure (AUC), determination of the total (cumulative) amount of exposure over time is also essential so that exposure can be related to induced effects [212,362]. Target site compound levels potentially connect the target site of both chronic toxicity and carcinogenicity; for example, essentially 100% of a compound absorbed from the intestine passes through the liver (portal circulation), and most biotransformation takes place in this organ (first pass). Highly lipophilic TSs enter directly the intestinal lymphatics bypassing portal circulation. To accomplish accurate extrapolation of systemic exposure over time, human time equivalent (HTE) values are employed (Table 25.8). At 12 months of dosing, these values amount to an HTE of 38 years for the mouse and 34 years for the rat; and at 24 months, to values of 76 and 64 years for the mouse and rat, respectively [191,194].

TK data are critical not only for delineating blood levels but also for understanding the effects of exposure, such as whether the parent or a metabolite is the main biologically active moiety, if the plasma concentration reflects the cellular site of action, and the nature of interspecies concentration (blood)–response (site) relationship [191,304,348]. Furthermore, biotransformation can also distort concentration–response homeostasis. Here, at high exposures, together with absorption and elimination being saturable (i.e., capacity limited), biotransformation can also significantly alter what is bioavailable at tissue target sites, including through the process of bioactivation, which is especially important in interspecies extrapolation [57]. Moreover, saturation of biotransformation and shunting through secondary routes of biotransformation should also

TABLE 25.9
Comparative Adult Tissue Volume Ratios

	Volume Ratios		
Tissue	**Mouse**	**Rat**	**Human**
Muscle	0.05	0.10	0.01
Adipose	0.01	0.10	0.02
Heart	3.00	1.40	0.50
Lung	36.70	1.10	0.18
Liver[a]	0.85	0.24	0.59
Kidney	2.35	3.50	2.50
Intestine	0.60	1.30	0.33
Plasma	4.40	4.36	1.19

Sources: Data from Iatropoulos, M.J., Comparative histokinetic and xenodynamic considerations in toxicity, in: *Drug Toxicokinetics*, Marcel Dekker, New York, 1993; Iatropoulos, M.J. et al., *Scand. J. Lab. Anim. Sci*., 13, 339, 1996.

Note: Volume ratio is calculated by dividing plasma flow rate of the tissue by its volume.

[a] 77.8% of the volume comes from the portal and 22.2% from the arterial circulation.

TABLE 25.10
Comparative Adult Tissue Relative Weights[a]

	Percent Relative Weights		
Tissue	**Mouse**	**Rat**	**Human**
Muscle	43.5	42.5	41.4
Adipose	10.5	11.0	21.4
Heart	0.3	0.4	0.5
Lung[b]	0.5	0.5	1.7
Liver	4.4	4.8	2.8
Kidney	0.8	0.8	0.4
Total of six tissues	60.0	60.0	68.2

Sources: Adapted from Iatropoulos, M.J., Comparative histokinetic and xenodynamic considerations in toxicity, in: *Drug Toxicokinetics*, Marcel Dekker, New York; Iatropoulos, M.J. et al., *Scand. J. Lab. Anim. Sci*., 13, 339, 1996.

[a] In percent of body weight.

[b] Dry weight.

be taken into consideration [55,83,142,161]. Knowledge of disposition (including species differences in xenobiotic biotransforming enzymes) of the TS is essential to interpreting the basis for an increase in neoplasia and for extrapolating to humans.

Finally, in the conduct of TK studies, it is imperative to be aware that controls can be contaminated, and precautions to prevent such contamination, as described above, have been developed [329].

QUALITY CONTROL OF THE TEST SUBSTANCE

The TS should, of course, be identical to the final product or article in commerce and of a high quality and stability. This requires that it be manufactured in the same way and contain the same concentrations of impurities as the final product. Impurities in excess of 0.1% need to be individually identified. For pharmaceuticals, the active substance used in the preclinical and clinical tests should be preferably the same as in the marketed product. The product should have a well-defined and described scale-up process [213]. Most medicines will be formulated with excipients, and the noncarcinogenicity of these is expected to be established [458]. In other situations, as with agricultural chemicals and cosmetics, the technical-grade product or a representative technical grade of active ingredients is tested. In some instances, complex mixtures are tested (e.g., polychlorinated biphenyls). This aspect is discussed further in the Complex Mixtures section.

Test Substance

Chemicals or Small Molecules

In some situations, it is desirable or necessary to test pure chemicals, as with candidate medicines or food additives. With isomeric compounds, if the two enantiomers exhibit inversion in vivo or have the same biological activity, then they are considered one entity, and carcinogenicity testing of the racemate is appropriate.

Biopharmaceuticals

Biopharmaceuticals include recombinant human proteins, humanized monoclonal antibodies, oligodeoxynucleotides, and genes. Recombinant human proteins are being produced by a variety of techniques, often in bacteria, where glycosylation does not occur. Quality control of biopharmaceuticals, as with small molecules, is necessary to establish that they are actually what they are expected to be at all times during the study. For this purpose, all relevant information, such as synonyms, trade names, structural and molecular formulas, and weights, as well as methods of analysis and chemical and physical properties of the pure substance, should be available.

Complex Mixtures

Many human contacts with chemicals involve mixtures rather than single chemicals (e.g., polychlorinated biphenyls); yet, the scientific data for some mixtures are generated mainly from studies of individual components. Mixtures can be comprised of chemicals with several isomers, chemicals with major contaminants, hazardous waste in solid or liquid form, and air pollutants. The mixtures can either have a common source (e.g., tobacco smoke, aluminum production, and coal tars) or be formulated deliberately (coal gasification, footware-processing work exposure). Several such mixtures are recognized as human carcinogens (e.g., tobacco smoke, coal tars, and diesel engine exhaust). A daunting challenge has been to ascertain the role of individual components in the carcinogenicity of such mixtures [202]. To accomplish this, complex mixtures can be fractionated and characterized into chemically defined entities that can be individually tested. Nevertheless, in the mixture, individual chemicals can enhance or inhibit the activity of others, as discussed in the Interactive Carcinogenesis section.

Impurities or Contaminants

It is highly desirable that all TS should be pure chemicals of analytical grade, as even traces of impurities of <1% can produce confounding effects. An example of this is *o*-toluenesulfonamide, an impurity of saccharin present in the materials used in early carcinogenicity studies [313]. Impurities can occur in the starting materials to be used in the formulation or in materials used in the manufacturing process of the TS [121,229,453]. Impurities in excess of 0.1% should be thoroughly identified. If the TS with the identified impurity is intended for long-term use, such as with a high dose of active compound, then mutagenicity and multiple-dose toxicity studies (up to 3 months) should be performed. If the results of the mutagenicity assays are positive, carcinogenicity testing of the impurity should be considered [213].

Preparation of Dose

The most critical aspects in preparing a TS for dosing are the identity of all ingredients, homogeneity of the product, particle size, stability of all active ingredients, and vehicle (carrier) to be used [121,229]. All batches of dosage preparations must be analyzed for concentration to confirm accurate preparation prior to use. Homogeneity of the TS–diet mixture and the stability of the TS in diet under the intended in-study conditions should be established prior to the start of an RCB. During the conduct of the RCB, samples of the TS–diet mixture are taken approximately every 6 months for analysis and correction if necessary. It is recommended that the same batch be used for the entire RCB. This presupposes that there is a validated method of analysis for the active ingredients. Quality control data as described earlier should be provided, and monitoring should be conducted regularly throughout the duration of the study.

ROUTE OF ADMINISTRATION

The route of administration should be appropriate to potential human contact and reflect knowledge about comparative bioavailability; thus, chemical disposition data are essential

and should be used in designing the 2-year RCB [191,194]. The most common route is the oral, followed by dermal, inhalation, or parenteral administration. The selection of a delivery system is crucial for all routes as it has the potential to significantly affect bioavailability. Second, producing uniform and homogeneous TS exposure throughout the bioassay is equally important. This requires the availability of a validated analytical methodology [212,396].

Oral

The oral route of administration is generally used for medicines and food additives for which ingestion is the usual route of contact for humans. For oral administration, the TS can be admixed in the feed or given by intragastric instillation (gavage). Comparable systemic exposures can be achieved with either, but with the intragastric route, the bolus dose results in a higher blood C_{max} [259]. With feed additives, attention must be paid to possible inappetence due to poor palatability.

The concentration of TS in the diet is adjusted to compensate for changes in body weight. During the rapid growth phase, the concentrations are adjusted biweekly first, weekly, and later monthly after the growth plateau has been reached. Here, TS stability and homogeneity data are essential. Periodically during the study, samples of TS–diet mixtures should be analyzed to confirm intended concentrations and enable corrective action to be implemented. In general, this is the most cost-efficient mode of dosing with most TS.

Intragastric administration (gavage) affords the most precise oral delivery of the TS. For this route of administration, a vehicle is required, such as carboxymethylcellulose (CMC). Edible oils (corn, olive) have been used, but these present problems [50], including effects on body weight [171]. For a TS that cannot be dissolved in water or CMC, polyethylene glycol 400 or a uniform suspension in CMC can be used. For a TS that is unstable or volatile in diet, microencapsulation can be used. For more details, see the Feeding Procedures section. In general, the volume of the TS preparation in rats is 3–5 mL/kg body weight. In mice, a volume of up to 10 mL/kg has been shown to be desirable as this enables more accurate delivery of the dose. In addition, dilution enhances absorption and decreases local irritation, especially with weak bases or acids that are the form of many TSs [35]. If deaths are due to gavage accidents, then a monitoring program should be implemented. In general, the baseline rate for accidents per technician should not exceed 1 per 10,000 gavages.

Dermal

Under certain circumstances of dermal contact by humans, such as with cosmetics, TS is delivered by the dermal route. Bioactivation of activation-dependent carcinogens does occur in the skin, although not to the degree as in the liver or intestinal tract [50]. This route is also used for photochemical carcinogen testing (see the following). Dermal application is usually to the superior dorsal area of the back (interscapular), where the skin is clipped at least weekly, 24 h prior to application (unless hairless mice are used) [268]. In dermal studies, animals are routinely housed singly to minimize ingestion by other cage mates. The TS (0.25–1 mL) is applied topically over the clipped area at intervals (e.g., two or three times weekly) to allow for recovery (especially when the TS is irritating). Skin penetration varies with species, chemistry of the TS, and vehicle [388]; thus, it should be considered in dermal dosing by weight or surface area that rats are large enough to vary significantly in size, whereas mice are not. Any dermal study should be preceded by or accompanied by measurement of skin cell proliferation, because skin neoplasms can be elicited by nonspecific enhancement of cell proliferation.

Inhalation (Intratracheal)

With some TSs, inhalation administration is indicated. The TS can be delivered to animals in chambers for 8 h/day [180] or by nose-only devices for several hours [402]. After delivery, the animals can either remain in the chamber or be taken to an adjacent room. Either way, constant airflow through the chamber during and after dosing prevents the build-up of ammonia. Single-cage occupancy is recommended to avoid ingestion due to grooming and licking. The cages should be rotated within the chamber periodically. Inhalation administration is an expensive, labor-intensive route of delivery that requires frequent monitoring of achieved concentrations. Sampling should be done from several fixed locations in the chamber after documenting the homogeneity of the test atmosphere [288]. With nose-only or head-only delivery devices [402], major advantages include minimization of external contamination and effective monitoring of respiratory parameters. The major disadvantages include restraining the animals, which leads to alteration of many physiological parameters and entails substantial manpower requirements.

Intratracheal administration can be conducted under anesthesia to achieve chronic delivery (up to 2 years) directly into the bronchial passages once or twice weekly [401]. This route has been used for NSPs [27,301,333,286]. Here, in addition to environmental control group, a second control group receiving vehicle should be added.

Parenteral Injection

In special cases, such as when the TS is broken down in the gastrointestinal tract, intraperitoneal, intramuscular, or subcutaneous injections are employed. Here, factors such as molecular size and pH have the potential to affect absorption and cause irritation [15]. Again, if a carrier is used, then a second control group administered the vehicle is needed. The delivery regimen here is limited to two or three times per week with rotation of injection sites. With dermal injection, subcutaneous sarcomas have been induced by the irritant or physical properties of the TS [155]. An insulin analog, insulin glargine, which produced injection site irritation and inflammation, was found to be associated with increases in malignant fibrous histocytomas at the injection site in both rats and mice [408].

CLINICAL AND PATHOLOGICAL EXAMINATION

The RCB is not an extended chronic toxicity study; standard chronic toxicity assays (6 and 9 or 12 months, depending on requirements) involve more clinical observations than are necessary or appropriate for the RCB. In the RCB, animals should be observed at the beginning and at the end of each work day. Attention should be paid to signs of dermal irritation, as this may lead to skin neoplasia. Unwell animals should be euthanized before they become moribund or die and are lost to autolysis (or cannibalization, if group caging is used). Real-time automated programs for carcinogenicity studies have been developed [330], which allow for monitoring, at least biweekly, the appearance, location, and growth of palpable cutaneous or subcutaneous masses. Standard parameters measured in the RCB include body weight, food consumption, temperature, health status, and mortality.

Body Weight and Survival

In an RCB using an MTD, controls can show greater weight gain and often poorer survival than the high-dose group in which body weight is reduced both by stress/toxicity, and by secondary effects resulting in reduced food consumption or energy utilization [277].

Sustained body weight gain suppression can lead to stress responses resulting in general protein catabolism and concomitant upregulation of heat shock protein (Hsp) 70 in the liver [196]. Over time, this results in systemic antigluconeogenesis, fat anabolism, and reduction of intermediary metabolism [192]. Here, there is a significant species difference between rodents and humans—namely, in rodents, subtle sustained hypoglycemia resulting from systemic antigluconeogenesis does not trigger an increase in food consumption [185,422]. As a consequence, the increased protein catabolism and body temperature, with concomitant slow decrease in tissue hydration, cannot be compensated for, leading to a chronic disruption (deficit) in energy homeostasis and metabolism, and associated interference with their feedback loops.

Intercurrent Diseases

Laboratory strains of rats and mice are susceptible to a variety of diseases, both genetic and acquired. The genetically determined conditions, importantly, include predisposition to the development of neoplasms (as discussed in the Species and Strain section). Also, pathologies such as amyloidosis in mice and chronic progressive nephropathy in rats are common. These conditions increase with age and can complicate long-term studies. Acquired diseases, such as sialoadenitis and murine hepatitis, can be minimized by proper animal husbandry. Many strains of mice harbor *Helicobacter hepaticus* in the gastrointestinal tract. Infection with this organism can lead to an increased incidence of liver neoplasms, particularly in male mice [159]. In hamsters, the chronic form of proliferative ileitis (wet-tail disease) caused by *Lawsonia intracellularis,* enhances the susceptibility of the small intestine to carcinogenicity resulting even in adenocarcinomas [233,502]. Satellite sentinel animals are included in all chronic studies to effectively monitor intercurrent acquired diseases.

Clinical Pathology

Several regulatory agencies have suggested the monitoring of continuous variables including hematology, clinical chemistry, urinalysis, and organ weights [222,447]. It must be realized that, in spite of initial randomization, aged rodents are no longer homogeneous because of nonrandom attrition and development of diseases in the second half of the in-life phase. Although such measures may be helpful at times, they need not be implemented without a specific reason (e.g., hematology smears to assist in the diagnosis of leukemia).

Anatomic Pathology

Pathology is an integral part of the protocol design and plays a pivotal role throughout the conduct, evaluation, and interpretation of carcinogenicity studies (as discussed in the Good Laboratory Practice section) [33,76]. In general, emphasis is placed on routine methods, but special pathology methods may be required depending on the target organs. Whatever can be anticipated from previous subchronic and chronic studies (e.g., effects on endocrine status) should be addressed in the protocol for pathology. Furthermore, microscopic changes must be correlated with clinical observations, body weight effects, survival patterns, clinical pathology data, macroscopic findings, and other information. GLPs and statistical considerations are discussed in separate sections in this chapter.

All animals euthanized or found dead are submitted to a complete necropsy. At both unscheduled and scheduled necropsies, the pathology team should be prepared for potential outcomes by participating in a prenecropsy briefing, at which all known clinicopathological correlations are discussed. At necropsy, body weights are obtained. Examination of all recorded palpable masses constitutes an initial procedure with the examination of all body orifices and skin. A ventral midline incision, with reflection of the skin to display subcutaneous tissues, initiates the opening of the abdominal cavity, followed by the thoracic and finally the cranial cavities. All macroscopic lesions are described as to their location, size, shape, consistency, and color. In general, organs should be examined in situ as well as after removal. Artifactual tissue damage (e.g., crushing or tearing of tissues) should be avoided or minimized and noted. Tissues are cleaned by rinsing in physiologic saline solution (tap water is not acceptable because the low osmolarity damages cells). Alimentary tract hollow organs are opened for better fixation and are examined after removal of their contents (their anatomic integrity should be maintained, if possible). Individual lungs are inflated (with 10% neutral buffered formalin: ~4 mL in rats and ~2 mL in mice), taking care not to overinflate, and, if indicated, the urinary bladder also (with formalin ~0.2–0.5 mL), after which the trachea and urethra are ligated to maintain the inflated state. Lesions and neoplasms are dissected to

include regional lymph nodes, if possible, and a small portion of surrounding (normal) tissue. At least 55 standard tissues and lesions are sampled, trimmed, and processed for histopathological examination, including blood and bone marrow smears [58,133,289] and brown adipose tissue (BAT; either mediastinal or periadrenal), which is emerging as an important, but neglected, tissue [196]. Any significant deviation from these procedures amounts to censoring, which potentially compromises the integrity of the study (see the GLP and Statistical Analyses sections). Organ weights are usually not taken in carcinogenicity studies because of the variability in weights caused by disease, neoplasms, or body weight variations. In some cases, selected organs are weighed [447,448], usually including the adrenals, brain, heart, kidneys, liver, lungs, spleen, testes (with epididymides), and uterus (including horns). Where organ weights are taken, percentage organ to body weight values are calculated [133].

The preparation of routine microscopic slides should be in accordance with established SOPs. Each slide should be matched with blocks and sampled tissues or macroscopically observed findings. Special methods, such as immunostaining for proliferating cell nuclear antigen or Ki67, compatible with formalin fixation, should be either described in detail in the protocol or be part of an SOP. Increasingly, these methods have shown utility, as they are capable of identifying cell proliferation, preneoplasia, and early neoplasia [48,195,331,510].

During microscopic examination, an open slide evaluation is recommended initially [189,190,327]. It consists of evaluation of the concurrent control groups first and then the high-dose groups. After this, the remainder of the dosed groups is evaluated, and the presence or absence of a dose–effect pattern is established. Open evaluation is preferably conducted by one pathologist. If the study entails more than 1000 animals, then one pathologist could read the males and a second the females to reduce the length of time to complete the histopathologic evaluation. Open evaluation is also performed when quantitation is performed (e.g., immunohistochemical assessment of cell proliferation). Under certain circumstances, when a reevaluation of certain tissue-specific lesions is necessary, a blinded microscopic examination of selected target tissues may be performed. In this type of examination, all slides are reevaluated in a blinded manner in a random sequence by a second pathologist, preferably one not previously familiar with the slides. Also, the valuable practice of peer review can be utilized. This consists of an independent examination by a second pathologist of all tissues from a representative sampling of randomly selected animals of both sexes from the control and high-dose groups and a representative sampling of proliferative lesions to substantiate the data from the initial evaluation for neoplasms and other proliferative lesions. Here, it is also recommended that the evaluation be open and that all changes (not only microscopic) are taken into consideration.

It is vital to record all pathology data in a consistent manner and to depict them as individual data in appendices. The toxicologic pathology nomenclature employed should be in accordance with the newly adopted internationally harmonized pathology nomenclature [284]. A summary of all dosing-related data should be in tables. An appendix depicting all missing tissues is highly desirable. Neoplastic diagnoses should adhere to an accepted nomenclature of histopathologic terms, according to recommendations made by the Society of Toxicologic Pathologists [76], with particular care to distinguish among proliferative non-neoplastic lesions, benign neoplasms, and malignant neoplasms [78,117,118,151,156,290]. The pathologist integrates all clinical, structural (macroscopic and microscopic), and functional (cellular and biochemical) data. Moreover, the pathologist ensures proper accounting of macroscopic and microscopic lesions and changes (as detailed in the GLP and Statistical Analyses sections).

The pathologist also has the responsibility to classify neoplasms as incidental or fatal. This is done by giving a Peto score [353] to all tumors that occur in preterminal decedents, including palpable tumors. Accidental deaths are classified as incidental for all animals that die or are euthanized moribund. After the terminal necropsy has commenced, these animals are considered part of the terminal necropsy, but the tumors are considered incidental [353].

In pathological evaluation, the most common deficiencies in GLP are as follows: (1) Macroscopic observations are not fully provided and the exposure-related ones are not compared side by side with microscopic findings; (2) macroscopic and microscopic pathology data do not match, and no explanation is provided; (3) organ weights in the study notebook and report do not match; (4) euthanasia dates preceding completion of the study are not in the final report, and no explanation is given; (5) differences between forms of data recording by the pathologist and those in the SOPs are not explained; (6) individual animal data in the notebook and data in tabular form in the report on the corresponding animal are not the same, and no explanation is provided; (7) there is a lack of uniformity in pathology nomenclature, and no explanation is provided; (8) there is lack of lesion accountability, and important tissues are missing without any explanation being given; (9) the method of slide evaluation is not stated (e.g., open, peer-reviewed); and (10) there is a lack of initialing and dating in various records such as macroscopic data, tissue trimming, microscope evaluation, and tissue recuts. The failure to provide an explanation for any of these deficiencies can compromise the RCB and/or require convening of a Pathology Working Group to address discrepancies.

In the case of medicines, where clinical trials are often ongoing at the time an RCB is conducted, unexpected tumor findings, once substantiated by histopathology, need to be reported to the appropriate regulatory agency as soon as possible.

RODENT CANCER BIOASSAY EVALUATION

Tumor Increases or Decreases

If the RCB has been conducted properly, it should provide adequate evidence to assess whether dosing has led to identification of carcinogenic activity or not, that is, presence or absence of statistically significant (see Statistical Analyses)

increases in neoplasia. A conclusion of lack of carcinogenic activity requires survival of an adequate numbers of animals with sufficient relevant exposures, usually at the MTD, with no evidence of neoplasm increases according to the criteria discussed above in the section Chemicals with Carcinogenic Activity. A conclusion of a positive outcome is often less rigorous; that is, in the interest of a conservative approach to hazard identification, a statistical increase in neoplasms is often accepted as valid in spite of artifacts in the study such as excessively reduced body weight gain or poor or unbalanced survival. It is important to consider the possibility that a neoplasm increase can be a consequence of the stress or toxicity of the dosing conditions [155] and not of a chemical action of the TS. Moreover, it is possible that the differences occurred by chance alone. This can be controlled to some extent by the use of two control groups to document in-study random variation [14,170]. A statistically significant greater incidence in neoplasms in a dosed group that does not exceed one of the control groups can be regarded as a *numerical imbalance*, not a true increase.

A critical aspect in the evaluation is the interpretation of pathological diagnoses. Findings of increases in malignant neoplasms are universally accepted as evidence of carcinogenic activity. Combining malignant and benign neoplasms of the same cell type of origin is also widely accepted, and guidelines have been published by the NTP [290]. It should be noted that in some tissues, certain benign tumors are not related to malignancies (e.g., benign mammary fibroadenomas and malignant mammary adenocarcinomas). When evaluating increases in neoplasms, attention must be paid to pathology in the tissue that is the site of neoplasms because cell injury and compensatory cell proliferation can facilitate neoplastic development (see the Cancer Hazard and Risk Assessment section below).

When assessing the incidences of neoplasms in groups, both the incidence in animals surviving to termination and the combined incidences of decedent and terminal animals are considered. Evaluation should be made with regard to the ranges of commonly occurring neoplasms (Tables 25.11 and 25.12), as well as the percent incidence profiles expressed by time of death (i.e., the neoplasm distribution) (Table 25.6) [14,28,40,59,89,169,175,283,293,360,369,411, 413,414,430,470,307]. Among the commonly occurring neoplasms in rats, pituitary, mammary gland, and skin

TABLE 25.11
Percent Incidence Ranges of Common Spontaneous Neoplasms in 104-Week-Old Rats

	Males Strain		Females Strain	
Sites/Neoplasms	**SD[a] (%)**	**Wi[b] (%)**	**SD[a] (%)**	**Wi[b] (%)**
Pituitary gland adenoma	1–70	22–51	26–93	2–61
Pituitary gland carcinoma	1–36	2–20	1–58	1–2
Pancreas islet adenoma	2–26	2–20	1–14	<1–2
Pancreas islet carcinoma	1–14	1–2	1–6	<1–2
Pancreas acinar adenoma	1–11	2–7	1–3	2–5
Pheochromocytoma	1–29	2–13	1–18	2–4
Thyroid C-cell neoplasia	2–30	6–23	3–28	5–24
Thyroid follicular adenoma	2–12	2–13	1–6	2–10
Skin fibroma	1–11	2–11	1–4	2–5
Skin keratoacanthoma	1–10	2–15	1–3	1–4
Mammary gland fibroadenoma	1–6	1–4	13–62	11–34
Mammary gland adenocarcinoma	1–4	<1–1	9–58	2–13
Mammary gland adenoma	1–2	<1–1	1–32	<1–1
Adrenocortical adenoma	1–8	2–7	1–34	2–4
Endometrial sarcoma	NA	NA	1–18	<1–1
Endometrial hemangioma	NA	NA	1–15	17–37
Endometrial polyp	NA	NA	1–10	2–17
Hepatocellular adenoma	1–8	<1–1	2–13	<1–1
Lymph node hemangiosarcoma	<1–1	2–15	<1–<1	1–2
Lymph node hemangioma	<1–1	2–13	<1–<1	2–11
Leydig cell testicular neoplasia	1–9	2–11	NA	NA
Lymphosarcoma, multicentric	1–6	2–10	1–10	2–6
Thymoma, benign	<1–1	2–5	<1–<1	2–10
Vaginal adenoma	NA	NA	<1–<1	2–10

Note: NA, not applicable; SD, Sprague–Dawley rats; Wi, Wistar rats.

[a] Data from Christian et al. [59], Brix et al. [40], Giknis and Clifford [146], and McMartin et al. [293].

[b] Data from Bomhard and Rinke [28], Eiben and Bomhard [99], Giknis and Clifford [145], Poteracki and Walsh [360], Tennekes et al. [413,414], Walsh and Poteracki [470].

TABLE 25.12
Percent Incidence Ranges of Common Spontaneous Neoplasms in 100-Week-Old CD-1 Mice

Sites/Neoplasms	Males	Females
Alveolar/bronchiolar adenoma	2–42	2–27
Alveolar/bronchiolar carcinoma	1–26	1–18
Hepatocellular adenoma	3–28	1–8
Hepatocellular carcinoma	2–16	1–4
Thymoma, malignant	2–15	1–2
Lymphoma, multicentric	4–28	2–50
Histiocytic sarcoma	1–8	2–18
Hemangiosarcoma	2–12	2–12
Pituitary adenoma	2–3	1–14
Harderian gland adenoma	2–14	1–8
Skin neurilemmoma, malignant	1–2	2–14
Endometrial polyp	—	2–17
Uterine leiomyoma/sarcoma	—	3–10

Sources: Data from Giknis, M.L.A. and Clifford, C.B., *Spontaneous Neoplastic Lesions in the Crl:CD-1® (1CR)BR Mouse*, Charles River Laboratories, Worcester, 2000; Maita, K. et al., *Toxicol. Pathol.*, 16, 340, 1998.

neoplasms occur predominantly after 50 weeks, whereas lymphoma occurs almost equally before and after 50 weeks (Table 25.6) [404]. In mice, lung, mammary gland, and liver neoplasms occur after 50 weeks, whereas lymphoma or leukemia occurs almost equally before and after 50 weeks (Table 25.6) [404].

The primary comparator for dosed groups is the concurrent study controls, of which there can be one or two groups (see Groups and Identification). Findings also can be discussed in the context of historical or published control data (see Tables 25.11 and 25.12) [14,89,139,157,260,369,307]. For any meaningful comparison with historical data, these must be obtained from the same strain and breeder, the same test facility and source of nutrition, and housing conditions. The historical control data should not be more than 5 years older than the current study and should not show changes in tumor incidence indicative of a genetic drift. Control data from the literature usually lack the comparability and detail to add much weight to the conclusions. This is reflected in the wording of EMA's Note for Guidance on carcinogenic potential: "Data from the literature might be added if thought to be informative." Whether any true increase in neoplasia has relevance for human cancer hazard is discussed in the Cancer Hazard and Risk Assessment section.

A controversial issue is the interpretation of a reduced incidence of a neoplasm in the RCB [81], which is quite frequent [173]. One consideration is that the reduction should not be attributable to nonspecific weight gain suppression (as discussed in the Feeding Procedures section). A variety of mechanisms for specific anticarcinogenesis have been delineated (see the Anticarcinogenesis section). Also, tumor decreases may reflect a systemic effect of the TS.

Statistical Analyses

Methods for statistical analysis were detailed in the previous version of this chapter [492] and are described in Chapter 9 [134]. Authorities have provided guidance on appropriate use of statistics [140,200,445,455]. The object of statistics is to substantiate whether dosing of TS has elicited a neoplastic response. Other than dosing, two other factors can underlie a numerical imbalance in neoplasia. One is bias (a systematic difference other than what is caused by dosing), and another is chance (a random difference). As chance cannot be completely excluded, especially when 50 or more tissues are being analyzed, using two control groups can provide a measure of variation between small groups. The probability of chance can be statistically measured; the smaller the probability, the higher the confidence [271]. Of the two types of mathematical models used in statistics, the stochastic one that contains uncertainties or random variables is mainly used to analyze RCB.

Theoretically, at least, randomization eliminates nonsystematic bias, but if there is another major source of variation (e.g., sex of the same strain or batch of the same strain), then a stratified randomization is more appropriate. To achieve that, separate randomization within each level of the stratifying variable (e.g., cage position, order of weighting, order of killing at termination) can be performed [271]. There are three aspects that determine the nature of collected data: the biological system, the study design, and the methodologies applied. Censoring of any of these must be minimized. In general, dosing variables are independent, and effect variables are dependent [135,271]. To enhance the identification of a dosing effect, the dose–effect trend is examined [412]. Furthermore, if there are differences in survival, age adjustment to avoid bias is necessary. Even if there are no meaningful survival differences, age adjustment increases the power of detecting differences between groups, as well as sharpening their contrast by avoiding dilution of pivotal (key) data with less important data. Age adjustment is effective when the context of observation is taken into account, that is, the neoplasm is judged by the pathologist to be fatal or incidental [353]. Finally, when multiple comparisons are to be made with unequal numbers in the groups, a number adjustment should be employed [132].

In the past, the single most important aspect in the analysis of RCB was a simple quantal response, that is, either neoplasia occurred or it did not. Currently, the mechanisms underlying neoplasia induced by chemicals are more fully understood and must be given individual consideration. The first statistical test used should be one that makes a global assessment, not a series of pairwise comparisons. The nature of the data should be established (i.e., whether it is continuous or discontinuous, scalar, rank, or quantal), and the distribution of these data should be examined [271].

In an RCB, the time course of adverse effects is of importance; consequently, life-table or Kaplan–Meier product limit methods are employed to determine the duration of survival and time until neoplasms develop, as well as the

probability of survival or time course of neoplastic development [74,78,353]. The presentation of life-tables should depict sex and administered dose and include the time of selected interval, as well as the number of animals that were alive during the interval, excluding the animals that were either withdrawn (e.g., interim euthanasia) or died during the same interval. Tumors should be classified as incidental or fatal (*Peto score*) (see earlier in the Anatomic Pathology section) [353]. For continuous outcome measures, which can be either scalar or ranked, group means are compared against the control mean at each termination point using a one-way analysis of variance (ANOVA) followed by Dunnett's method for multiple comparisons, which is a powerful post hoc test [96]. Moreover, a square-root transformation can be added to stabilize the variance. In addition, the continuous mean outcome levels are compared among termination times at each exposure level using one-way ANOVA followed by Tukey's multiple comparison procedure [124].

To assess the course of dose–effect (its linearity), ordinary least-squares regression analysis can be used fitting the outcome level vs. exposure and squared exposure terms. For the incidence of specific site neoplasia comparing all test groups, the Pearson chi-square test followed by pairwise comparisons of each exposure group with control, adjusted for multiple comparisons, can be applied [131]. For incidence trend analysis, the Cochran–Armitage test, partitioning the chi-square statistic into the overall trend and departure from linearity (p nonlinear) can be tested [123]. Furthermore, survival data can be used by applying log-rank test for both homogeneity and exposure-related trend [167,168]. Neoplasm data can be analyzed using a survival-adjusted trend test that discriminates among fatal, incidental, and palpable neoplasms (see earlier in the Anatomic Pathology section) [353]. If one or more tumor types in a valid RCB show a significant positive trend in incidence rates, the significance level (p-value) for rare (≤1%) neoplasms would be 0.025 and for common neoplasms 0.005 [455]. For pairwise comparisons (control vs. high dose), the significance of rare neoplasms would be 0.05 and of common neoplasms 0.01 [455].

Thus, for the determination of statistical significance, decision rules have been developed by the U.S. FDA CDER [275,455]. Tumor types are classified as common (>1% background incidence) or rare (≤1% background incidence), according to Haseman [166,167], on the basis of historical background incidences. The significance levels are as follows:

	Pairwise Difference	Trend
Common tumor	$p < 0.01$	$p < 0.005$
Rare tumor	$p < 0.05$	$p < 0.025$

These significance levels can be applied only in case two standard 2-year carcinogenicity studies have been performed. They aim to limit the false-positive rate to 10% [275,455].

When animal weights differ across dose groups, as is usually the case with high-dose testing, such differences can contribute to differences in neoplasm incidence (see the Feeding Procedures section). Analyzing tumor incidence within body weight strata can reduce the bias resulting from weight differences [141]. The bioassay report (see later) must contain a critical interpretation of analyzed data. The final interpretation should be based on both biological and statistical considerations [118,452]. The outcome can indicate either positive or negative concurrence of differences.

Eight considerations have proven to be very helpful in evaluation and interpretation using statistical methodology: (1) exposure–effect relationship, (2) incidence of proliferation and preneoplastic and early neoplastic markers at the target site of neoplasia, (3) presence of sex and species similarities or differences at the target sites, (4) convergence in target sites of nonproliferative chronic toxicity and neoplasia, (5) combined neoplasia increases in tissues affected by chronic toxicity, (6) neoplasms of similar histogenetic target sites in other sexes or species, (7) concurrent and historical control data, and (8) relative survival of control and exposed groups. Statistical analysis of all these aspects helps with the final interpretation.

BIOASSAY REPORTING

The final report of an RCB is submitted to the regulatory agency under whose purview it was performed [67,84,110,111,442]. The final study report consists first of an introductory section containing the compliance statement signed by the study director, followed by the quality assurance and study identification statements. The study identification statement provides the study title and number, the TS, the testing facility, the test facility manager, the sponsor, the study director, the principal investigator of all study aspects, the exact specific study timetable, and approved signatures from all final report authors, including the study director and investigators of all aspects of the study (e.g., analytical, toxicokinetics, during-life observations, pathology, and statistics). The first part of the final report itself is the summary, which is an abstract of the entire study. It contains, in this order, an introduction, a listing of the materials and methods, the results, and the conclusions. The summary is followed by a summary table that depicts all pertinent findings in tabular form.

After the summary section, an extensive introduction explaining the origin and purpose of the RCB is provided, followed by listings of test animals, test materials, methods, results, discussion, conclusions, and references. All sections should fully describe all methods used and all data obtained. All individual data (e.g., analytical, body weight, during-life, necropsy, and microscopy) should be in appropriate appendices. All relevant summary data (e.g., analytical and body weight) should be in tables. Numerical incidences should precede percent incidences. Tables and graphs presenting special issues and arguments should be included in the text (text tables or text graphs). Appropriate statistical analysis of correlation of survival patterns, clinical observations, body weight gain pattern, and TK data with macroscopic and microscopic findings should be conducted. All of these considerations have been discussed in other sections in detail.

An effective way to summarize the findings is a format used in Europe known as the tabulated study report. All relevant data are presented in standardized tabular form without narrative. This corresponds to the summary tables of the final report described earlier.

ASSESSMENT AND CLASSIFICATION OF EVIDENCE OF CARCINOGENICITY

The findings of an RCB are subject to evaluation and classification, either by the regulatory body to which it is submitted or by other entities. In the United States, the results of RCB on pharmaceuticals tested under an investigational new drug application approved by the FDA/CDER are submitted to the Reviewing Division, which then evaluates them, often with the participation of the CDER CAC. The CAC consists of a chair, an executive secretary, and members from several divisions, the Office of Epidemiology and Biostatistics, the Office of Testing and Research, and the Office of Pharmaceutical Sciences. The Reviewing Division (of the FDA) notifies the sponsor when a CAC meeting is scheduled after all RCB studies are submitted, and the sponsor may attend. The final interpretation of the results will appear in the labeling of the medicine, if approved. The FDA normally describes the RCB data usually with the comment that human relevance is unknown and description of the multiples of exposure in the rodents compared to humans.

In the EU, the nonclinical studies on carcinogenicity of pharmaceuticals are evaluated as part of the assessment of the application or marketing authorization by the Committee for Medicinal Products for Human Use (CHMP) of the EMA. CHMP consists of one delegate from each of the 27 EU member states, one delegate each from Iceland and Norway as members of the European Economic Area, and five additional co-opted members who are elected by the delegates from the EU member states. The CHMP can decide to ask for an assessment by its safety working party. Carcinogenicity in rodents and its possible relevance for humans are considered as part of the benefit–risk assessment. The key findings will be included in the Summary of Product Characteristics and the European Public Assessment Report.

At the U.S. EPA, an ad hoc CAC of the EPA Science Advisory Committee evaluates the submitted dossier. The agency formerly used an alphabetical/numerical classification ranging from group A (human carcinogen, based on animal data) to group E (noncarcinogen) (Table 25.13) [442,444], but this has been changed to a narrative classification [443,444], which allows the incorporation of mechanistic data, similar to the IFSTP classification described below.

RCBs conducted by the NTP are reviewed by a peer review panel and published as technical reports. They are used by the EPA, FDA, and OSHA for regulatory action [442]. The NTP uses a classification system of no, limited (some), or clear evidence of carcinogenicity [322]. The NTP also publishes a biennial *Report on Carcinogens* [323].

In the EU and Japan, similar classification schemes are used by various health boards and the CHMP of the European Medicines Agency [84,97,112].

TABLE 25.13
Classification of Carcinogens

IARC	HWC	EPA	IFSTP
Group 1	Group I	Group A	Group 1
Group 2A	Group II	Group B1	Group 2a
Group 2B	Group III	Group B2	Group 2b
—	—	Group C	—
Group 3	Group IV	Group D	Group 3a
—	—	—	Group 3b
—	—	—	Group 3c
Group 4	Group V	Group E	Group 4

IARC Group 1, HWC Group I, EPA Group A, IFSTP Group 1: The agent is carcinogenic to humans. There is sufficient evidence in humans of a positive relationship between cancer and human exposure such that chance, bias, and confounding variables can be reasonably ruled out.

IARC Group 2A, HWC Group II, EPA Group B1, IFSTP Group 2a: The agent is probably carcinogenic to humans. There is limited evidence in humans of a positive relationship between cancer and human exposure, but chance, bias, and confounding variables cannot be ruled out. There is sufficient evidence of carcinogenicity in animals based on an increased incidence of benign and malignant neoplasms in at least two species or in two independent studies.

IARC Group 2B, HWC Group III, EPA Group B2, IFSTP Group 2b: The agent is possibly carcinogenic to humans. There is either limited evidence or absence of data in humans; there is either sufficient or limited and weak evidence of carcinogenicity in animals (e.g., presence of other relevant data, genotoxic agents cause only benign tumors or increases in certain spontaneous neoplasms).

EPA Group C: The agent is possibly carcinogenic to humans. There is either an absence of data in humans or limited evidence of carcinogenicity in animals (e.g., agents that cause only benign tumors, or neoplasm incidence increases are marginal and not consistent).

IARC Group 3, HWC Group IV, EPA Group D, IFSTP Group 3a: The agent is not classifiable as to its carcinogenicity in humans.

HWC Group IV: The data are inadequate for evaluation, or these agents cannot be classified in other groups.

IFSTP Group 3a: The experimental data of epigenetic carcinogens show a threshold level within the range of human exposure.

IFSTP Group 3b: The experimental data of epigenetic carcinogens show a threshold level beyond the range of human exposure.

IFSTP Group 3c: The experimental data of epigenetic carcinogens show that their mechanism of action is not applicable in humans.

IARC Group 4, HWC Group V, EPA Group E, IFSTP Group 4: The agent is probably not carcinogenic to humans. There is evidence suggesting a lack of carcinogenicity in humans (even if inadequate) and in animals (negative animal studies). In IFSTP Group 4, the suspected carcinogens have not been sufficiently tested.

Notes: U.S. EPA, U.S. Environmental Protection Agency [437]; HWC, Health and Welfare Canada [178]; IARC, International Agency for Research on Cancer [201]; IFSTP, International Federation of Societies of Toxicologic Pathologists [118]; —, no corre-spondence.

The IARC convenes working groups several times each year to evaluate groups of chemicals with published carcinogenicity data. They issue the IARC monographs and IARC biennial reports with evaluations of experimental and human data. The grouping ranges from Group 1 (carcinogenic to humans) to Group 4 (evidence suggesting lack of

carcinogenicity) (Table 25.13). A deficiency in this classification is that Group 3 contains chemicals with data that are considered not likely to be a human cancer hazard.

The IFSTP [118] has proposed a classification as follows: (1) carcinogens for humans based on epidemiological data, (2) genotoxic carcinogens for animals based on experimental data, (3) epigenetic carcinogens for animals based on experimental data, and (4) suspected carcinogens insufficiently tested. This is the only classification that explicitly incorporates mechanistic distinction (Table 25.13).

CANCER HAZARD AND RISK ASSESSMENT

The first step in cancer risk assessment is hazard identification [221,319], which involves an RCB to identify dosing-emergent neoplasms (see Rodent Cancer Bioassay Evaluation section). Using dose–effect data from the bioassay and potential human intake, a cancer risk can be assessed [319], often involving allomorphic scaling to extrapolate the rodent exposure to human [440]. To identify a potential human cancer hazard, the RCB results should be interpreted together with other mechanistic data [118].

If the tested chemical is clearly DNA-reactive, then a neoplasm increase strongly implies a potential hazard to humans [280,481,488,492]. On the other hand, it is now recognized that epigenetic carcinogens may exert the effects underlying tumorigenicity only in particular rodent species (e.g., $\alpha_2\mu$-globulin nephropathy inducers in male rats) or only at high toxic doses (e.g., nitrilotriacetic acid–induced nephropathy). Such effects either are considered to be irrelevant to human hazard [439] or can be subjected to a margin of exposure (MOE) risk assessment [444]. The MOAs of carcinogenicity of epigenetic (nongenotoxic) agents involve a variety of secondary organ and tissue target sites, with indirect interference with the organ or tissue homeostasis. Sustained adaptive effects [501] and disruption of endocrine, paracrine, nervous, and immune systems can be involved in the pathogenesis of neoplasia induced by such agents. Accordingly, the carcinogenetic effects of epigenetic agents are typically species, sex, and tissue specific.

These indirect epigenetic mechanisms involve key events such as enhancement of cell proliferation, disruption of hormonal feedback pathways, inhibition of trophic activity in tissues (including long-standing tissue ischemia), immune surveillance dysfunction, inhibition of enzymatic reaction/activation in cells, sustained exaggerated pharmacological effect, and sustained accumulation of normally low levels of endogenous products. All of these effects result in sustained cellular stress and/or toxicity, leading to compensatory proliferation, which is a common pathway through which chemicals with diverse cellular effects ultimately elicit or enhance neoplasia [62,72,195]. The effects that lead to compensatory cellular proliferation usually require high levels of exposure and exhibit no-observed-adverse-effect level (NOAEL). It is probably for this reason that, of the NCI/NTP rodent carcinogens identified, 6% had increased incidences of neoplasms that were limited to the top dose for all sites of tumor increases [172]. Examples of rodent neoplastic effects that are considered to be either not relevant, or possibly not relevant to human hazard, along with some MOA, are described in more detail in the next section.

It has been speculated that carcinogens that are highly potent (i.e., short latency, multiple tissue targets, and activity in more than one sex and species) are likely human carcinogens [12]. This reflects the fact that such chemicals are usually DNA-reactive. However, epigenetic carcinogens can also exhibit these features, and accordingly more information is needed for classification beyond the tumor profile. In this regard, understanding the chemical MOA pathway is essential to determining the relevance of tumor findings to human hazard [29,30,297,392,405].

Finally, in order to effectively extrapolate tumor findings from rodents to humans, an additional scaling factor based on the totality of the general cancer biology behavior of the rodents compared to humans is worthy of consideration. The differences in percent incidences of selected common neoplasms in all three species are provided in Table 25.14.

TABLE 25.14
Comparison of Percent Incidences of Selected Common Human and Rodent Neoplasms That Occur in All

	Humans[a]				SD Rats[b]				CD-1 Mice[c]			
	Males		Females		Males		Females		Males		Females	
Site	Percent	Rank	Percent	Rank	Percent	Rank	Percent	Rank	Percent	Rank	Percent	Rank
Lung	0.032	2	0.027	2	0.1	23	0.1	23	15	2	15	2
HLR[d]	0.011	6	0.010	5	2	10	2	11	8	3	22	1
Breast	NA	NA	0.071	1	NA	NA	58	2	NA	NA	6	3
Liver	0.004	9	0.002	13	5	6	3	6	18	1	3	5

Note: NA, not applicable.

[a] Data from American Cancer Society [232]; in humans, the percent figure reflects new cancer cases estimated from 2004 U.S. population.

[b] Data from Christian et al. [59], Giknis and Clifford [146], and McMartin et al. [293].

[c] Data from Giknis and Clifford [144]; in rats and mice, the percent figure reflects spontaneous neoplasms observed in excess of 1000 control animals.

[d] Hematolymphoreticular tissue neoplasms correspond to the diagnosis of lymphoma in humans.

For example, in humans of both sexes, lung neoplasia is a very common neoplasm, yet the percent incidence is only 0.032 M/0.027 F of the estimated cases from the U.S. population, whereas in mice it is 15%, and in rats it is 0.1%, somewhat closer to the human incidence. The interrodent percent difference (150 times) can be explained using the comparative adult tissue volume ratios (Table 25.9), which show that the mouse ratio is 33 times higher compared to the rat ratio. Thus, one should consider many species differences before ascertaining neoplasm incidences and *lifetime* risks (Table 25.14).

MODES OF ACTION

As discussed in the preceding sections, various criteria are applied to the assessment of carcinogenic activity. This includes MOA, where such information is available. Among the many established MOA (see Types of Carcinogens), DNA reactivity is widely regarded as relevant to human hazard. In contrast, for several neoplastic responses in rodents, sufficient mechanistic information has accrued to support general recognition that their underlying MOA are species specific and do not operate in humans. Others arise through MOA that are possibly or unlikely to be relevant to humans.

Modes of Action Not Relevant to Humans

Rat Gastric Neuroendocrine Neoplasm (Carcinoid) Elicited by Suppression of Gastric Acid Secretion

Hyperplasia and neoplasia of gastric neuroendocrine (NE) cells (enterochromaffin-like cells) are stimulated by gastrin in rats and to a lesser degree in mice. Elevations of gastrin are elicited by reduced gastric hydrochloric acid production, which can be caused by either gastric antisecretory medicines such as proton pump inhibitors (e.g., omeprazole) or histamine (H_2) antagonists (e.g., cimetidine) [42,300]. Chemicals that cause gastric atrophy (e.g., alachlor and butachlor) also have elicited this neoplasm (Table 25.2) [415]. Rats have a high density of gastric NE cells, achieve high levels of gastrin (over 1000 pg/mL), and are very responsive to elevation of gastrin [424]. For most hypoacidity-producing agents, female rats are more susceptible than males to the development of NE cell neoplasms. NE cell neoplasms have been observed in patients with multiple endocrine neoplasia syndrome (MEN-1), associated with elevated gastrin, but not with antiulcer therapy [300]. Significant NE cell proliferation in humans is seen only with gastrin, levels above 400 pg/mL, and this can be controlled in the clinical setting. Thus, this tumor response does not denote a practical human hazard [8,130,209].

Rat Kidney Neoplasm Resulting from $\alpha_{2\mu}$-Globulin Nephropathy

A variety of chemicals induces kidney neoplasms in male rats, especially F344, which excrete $\alpha_{2\mu}$-globulin in the urine. This protein is associated with hyaline droplet formation, atypical hyperplasia of the epithelium of the P_2 segment of the proximal tubules, and neoplasia. Male rats are proteinuric compared to humans, and no human renal protein is similar to $\alpha_{2\mu}$-globulin [439]. This MOA was confirmed with D-limonene administration to $\alpha_{2\mu}$-globulin transgenic mice [273]. Accordingly, it has been accepted that renal tubule neoplasms produced as a result of the $\alpha_{2\mu}$-globulin accumulation mechanism are not an appropriate endpoint for human hazard identification [8]. Agents associated with this neoplastic effect include D-limonene and trimethylpentane (Table 25.2) [347,415,439]. Likewise, an IARC working group concluded that an agent that acts solely through $\alpha_{2\mu}$-globulin nephropathy in the production of renal cell neoplasms alone in male rats is not a cancer hazard to humans [374].

Rat Mesovarial Leiomyoma

Smooth muscle tumors of the ovarian suspensory ligament have developed in female rats after long exposures to β_2-adrenoceptor stimulant medicines (Table 25.2) [8,243]. This neoplasm is rare in humans, and the agents that have induced it in rats (soterenol, mesuprine, zinterol, terbutaline, reproterol, and salbutamol) are not associated with cancer in humans. Hence this neoplasm is not relevant for humans.

Rat Urinary Bladder Transitional Cell Neoplasm Resulting from Luminal Milieu Modification

Many investigations have used rat models for urothelial neoplasia. In early studies, it was recognized that placement of inert pellets in the bladder lumen would elicit increases in urothelial neoplasia [60]. Rats have been shown to be more sensitive than mice to urothelial damages, apparently because the rat bladder urothelium lacks tight junctions, thus rendering the superficial layer ineffective as an intraluminal barrier and leaving the underlying layers vulnerable to chronic stimulation [193,265]. Moreover, rats, particularly males, have more intraluminal proteins, silicate precipitation, crystal formation, and urolithiasis than other species [61,63]. In particular, rats, unlike humans, develop calcium phosphate urinary precipitates [374]. The consequences of this are exacerbated by the fact that the bladder is horizontal in rodents (and dogs, which have also been used to study bladder carcinogenesis) and does not empty as effectively as the vertical human bladder, despite the fact that rats urinate frequently. Thus, rats are more prone to chronic cell damage to the bladder urothelium, which results in cell proliferation and neoplasia [62,193]. This effect does not occur in humans [62,100,506]. An IARC working group has concluded that production of bladder cancer in rats under conditions of formation of calcium phosphate–containing urinary precipitates is not predictive of cancer hazard to humans [374]. Conditions and chemicals associated with this neoplastic effect include crystalluria (PPARγ agonists—proglitazone, pioglitazone; PPARα/γ agonist—muraglitazar) and urine pH extremes (melamine, saccharin, carbonic anhydrase inhibitors, and dietary phosphates) (Table 25.2) [60–63,100,193,265,374,410].

Rat Vaginal–Cervical Granular Cell Neoplasm

A proliferative lesion of *granular* cells with granular eosinophilic cytoplasms occurs in the vaginal–uterine cervical regions of female Sprague–Dawley, Donryu, and Wistar rats [73,386]. This lesion is probably under hormonal influence, mainly estrogen. Granular cell aggregates occur rarely in the vulva of women, but no evidence suggests that the pathogenesis is similar to that of rat granular cell tumors. Thus, this lesion is considered not relevant for humans.

Rodent Liver Neoplasm Elicited by Hepatic Peroxisome Proliferator–Activated Receptor α Agonists

A wide variety of chemicals elicit increases in rodent liver tumors associated with the proliferation of peroxisomes [205]. Rodents are more susceptible to induction of hepatic peroxisome proliferation by PPARα agonists than primates or humans [53,205,498], apparently because of high expression of the PPARα in rodent liver [427]. Perhaps related to this, it has been reported that, in cultured rat hepatocytes, PPARα agonists enhanced DNA synthesis and suppressed apoptosis, whereas in human hepatocytes, DNA synthesis was suppressed and apoptosis enhanced [351]. The mechanism of carcinogenicity of PPARα agonists (Table 25.2), while not fully understood, appears to involve sustained hepatocellular proliferation as a key event [150,256]. No PPARα agonist is associated with cancer in humans, and an IARC group has recommended that a liver neoplasm response in mice or rats secondary only to peroxisome proliferation could modify the evaluation of carcinogenicity [205].

Rodent Subcutaneous Sarcoma at Injection or Implant Site

Rodents, especially males, are highly susceptible to the development of subcutaneous neoplasms, both as background and induced occurrences [154]. These neoplasms are variously diagnosed as fibrous histiocytomas, fibrous sarcomas, liposarcomas, and leiomyosarcomas. They can arise from a solid-state effect known as the Oppenheimer effect [339] with implantation of solid materials. Such sarcomas are also induced by subcutaneous injection in rodents of nongenotoxic irritant materials such as iron-dextran [154]. No material producing such an effect in rodents (including recombinant human insulin [356]) has been associated with cancer in humans [37].

Rodent Thyroid Neoplasm Resulting from Thyroid–Pituitary Feedback Homeostasis Disruption

Few DNA-reactive carcinogens elicit thyroid neoplasms, probably because bioactivation does not occur to a significant extent in this gland. On the other hand, disruption of thyroid–pituitary feedback homeostasis is a common mechanism of thyroid carcinogenesis in rodents, particularly rats [8,52,417]. Reduced thyroid hormone levels, either through inhibition of iodide uptake (e.g., perchlorate), inhibition of hormone synthesis by antithyroid agents (e.g., propylthiouracil), or increased clearance as a result of enhanced conjugation (e.g., phenobarbital), can lead to a feedback increase in thyroid-stimulating hormone levels, which produces thyroid follicular cell hypertrophy, hyperplasia, and eventually neoplasia. Species differ in their susceptibility to disruption of thyroid economy, with the rat being particularly sensitive [102]. Several inducers of hepatic thyroid hormone conjugation in rats (which often is also associated with increased liver neoplasms), as discussed in the next group of MOAs, do not affect mice [469]. Only radioactive chemical exposure is known to cause thyroid follicular neoplasms in humans [374]. Accordingly, it has been concluded that chemical-specific data on thyroid effects in rodents can be applied to risk assessment [183], and that agents (Table 25.2) that cause thyroid neoplasia through an adaptive hormonal mechanism belong to a different category from those acting through genotoxic effects or involving pathological response to tissue injury [374]. Thus, these epigenetic agents are considered to pose no cancer hazard to human [8].

Modes of Action Possibly Not Relevant to Humans

Several MOAs of epigenetic oncogenesis in rodents are possibly not relevant to human cancer hazard [8]. If only neoplasms arising through one of these MOA is elicited by the TS, then the effect is likely not relevant to humans.

Mouse Ovary Tubular Adenoma

This is a benign neoplasm with tubular, stromal, or mixed components, which occurs mainly in mice [7,51]. Tubulostromal adenomas have been observed with cytotoxic agents but are not seen in other laboratory animals or humans and are considered to be possibly not relevant to humans [7,8,51].

Mouse Urinary Bladder Mesenchymal Lesion

This lesion occurs in the posterior portion of the urinary bladder close to the trigone area and has been known under various names for some time [236]. Recently, the lesion has been referred to as a *mesenchymal tumor* [46,160]. The lesion has been found in mice given agents that bind to progesterone (mainly) and estrogen receptors (e.g., many oral contraceptives) [291]. Persuasive evidence has been provided [236,237] that the lesion represents a decidual reaction of mesenchymal cells carrying or developing progesterone receptors. No known counterpart of this lesion has been described in humans; therefore, its significance is questionable.

Rat Adenohypophysis Neoplasia

Most pituitary neoplasms are adenomas in both rodents and humans [20,156,294]. These common fatal neoplasms affect female rats more often than males and occur late in life [404] (Tables 25.6, 25.7, and 25.11). The majority of these neoplasms contain prolactin (PRL) cells suggesting that decreased dopamine content in old age is involved in the production of these neoplasms [365]. In addition, AL fed rats develop more pituitary neoplasms, at 2 years, compared to DR (by 20%) counterparts [22,425]. Furthermore, in the DR rats, PRL, estradiol, luteinizing hormone (LH), GH, and IGF-1 levels were reduced [303]. Nevertheless, the majority

of the background pituitary neoplasms contain PRL [315]. To date, neuroleptics (dopamine inhibitors), long-term dosing with estrogens, gonadotropin-releasing hormone (LHRH)–like drugs (goserelin, leuprolide, and calcitonin), as well as gonadectomy, thyroid ablation, iodine deficiency, dosing with goitrogens and ionizing radiation have been associated with increased pituitary neoplasms (Table 25.2) [149]. Based on all these, in rats, these neoplasms are the result of hormonal feedback interference, which is not operational in humans, where in women, hyperprolactinemic amenorrhea is often associated with pituitary neoplasia [13,3]. Thus, in humans, despite chronic dosing of contraceptive steroids or estrogen replacement therapy (monitored through imaging), no causal relationship has been established for these neoplasms [13,3].

Rat C-Cell Thyroid Neoplasia

Thyroid C-cells (clear, parafollicular, or calcitonin-producing cells) are more numerous in laboratory animals than in humans. C-cell neoplasia occurs both as background (Tables 25.7 and 25.11) and as exogenously induced findings in RCB with rats (most commonly), mice, or even hamsters [302,52,86,36]. Hyperplastic and neoplastic C-cells produce calcitonin as well as neurotensin and somatostatin [520,358]. C-cell hyperplasia preceding neoplasia has been observed in rats, but not in mice or hamsters [87,467]. C-cell hyperplasia in both rats and humans is accompanied by blood calcitonin elevation [302,52,36]. The C-cell medullary carcinoma variety occurs in the susceptible Long-Evans rat strain [86]. Administration of radioactive iodine, which induces follicular cell neoplasia, reduces the number of C-cell neoplasms compared to controls [342]. Finally, dosing with exenatide, an antidiabetic nongenotoxic drug given at up to 130 times the human exposure, as an s.c. injection produced benign C-cell adenomas in female SD rats [357]. Thus, C-cell hyperplasia and neoplasia, induced by nongenotoxic compounds, appear not to represent a human cancer hazard.

Rat Clitoral Gland Neoplasm

This rare neoplasm occurs sporadically and, if found in a TS group, can be a subject of concern. No chemical has been shown to reproducibly induce these tumors, and no underlying mechanism has been identified.

Rat Hibernoma (Brown Adipose Tissue Tumor)

Neoplasms of BAT occur in the mediastinum or periadrenal area of rats. This tissue is now considered to be an endocrine organ [196,246]. Several chemicals have been reported to produce a flat low-incidence pattern, mainly in male rats, including some antihyperglycemic thiazolidinediones (TZDs), imidazopyridine hypnotics [356], phentolamine (PHEN), a nonselective α-adrenergic receptor antagonist [196,361], and triazolone herbicides. To date, no chemical has been conclusively shown to induce BAT preneoplastic lesions or early (small in situ) neoplasms. Some TZDs, which have conclusively induced dose-related BAT hyperplasia in both rats and mice, do not stimulate progression of this chronic hyperplasia into preneoplasia or neoplasia after 2 years of continuous exposure [179]. Moreover, in a study with 12 month exposure to PHEN, focusing on the structure, function, and pathology of BAT in younger (6 weeks of age) and older (36 weeks of age) rats, no increased BAT hyperplasia, decreased BAT apoptosis, or preneoplastic lesions were present [196]. In addition, PHEN doses after 12 months of administration increased rectal temperature, decreased epinephrine (EPIN), and norepinephrine (NEPI) levels, but did not induce BAT hyperplasia. Temperature, EPIN, and NEPI changes were more pronounced in younger rats but were not present after 4 weeks of recovery. Hypothermia and sustained increases in EPIN and NEPI are considered important conditions for the induction of BAT hyperplasia. BAT neoplasms are very rare benign neoplasms in both rodents and humans [196]; however, this tissue has not been routinely sampled in RCB, and hence BAT neoplasms are recorded only when macroscopically evident. To date, malignant hibernomas have not been conclusively described in humans [103,245,478]. Review of TZD-induced hibernomas in rodents [179] further supports that they are also benign and as such of limited significance to humans, since gluconeogenesis, protein catabolism, intermediary metabolism, and energy (body temperature) homeostases and their feedback loops are different between rodents and humans (see in the Body Weight section).

Rat Mammary Gland Fibroadenoma

This is a benign neoplasm with a minor glandular epithelial component and a predominant pericanalicular type of proliferation of connective tissue. It bears little resemblance to the common intracanalicular type of fibroadenoma seen in women [377,380], which is hormonally responsive [16]. Fibroadenoma is the most common background breast neoplasm in all the routinely used rat strains (Tables 25.7 and 25.11), occurring usually >50 weeks of age (Table 25.6) and does not progress to malignancy. Thus, combining fibroadenomas and carcinomas is not appropriate, and fibroadenomas by themselves are of questionable human relevance. Fibroadenomas in rats are stimulated by PRL [192,17,120], whereas in humans, stimulation is also by estrogen (mainly) and progesterone [17,192,370].

Rat Mononuclear Cell Leukemia

MCL, also referred to as large granular lymphocyte leukemia, is a neoplasm that initially develops in the spleen and then in the liver, lungs, lymph nodes, and bone marrow. It occurs at over 18 months of age in high incidence (62% in males and 42% in females) in F344 rats [174] and is lethal (Table 25.7). No chemical has been demonstrated to reproducibly induce this neoplasm, so nothing is known about its pathogenesis. A number of chemicals (furan, C.I. Direct Blue 15, iodinated glycerol, diisononyl phthalate, and dimethylmorpholinophosphoramidate) are reported to be associated with increased incidences of MCL [101,276]. These have not been implicated in human cancer.

Rat Pancreatic Islet Cell Neoplasia

All species of rodents (but especially rats) develop later in life background pancreatic islet cell neoplasia (Tables 25.7 and 25.12)

consisting of insulin- (mainly) but also glucagon-, somatostatin-, and polypeptide-positive containing cells [378,517,308,88]. In humans, this neoplasm is rare (~1% of all pancreas neoplasms) [334]. Differences in endocrine and exocrine pancreas homeostases have been discussed in the Feeding Procedures, Body Weight, and RCB Evaluation sections. Namely, sustained hypoglycemia resulting from systemic antigluconeogenesis does not trigger an increase in food consumption in rodents [185,422], resulting in a chronic disruption (deficit) of energy homeostasis and metabolism. Experimentally, islet cell neoplasia has been produced in rats by streptozotocin or alloxan with or without nicotinamide or picolinamide [516], although the MOA has not been thoroughly elucidated. Probably, it is based on species-specific hormonal feedback interference, which is not operational in humans.

Rat Scrotal Vaginal Tunic Mesothelioma

This mesenchymal lesion, which includes hyperplasia and neoplasia [292], arises from the serous membranes of the scrotal tunica vaginalis testes. It is common (about 3%) in F344 rats [174] (Table 25.7). Because the scrotal lesion often arises in association with testicular neoplasms, especially the commonly occurring Leydig cell neoplasms [285], which assume a large size, an element of physical irritation may be involved in the pathogenesis. Thus, chemicals that induce only this tumor type are likely irrelevant to human hazard. Chemicals that produce increases in this tumor (e.g., acrylamide, potassium bromate, and pentachlorophenol) are structurally diverse, and no MOA has been established. In humans, none of these is associated with mesothelioma or any other cancer [211].

Rat Skin Fibromas

This subcutaneous benign, well-localized background neoplasm (Tables 25.6, 25.7, and 25.11) is difficult to distinguish from mammary fibroadenoma where glandular elements have atrophied. It is more common in males than females in all strains and occurs later (>50 weeks). If some of these have assumed malignant features, then careful examination is warranted, since recently, studies with PPARα/γ agonists have increased fibrosarcomas. Nevertheless, their human relevance is uncertain [461].

Rat Splenic Sarcomas

Following chronic administration of aniline (and aniline-based compounds), *p*-chloroaniline, *o*-toluidine, D and C Red No. 9 dyes, splenic sarcomas were observed in F344 rats [473,480]. The underlining MOA with anilines was postulated to involve methemoglobinemia, splenic erythrocyte sequestration with congestion, iron accumulation, and resulting oxidative stress, which produces increases in interleukin 1a, b, TNFα, and enhanced fibrogenesis and neoplasia [247,471]. With the antimalarial (together with pyrimethamine) and antileprosy drug dapsone (4,4′-diamino-di-phyenylsulphone) in an RCB in male rats, splenic congestion, necrosis, and sarcomas were evident [473,480]. Yet, years of therapeutic use produced no splenic sarcomas. Accordingly, this neoplasm is possibly not relevant for humans.

Rodent Adrenal Medulla Neoplasms (Pheochromocytomas)

The most common neoplasia of the adrenal medulla in rodents is pheochromocytoma (or chromaffin cell neoplasm). They occur most commonly in the hamster, followed by the rat and mouse (Table 25.7) [156,174]. In many situations, pheochromocytomas have been associated with systemic hypoxemia, which stimulates catecholamine secretion from the adrenal medulla where chronic endocrine hyperactivity may lead to compensatory adrenal medullary hyperplasia and neoplasia, and indeed in rats, pheochromocytomas are often found in a background of diffuse medullary hyperplasia [156]. Agents producing this feedback interference include lactose, sugar alcohols, and Ca^{2+} [344,420]. Pheochromocytomas elicited through this type of interference are most likely not relevant for humans [158].

Rodent Endometrial Neoplasia

Endometrial carcinoma was produced in an RCB in rats dosed with bicalutamide, a nonsteroidal antiandrogen [223]. This drug was exclusively used in men to treat prostatic cancer, and consequently the neoplastic finding in female rats was considered irrelevant. Moreover, chronic exposure to pergolide, a dopamine receptor agonist, produced endometrial neoplasia in rats and leiomyomas/sarcomas in mice. It was believed that both neoplasms reflected PRL inhibition and disruption of the estrogen/PRL feedback homeostasis [8,356], which is not operational in humans.

Rodent Forestomach Squamous Cell Carcinoma

The rodent forestomach is a portion of the stomach situated between the esophagus and glandular stomach, which is lined by squamous epithelium. It does not exist in humans. Squamous cell carcinoma of the forestomach is a rare neoplasm, with the exception of $B6C3F_1$ mice (Table 25.7). A number of DNA-reactive agents have induced these neoplasms in rodents [263], usually through direct contact from gavage administration. Mostly, nongenotoxic agents have produced increases in this neoplasm (Table 25.2). The epigenetic mechanism appears to involve chronic irritation leading to a promoting action, which requires high exposure, as shown for butylated hydroxyanisole [209,506,508]. An IARC working group [209] concluded that epigenetic agents that produce only forestomach tumors after prolonged exposure may be of less relevance to humans, since human exposure would need to surpass time-integrated dose thresholds in order to elicit the carcinogenic response. Indeed, none of these chemicals has been associated with cancer in humans.

Rodent Harderian Gland Neoplasia

Spontaneous neoplasms of this merocrine ocular gland in hamsters, rats, and more commonly mice (at an incidence of 0.5%–15%) [156,174,261]. Adenocarcinomas of the Harderian gland have been reported with a number of genotoxic

(ganciclovir-IV) [357] and nongenotoxic (zoledronic acid) agents in mice [356]. The relevance of neoplasms of this gland, which is not present in humans, is at best questionable.

Rodent Hemangioma/Hemangiosarcoma

This vascular neoplasm is a common background neoplasm in mice (Table 25.12) and less so in rats (Table 25.11) and occurs rarely in humans. In CD-1 mice, it is usually multifocal and affects many organs almost equally in males and females. The range of incidence is 6%–33% in males and 7%–32% in females [144]. Organs commonly involved (>2%) include spleen, liver, lymph nodes, testes, skin, and pancreas in males and uterus, spleen, liver, ovary, lymph nodes, and heart in females [144]. Increases in vascular neoplasia and hemangiosarcoma (HS) have been found in rodents with both DNA-reactive (e.g., vinyl chloride, vinyl bromide, and thorotrast) and epigenetic (e.g., pregabalin, elmiron PPAR α and/or γ agonists, and 2-butoxyethanol) agents [64,77,162,227,267,332]. A common MOA appears to exist for epigenetic induction of HS in rodents, consisting of convergence of key initiating events that include tissue hypoxia, hemolysis, decreased respiration, and adipocyte growth, all leading to dysregulated angiogenesis and/or erythropoiesis [64,77,267]. In humans, no comparable species-specific multifocal lesion has been reported, and no inducing agent has been established [67,77,429]. To date, chemical-induced hemangiosarcomas (primarily in the liver) are produced by vinyl halides and thorotrast (both genotoxic) in both rodents and humans [77].

Rodent Histiocytic Sarcoma

This neoplasm of histiocytes, found in older mice and less commonly in older rats, affects mainly the liver and uterus [429]. No chemical is known to produce an increase in only this neoplasm, and therefore it is possibly not relevant to humans [117,429].

Rodent Liver Neoplasm Induced by Phenobarbital-Like Enzyme Inducers

A variety of compounds elicit an increase in liver neoplasms in mice and rats through an MOA involving liver hyperplasia (Table 25.2) [489]. The prototype compound is phenobarbital [483]. Consideration of kinetic and dynamic factors led to the determination that the MOA was unlikely in humans [187,483]. Indeed, extensive epidemiologic data reveal no increased risk of cancer [338].

Rodent Osteomas/Osteosarcomas

Primary bone neoplasia in rodents and humans is uncommon. Background bone neoplasms in rodents are likewise rare [350,379]. Bone neoplasia has been induced in rodents by ionizing radiation, vinyl chloride, and nitrosamines [153,349]. Chronic administration of diethylstilbestrol (DES) or 17β-estradiol to mice also produced bone tumors [181]. Also, administration of an antiosteoporosis drug, teruoaretude/rh PTH, in an RCB in rats, produced dose-dependent hyperostosis, osteosarcomas, as well as osteomas and osteoblastomas [356,186,463,464]. In addition, hyperostosis and bone neoplasms (osteomas and osteosarcomas) have been produced in rats after 2 years of glucocorticoid administration [521]. Finally, proestrogens and prostaglandin E–like compounds in RCB have produced hyperostosis and osteosarcomas in mice [238,359]. In all these cases, with nongenotoxic and endocrine-active compounds, the initial MOA seems to be sustained (chronic) interference with endocrine feedback homeostases of bone growth patterns, which are not operational in humans.

Rodent Testicular Leydig Cell Neoplasm Resulting from Hormone Homeostasis Disruption

Leydig or interstitial cell neoplasms occur spontaneously in high incidence (>80%) in aged F344 rats (Tables 25.7 and 25.11) [174]. These tumors are invariably benign. In rats, they result from increases in LH [116] and in mice from increases in estrogen [363] or administration of estrogen agonists such as DES or tamoxifen [287,426]. The human counterpart is extremely rare, and no chemical that produces increases in rat testicular neoplasms (i.e., lactose, sugar alcohols, cimetidine, hydralazine, gemfibrozil, carbamazepine, vidarabine, isradipine, exogenous gonadotropins, LHRH analogues, flutamide, ergolines, and finasteride) has been associated with the induction of this or any other neoplasm in humans. The available information, therefore, suggests that nongenotoxic compounds that induce Leydig cell neoplasms in rodents do not represent a human cancer hazard [70].

Types of Cancer Hazards

Formerly, all rodent carcinogens were regarded as potential human cancer hazards. This concept was embodied in the Delaney Clause to the 1958 Federal Food, Drug, and Cosmetic Act (FFDCA), which provided that no chemical determined to be carcinogenic in animals could be allowed as a food additive, regardless of concentration. Subsequently, expanded understanding of mechanisms of carcinogenesis has led to refinements of hazard assessment [492]. In evaluation of drugs, the FDA and other regulatory bodies have long applied risk–benefit analysis. Beginning in 1992, the IARC accepted data on mechanisms as being relevant to the evaluation of the carcinogenic risk of a chemical to humans [203], and this has been further developed [209,374]. A critical aspect is the distinction between DNA-reactive and epigenetic carcinogens (see Types of Carcinogens section). This has been applied by the EPA in concluding that the rat kidney neoplasm response to $\alpha_{2\mu}$-globulin nephropathy inducers is not relevant to humans [439]. The potential hazards of a variety of DNA-reactive and epigenetic carcinogens were explored in detail by an international group of experts, drawing on comprehensive reviews of MOA of 10 prototype carcinogens both epigenetic and DNA-reactive [218]. These experts concluded in 1996, that the mechanism of carcinogenicity of a chemical can determine whether or not a risk to humans exists [218].

Currently, in the assessment of potential human cancer hazard, regulatory agencies often refer to findings implicating a chemical as a *genotoxic* carcinogen, usually with only an operational definition [66], namely, that the chemical produced a positive result(s) in some mutagenicity test [252]. All chemicals that are reliably positive in a variety of genotoxicity assays, especially including a DNA damage assay, are in fact DNA-reactive and thus belong to that category of carcinogen (see Types of Carcinogens). Many chemicals, however, are positive in only specific in vitro assays (e.g., phenobarbital and DES [201,208]), sometimes because of intrinsic spurious positive results [44]. Thus, the issue becomes a matter of which test results are to be accepted as evidence of genotoxicity. A scientifically sound approach is to define genotoxicity as a mechanism of carcinogenesis, that is, a genotoxic carcinogen is one that forms molecular lesions (such as DNA adducts) that lead to mutations in the cells that are the progenitor of neoplasms induced by the chemical [364]. Under this definition, genotoxic carcinogens would largely be confined to DNA-reactive agents, typically fulfilling the criterion for carcinogenic activity of an agent that induces malignant neoplasms not present in controls. Such chemicals often are multispecies carcinogens, inducing a high yield of neoplasms with short latent periods and often in several organs. For such carcinogens, assumption of human hazard is well founded, although evidence is accruing that even DNA-reactive carcinogens have thresholds at low levels of intake [496,507,510]. Moreover, some of the underlying MOAs of these chemicals are species specific and do not operate in humans because of differences in disposition (such as tamoxifen, which is poorly bioactivated and readily detoxified in humans) [31,98,335]. Epigenetic carcinogens do not directly produce DNA damage, but rather produce a cellular or systemic effect that is the basis for carcinogenicity. These generally do not constitute human hazards, as evidenced by the few associated with human cancer, notably hormones and immunosuppressants (Table 25.3), in spite of the large number of such agents with which humans regularly come in contact [207,491]. The lack of relevance stems from the fact that epigenetic effects are either rodent specific, as with the $\alpha_{2\mu}$-globulin nephropathy inducers [8,374,415,439], or require high and sustained duration exposure in rodents to elicit the cellular effect leading to carcinogenicity, as with butylated hydroxyanisole forestomach effects [218,482,483]. Epigenetic mechanisms (MOA) that either are not indicative of a cancer hazard to humans or are probably not indicative have been discussed earlier and addressed in numerous reviews [29,45,392]. These considerations should be applied in formulating hazard assessment.

Cancer Risk Assessment

In the United States, under the provision of the Delaney Clause to the 1958 FFDCA, a zero tolerance in food was established for chemicals determined to be carcinogenic in either humans or animals. This applies to residues of veterinary drugs used in food-producing animals [44,456]. Likewise, the EPA Cancer Principles of 1970 stated that no level of exposure to a chemical carcinogen could be considered toxicologically insignificant for humans. These are actually risk management approaches, rather than risk assessments. In contrast, in Europe, governmental agencies have not been required by regulations to impose standards of no exposure for carcinogenic chemicals and have used more flexible approaches than those imposed in the United States [504], although often this entails restricting external contact to that as low as reasonably achievable (ALARA), which again is a risk management procedure.

The prototype chemical safety assessment, introduced by Lehman and Fitzhugh [272] of the FDA, is the acceptable daily intake (ADI), calculated as the NOAEL divided by uncertainty factors (UFs), for example, 10 for intraspecies (individual) variation and 10 for interspecies extrapolation [496]. It is becoming increasingly accepted that for epigenetic carcinogens, even if a potential cancer hazard to humans is presumed, such a safety margin can be deployed [399,444,492]. For pharmaceuticals with animal neoplasm findings, the FDA/CDER calculates an MOE, which is the ratio of the AUC in rodents for the highest noncarcinogenic dose (nontumorigenic effect level, or NTEL) to the maximum AUC at the therapeutic dose [20,66], allowing for individuals who are poor metabolizers. Under ICH guidance [215], pharmaceuticals need not be tested for carcinogenicity at a dosage providing an exposure ratio greater than 25 [459], based on data showing that such exposure does not represent a cancer risk when the medicine is carcinogenic only at such exposure. In practice, many pharmaceuticals with carcinogenic activity at lower MOE have been approved for use [459]. It appears that the factor of 10 is generally regarded as an acceptable MOE [459].

In 1996, the FFDCA was amended by removing the zero-risk provision of the Delaney Clause and replacing it with a new standard of "a reasonable certainty of no harm" [67]. The new standard applies to pesticide residues in both raw and processed foods, allowing the presence of some residues of chemicals that have been shown to cause cancer in animals [433,434]. In the new EPA draft cancer assessment guidelines, the safety margin is referred to as an MOE using the NTEL [444]. In the EU, it has been proposed to incorporate potency considerations, including TD_{25} values, and to classify carcinogens into three potency groups for regulation [97]. The FDA CFSAN has published a *threshold of regulation* procedure for indirect food additives [451]. In this, based on a large database of chemical carcinogens, it was determined that an intake of 1.5 μg/person/day is unlikely to represent a cancer risk. This concept has been developed into a TTC [264,314]. The Joint WHO/Food and Agriculture Organization (FAO) Joint Expert Committee on Food Additives (JECFA) reviews safety studies on food additives and contaminants, and the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) reviews those on pesticides to set global exposure limits. These groups develop ADI and tolerable daily intakes, respectively. The JECFA uses the TTC approach for flavors with very low intakes (up to 1800 μg/day) [230]. Health Canada in

its Human Health Risk Assessment for Priority Substances under the Canadian Environmental Protection Act applies an exposure potency index calculated as an average exposure in the population divided by the dose in experimental animals that produces a 5% incidence of neoplasms.

Several expert bodies have developed approaches to evaluating and integrating data in risk assessment [4,29,30,297,392,405]. Another approach proposes a carcinogen "toxicologically insignificant daily intake (TIDI)" as the NEL for the molecular or cellular effects that are the basis for carcinogenicity divided by a safety factor similar to UFs [487,491,492]. This actually is more conservative than using the NTEL because the NEL for molecular and cellular effects leading to cancer is a more robust endpoint and is usually lower than the dosage required to elicit cancer, as illustrated in reviews of rodent carcinogenicity of phenobarbital [483] and butylated hydroxyanisole [482]. A similar approach for nongenotoxic carcinogens proposes using toxicogenomic data to establish a point of departure for cancer risk assessment [18].

For genotoxic or DNA-reactive carcinogens, authorities regulate such agents either by prohibiting human contact (risk management) or by using a linear no-threshold model for quantitative risk assessment [437,442,512]. Currently, the concept of TTC is being considered for genotoxic contaminants of medicines [239] and food substances [264]. This recognizes the accruing evidence that even DNA-reactive carcinogens can have thresholds [510,492,496]. The TIDI concept described above can also be applied to DNA-reactive carcinogens using an NEL for DNA binding in target tissues [491].

These approaches were all developed for individual chemicals. However, there are situations in which multiple exposures can occur, and these can pose risks of interactive carcinogenesis, as discussed below.

INTERACTIVE CARCINOGENESIS

Interactive carcinogenesis comprises the enhancement or inhibition of carcinogenesis by combined or sequential exposures to more than one carcinogen or to a carcinogen and a noncarcinogen, which modifies the effect of the carcinogen [486]. Various types of interaction can occur between chemicals [49,484], including syncarcinogenesis, neoplasm promotion, cocarcinogenesis, and anticarcinogenesis, and between chemical and physical agents, as with photochemical carcinogenesis. Studies of interactive effects for regulatory purposes are being increasingly applied. Also, in the testing of complex mixtures, these types of interactions can influence the outcome and warrant consideration.

Syncarcinogenesis

Syncarcinogenesis is the enhancement of carcinogenesis produced by concurrent or sequential administration of two carcinogens [389], usually of the DNA-reactive type. This interaction in the case of DNA-reactive carcinogens represents a summation of the genetic effects of each of the chemicals. Generally, the enhancement occurs only in a target organ where both carcinogens independently produce a neoplastic effect [494,502].

Promotion

Neoplasm PM is the enhancement of neoplastic development by a second epigenetic agent given after an initiating, typically DNA-reactive, carcinogen [19], when a sufficient interval has been allowed for acute molecular effects of the carcinogen (e.g., DNA adducts) to be processed. If the second chemical is administered when molecular lesions are still present, the enhancement may be due to cocarcinogenesis (see below). Promoting agents essentially facilitate clonal expansion of initiated preneoplastic cells and their evolution into neoplasms. The growth advantage of preneoplastic populations can be achieved either by an enhanced rate of cell proliferation or by a decreased rate of apoptosis in either the tissue or incipient neoplasms. Promoting agents are usually assumed to be noncarcinogens, but in fact most are weak carcinogens under some circumstances, probably because they facilitate neoplasm development from cryptogenically initiated cells, which are the source of background neoplasms. Thus, most, if not all, promoters are epigenetic carcinogens (Table 25.2), albeit weak ones. An essential characteristic of a promoting agent is that it is not DNA-reactive and is not an initiating agent; otherwise, its enhancement of the effect of an initiating agent is likely to be due to syncarcinogenesis, described above. For experimental demonstration that promotion is the MOA of a chemical that has been shown to induce neoplasms, the exclusion of both genotoxicity and initiating activity is paramount.

Cocarcinogenesis

Cocarcinogenesis is the enhancement by a noncarcinogenic chemical of the carcinogenicity of a carcinogen when the noncarcinogenic chemical is administered prior to or concurrent with the carcinogen or when given shortly after the carcinogen at a time when molecular damage is still present [486]. Cocarcinogens can enhance the uptake of the carcinogen, enhance its tissue localization, increase the proportion that is bioactivated, or enhance the induced neoplastic transformation, usually by transiently increasing cell proliferation. Cocarcinogens do not act as promoters, although most promoters have cocarcinogenic activity, often due to enhancement of cell proliferation, when administered concurrently or shortly after the initiating agent.

Anticarcinogenesis

Anticarcinogenesis is the reduction of occurrence of neoplasia and may involve the inhibition of carcinogenicity of a concurrently or previously administered chemical. Anticarcinogens are typically noncarcinogenic, although certain epigenetic carcinogens, such as phenobarbital [65] and butylated hydroxyanisole [509], are effective anticarcinogens when administered concurrent with the carcinogen. Three operational pathways for anticarcinogenesis that have

been recognized are prevention of formation of a carcinogen, blocking of the effects of a carcinogen, and suppression of neoplastic development [472].

Photochemical Carcinogenesis

Another specific type of interactive carcinogenesis is photochemical carcinogenesis, which is the combined skin carcinogenicity of a chemical and UVR [126,127,204]. Photochemical carcinogenicity can result from several types of interactions among the chemical, UVR, and the skin. Some chemicals, such as psoralens, can be photoactivated to DNA-reactive chemical species. Others, such as fluoroquinolones, can undergo photoactivation to generate secondary reactive molecular species such as reactive oxygen [499]. Also some chemicals can affect the structure of skin, causing, for example, thinning of the epidermis in the case of retinoids, which results in the sensitization of the skin to effects of UVR. Stimulation of epidermal cell proliferation can enhance photocarcinogenesis. Finally, immunosuppression can facilitate UVR-induced skin carcinogenesis [262]. An FDA evaluation of available data found that photosensitivity and photogenotoxicity assays did not necessarily predict effects in photocarcinogenicity studies [226].

Photosafety Testing

Photochemical carcinogenicity studies can be required for topically applied medicines and even for some oral medicines, as well as for topically applied cosmetics and consumer products when the compound absorbs UVR or localizes in the skin [449,455].

The FDA has issued a guidance for cutaneous photosafety testing [454]. The test species is usually the SKH1 albino hairless mouse, which has the advantage that it does not require hair clipping and allows easy detection of UVR-induced skin tumors. In a typical protocol [85,126,127,382], the TS is applied before UVR (at 290–400 nm by a UV solar simulator) on Monday, Wednesday, and Friday and after UVR on Tuesday and Thursday for 40 weeks, followed by a 12-week observation period without exposure. This pattern of dosing allows detection of photoactivated chemicals as well as those that may modulate photocarcinogenesis. Typically, separate groups are administered low and high UVR doses in addition to the TS. The endpoints of evaluation include skin neoplasm incidence, multiplicity, and latency. Measurement of epidermal cell proliferation to monitor for nonspecific enhancement that can lead to enhancement of skin cancer can contribute to understanding of photochemical effects.

CONCLUDING REMARKS

It has been evident for 40 years or more that the carcinogenicity of a chemical could be predicted from data other than an RCB [475]. Nevertheless, for a variety of reasons, the RCB remains an essential component in the safety assessment of chemicals. The RCB appears to be highly effective in identifying human cancer hazards in as much as, to our knowledge, no chemical that was negative in a properly conducted RCB has subsequently proven to be carcinogenic in humans, although in some cases (arsenic, benzene) human carcinogenicity was noted first. The basic methodology of the RCB has changed little since its standardization in the 1980s, but refinements in both the analysis and the interpretation have evolved. Various types of neoplastic responses in rodents are now accepted as not being relevant to human safety. Moreover, responses considered to be relevant are increasingly recognized as having thresholds, and hence, safe levels of contact can be established.

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QUESTIONS

25.1 What are the pertinent mechanisms of carcinogenesis operational in both rodents and humans?
25.2 What are some mechanisms of carcinogenesis operational only for rodents? Explain why.
25.3 Which neoplasms are of questionable significance to human cancer hazards? Explain why.
25.4 What are the types of cancer hazards?
25.5 What constitutes adequate exposure in a rodent cancer bioassay?

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26 Principles of Clinical Pathology for Toxicology Studies

Robert L. Hall and Nancy E. Everds

CONTENTS

Clinical pathology is an integral component of nonclinical safety assessment and toxicology studies designed to identify target organ toxicity and establish dose–response relationships. In the context of these studies, clinical pathology usually consists of relatively routine hematology, clinical chemistry, and urinalysis tests. The majority of parameters evaluated are identical to those used in human and veterinary medicine because the fundamental physiology and pathophysiology of blood and major organ systems are similar in most species used in toxicology studies. There are, of course, species differences for reference intervals, some methodologies, the value or appropriateness of individual tests, and interpretation of findings. Selection of tests for a toxicology study is dependent on several factors, including study objectives, test species, regulatory requirements, and test article characteristics.

Clinical pathology tests are best characterized as screening tools to identify general metabolic or pathologic processes and target tissues. Test results narrow the possibilities and help direct further studies, although specific diagnoses and precise mechanisms for a toxic effect are infrequently identified. Clinical pathology tests can also help determine the biological importance of effects associated with test article administration. Alterations in clinical pathology test results are typically not the only evidence of adverse or pathologically significant toxicologic effects. In-life clinical observations and anatomic pathology findings usually corroborate pathologically meaningful laboratory findings.

Interpretation of clinical pathology data from a toxicology study is considerably different from the assessment of data from an individual patient suffering from an unknown illness. The most obvious difference is that data from groups of treated subjects receiving increasing dose levels of a test article are compared with data from a group of age-, weight-, and sex-matched control subjects that are concurrently exposed to the same environmental and experimental conditions. For larger laboratory animals (e.g., rabbits, dogs, monkeys), pretreatment clinical pathology data for each individual are also available for comparison with posttreatment results. Finally, clinical pathology results from a toxicology study can be correlated with carefully recorded in-life and necropsy observations, organ weight data, and histopathologic findings from an extensive tissue list. Given the uniformity of the animals studied and the analytical precision of modern clinical pathology instrumentation, data from toxicology studies can identify subtle changes that would not be apparent for an individual patient. One of the most challenging aspects of clinical pathology data interpretation for a toxicology study is differentiating potentially harmful toxic effects from changes representing homeostatic, metabolic, or adaptive responses to benign test article effects or study-related procedures. Proper interpretation of clinical pathology results from a toxicology study requires not only an understanding of the tests but also knowledge of species differences, study design, unique study-related procedures, clinical observations, anatomic pathology findings, and the test article. Interpretation of one test result is frequently dependent upon the results of another test, and pattern recognition is essential.

This chapter addresses (1) experimental design considerations, including test selection, timing and frequency of testing, sources of variability in clinical pathology test results with emphasis on preanalytical factors, and quality control; (2) basic principles of clinical pathology data interpretation, including the use and misuse of reference intervals; and (3) the characteristics and interpretation of routine hematology, clinical chemistry, and urinalysis tests used in toxicology studies. For in-depth descriptions of clinical pathology tests, including methods, the reader is referred to Refs. [1–10].

EXPERIMENTAL DESIGN CONSIDERATIONS

The value of clinical pathology in toxicology studies is heavily influenced by the experimental design and technical skill of the laboratory. Selection and timing of appropriate tests, consideration of unique study procedures, reduction of sources of variation, proper sample collection and handling, and controlled analytical technique are all factors that determine the value of clinical pathology test results.

Test Selection

The selection of appropriate clinical pathology tests for a toxicology study is ultimately dependent on the study objective or purpose (Table 26.1). If the objective is simply to screen a number of similar chemical entities for potential hepatocellular toxicity, the laboratory evaluation might be limited to a few targeted tests such as alanine aminotransferase (ALT), glutamate dehydrogenase (GDH), and sorbitol dehydrogenase (SDH) activities. Conversely, if the study is part of the package of studies required to support government approval of a new drug or other chemical entity with potential human

TABLE 26.1
Examples of Basic Tests Applicable to Most Rat, Dog, and Monkey Studies

Hematology and Coagulation	Clinical Chemistry	Urinalysis
RBC count	Glucose	Color and clarity
Hemoglobin	Urea nitrogen (or urea)	Overnight or timed volume (e.g., 16 h)
Hematocrit	Creatinine	Urine specific gravity
MCV	Total protein	Reagent strip tests: pH, protein, glucose, ketones, bilirubin, urobilinogen, blood
MCH	Albumin	
MCHC	Globulin (calculated)	Microscopic examination of sediment: cells, casts, crystals, bacteria, sperm
RDW	Albumin-to-globulin ratio (calculated)	
RBC morphology	Cholesterol	
WBC count	Total bilirubin	
WBC differential count	ALT	
Platelet count	AST	
MPV	ALP	
Blood and bone marrow smears	GGT	
PT	CK	
APTT	Calcium	
Fibrinogen	Inorganic phosphorus	
	Sodium	
	Potassium	
	Chloride	

exposure (e.g., food additives, pesticides, chemicals used in manufacturing), several tests are required or recommended in study guidelines published by the presiding governmental regulatory agencies (e.g., U.S. Food and Drug Administration [FDA], U.S. Environmental Protection Agency [EPA], Japanese Ministry of Health, Labour and Welfare [MHLW]) or professional standards organization (e.g., the European Organisation for Economic Co-operation and Development [OECD]).[11] The various published guidelines are relatively similar with respect to recommended clinical pathology tests but have several differences and instances of ambiguous or inappropriate testing requirements.

In an effort to encourage global harmonization of regulatory guidelines, a Joint Scientific Committee for International Harmonization of Clinical Pathology Testing was formed in 1992 to provide recommendations for clinical pathology testing of laboratory animals used in regulated safety assessment and toxicology studies. The committee was comprised of representatives from 10 professional organizations located throughout the world, with scientific expertise in animal clinical pathology, and was independent of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). The committee prepared a document listing minimum recommendations for clinical pathology testing in regulated safety assessment and toxicology studies.[12] These recommendations are described in the following paragraphs, along with comments from the authors reflecting updated approaches and new methodologies.

With respect to hematology, the core recommended tests were total white blood cell (WBC) count, absolute differential WBC count, red blood cell (RBC) count, hemoglobin concentration, hematocrit (or packed cell volume), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), evaluation of RBC morphology, and platelet count. The importance of reporting absolute WBC differential counts rather than relative (percent) WBC differential counts was stressed. The method for evaluation of RBC morphology was not defined, but some laboratories examine blood smears for RBC morphologic characteristics such as size variation (anisocytosis, microcytosis, or macrocytosis), color (polychromasia), shape (poikilocytosis), and hemoglobin content (hypochromasia). Most laboratories assess RBC morphology using automated measurements such as MCV, MCH, MCHC, red cell distribution width (RDW), and hemoglobin distribution width (HDW). If automated methods are used for determination of hematology parameters, it is prudent to prepare blood smears in the event that the data indicate a need to examine the cells microscopically.

The Joint Scientific Committee did not recommend routine determination of reticulocyte count. At the time its paper was published, these counts were typically done manually and were relatively inaccurate, imprecise, and labor intensive. Accurate and precise automated reticulocyte counting is now a common feature of most hematology analyzers. Absolute reticulocyte count should be included as a standard hematology test. If a hematology analyzer is unable to perform an automated reticulocyte count, peripheral blood smears stained with a supravital dye (e.g., new methylene blue) should be prepared for possible manual enumeration of reticulocytes. Manual counts would then be indicated whenever a significant unexplained decrease in red cell mass existed.

The Joint Scientific Committee recommended that bone marrow smears be prepared at study termination for possible cytologic examination. Unexplained nonregenerative anemia, leukopenia, thrombocytopenia, and pancytopenia are possible reasons for performing bone marrow cytologic examinations.[13,14] Routine performance of cytologic examinations, myeloid-to-erythroid (M/E) ratios, or bone marrow differential cell counts is not recommended.

Prothrombin time (PT) and activated partial thromboplastin time (APTT), or appropriate alternatives such as the Thrombotest,[15] and platelet count were the core recommended tests for hemostasis assessment. If blood volume limitations or sample quality is a concern (e.g., multiple blood collections for a rat study; retro-orbital plexus/sinus collections), it may be necessary to perform PT and APTT only at study termination.

Current regulatory guidelines for carcinogenicity and oncogenicity studies recommend performing hematology tests (e.g., WBC differential counts) on some or all animals at set intervals (e.g., weeks 26, 52, 78, and 104). In contrast, the Joint Scientific Committee and a more recent best practice paper[16] recommended preparation of blood smears for all animals at unscheduled sacrifices (e.g., moribund animals) and at terminal sacrifice for possible examination as an adjunct to histopathology for the identification and differentiation of hematopoietic neoplasia. For example, if the histopathologist is unsure whether leukocytic infiltrates in multiple tissues represent leukemia or a leukemoid response (i.e., marked leukocytosis secondary to an inflammatory stimulus), the blood smear from that animal can be examined to help differentiate between the two conditions. WBC differentials at set intervals are not useful for detection of rodent hematopoietic neoplasms. These tumors become leukemic only late in the disease process,[17] so histologic evidence is sufficient for diagnosis.

With respect to clinical chemistry, the core recommended tests were glucose, urea nitrogen (or urea), creatinine, total protein, albumin, globulin (calculated from total protein and albumin), cholesterol, calcium, sodium, potassium, and selected tests of hepatocellular and hepatobiliary health and function. Measurement of at least two scientifically appropriate tests for hepatocellular evaluation (e.g., ALT, SDH, GDH, aspartate aminotransferase (AST), or total bile acids) and at least two scientifically appropriate tests for hepatobiliary evaluation (e.g., alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), 5′-nucleotidase (5′-N), total bilirubin, or total bile acids) was recommended. Because several acceptable tests are used to evaluate hepatic health and function, the Joint Scientific Committee recommended that each laboratory should have the freedom to choose those tests that best meet their individual needs and with which they have the most experience. For example, GDH is commonly evaluated in Europe, but the availability of commercial kits for this enzyme assay is limited in the United States. A recent publication from the American Society for Veterinary Clinical Pathology provides an additional description of clinical pathology indicators of hepatic injury in nonclinical studies.[18]

Core recommended urinalysis tests performed on an overnight or timed sample (i.e., approximately 16 h collection) were an assessment of urine appearance (color and turbidity), volume, specific gravity or osmolality, pH, and either the quantitative or semiquantitative determination of protein and glucose.

The Joint Scientific Committee listed several tests that are specifically not recommended for routine use in animal toxicology studies. These tests include ornithine decarboxylase, ornithine carbamoyltransferase, lactate dehydrogenase (LDH), creatine kinase (CK), serum or plasma protein electrophoresis, microscopic examination of urine sediment, and urinary mineral and electrolyte excretion (e.g., urine sodium, potassium, chloride, calcium, or inorganic phosphorus excretion). Although ornithine decarboxylase has appeared in the test lists of several regulatory guidelines, it has no value as a diagnostic clinical chemistry test.[19] This enzyme may have been included in the original FDA guidelines by mistake, and the error was repeated by other organizations. The FDA may have intended to include ornithine carbamoyltransferase, a liver-specific enzyme involved in the urea cycle that enjoyed limited popularity as a diagnostic test in the late 1970s. Ornithine carbamoyltransferase has never demonstrated a clear diagnostic advantage over other more common liver enzymes (e.g., ALT) and is rarely measured. LDH is similar to AST (i.e., nonspecific) but is generally more variable and not considered beneficial. CK may be helpful for evaluating test articles that cause muscle injury but is not considered necessary for most test articles. Blood collection techniques that potentially damage tissue and contaminate the blood sample with tissue fluids (e.g., cardiac puncture and retro-orbital plexus/sinus collection) can diminish the value of measuring muscle enzyme activities by increasing variability.

The routine use of protein electrophoresis is inappropriate for toxicology studies. As a diagnostic test for patients, serum or plasma protein electrophoresis is used to evaluate large, unexplained increases or decreases in globulin concentration. With respect to increased globulin concentration, protein electrophoresis is used to rule out a monoclonal gammopathy caused by some cancers of lymphoid origin (e.g., plasma cell myeloma). With the exception of monoclonal protein spikes due to a protein-based test article, these conditions are rare in toxicology studies. Regardless, analysis of stored serum samples can easily be accomplished if routine clinical chemistry tests indicate a need for electrophoresis.

Microscopic examination of urine sediment may be helpful for screening test articles that are known to cause severe renal or bladder toxicity, but histopathology is generally a more sensitive tool for detecting lesions of the kidney and bladder. In part, this is because the collection of high-quality urine specimens from many animals at one time is very difficult. On rare occasions, examination of urine sediment may be valuable for detecting the presence of crystals specific for a test article. Measurement of urinary mineral or electrolyte

excretion may be appropriate for test articles that are known to affect renal function (e.g., diuretics) or bone metabolism (e.g., parathyroid hormone), but as routine screening tests, these are excessive. If serum/plasma mineral and electrolyte concentrations are greatly affected by a test article and other causes for these findings are ruled out (e.g., vomiting, diarrhea, renal failure), an assessment of the renal handling of the mineral or electrolyte in question may then be valuable.

The recommendations of the Joint Scientific Committee and the requirements listed in the various regulatory guidelines should be viewed as a minimum database for screening the potential toxicity of a test article that will likely undergo extensive evaluation in studies of varying duration with multiple species before regulatory approval. Additional tests may be appropriate for the assessment of test article effects, depending on the study objectives and known characteristics of the test article. Platelet function tests (e.g., platelet aggregation and bleeding times) may be appropriate for evaluating drugs that target platelets. Analysis of methemoglobin concentration or the enumeration of Heinz bodies in erythrocytes can be valuable for assessing oxidative injury caused by a compound. Serum cardiac and skeletal troponin I and T can be useful markers of cardiac and skeletal muscle damage, respectively. Activity of plasma or RBC cholinesterase can indicate exposure to organophosphates or carbamates. Activity of brain cholinesterase is a measure of the toxic effect of these compounds. Determination of urinary enzyme activities and characterization of urinary protein excretion can be used for screening related compounds known or suspected to cause renal toxicity.[20–23] Various hormones may be measured when endocrine dysfunction is suspected, and cholesterol fractions may help define effects on lipid metabolism. Serum inorganic phosphorus and chloride concentrations are commonly measured as part of standard chemistry profiles in the United States and can be helpful when assessing changes in calcium and sodium concentrations, respectively.

The list of potentially valuable clinical pathology tests will grow because of industry and regulatory interest in developing improved biomarkers for both efficacy and toxicity. Novel parameters are being sought to provide more specific information about the status of individual organs and overall health. A nonstandard or novel test should be used for a specific purpose and only after the test has been shown to be useful for the species in question.

Frequency and Timing of Testing

Frequency and timing of clinical pathology testing are dependent on study objectives and duration, the biological activity of the test article, and the species tested. The Joint Scientific Committee made minimum recommendations[12] that may be modified because of these factors. With respect to regulated acute or single-dose toxicology studies, the regulatory guidelines have no clinical pathology requirements. Traditionally, in these studies, animals are dosed once at relatively high doses and sacrificed following a 1- or 2-week observation period to determine appropriate dose levels for future repeat-dose studies. Because the doses on these studies are higher than those of repeat-dose studies, information about acute high-dose effects can be gathered using clinical pathology tests. If clinical pathology tests are utilized, it is generally best to obtain samples approximately 48–72 h postdose. Waiting until the end of the observation period to obtain samples can allow recovery from acute effects. For example, at 2 weeks following the administration of near-lethal doses of carbon tetrachloride or mercuric chloride to rats, standard clinical pathology tests fail to recognize the effects of these compounds on the liver or kidney, respectively, because of the regenerative capacity and large functional reserve of these tissues. Occasionally, specific clinical pathology tests may be most appropriate within hours after dosing because the test article has a very short half-life or a limited duration of action (e.g., some cytokines, peptides, and organophosphates). Following administration of acute renal toxins, urinary enzyme activities tend to be greatest in the first 24–48 h postdose.[20] Alternatively, the effects of some test articles (e.g., cytotoxic chemotherapeutic agents) may take longer to reach their peak. For these test articles, it may be best to wait several days before performing clinical pathology tests or to take samples at multiple times to determine the time of greatest effect and pace of recovery (e.g., days 4, 7, 10, and 14).

Limited blood volume in mice dictates that blood sample collection for clinical pathology is usually practical only when the animals are sacrificed. Because the blood volume of a 30 g mouse is less than 2 mL, it is difficult to acquire a full milliliter of blood from a single mouse, regardless of the blood collection technique. If several tests are required or desired (e.g., full hematology and clinical chemistry profiles), it may be necessary to specify certain animals for hematology tests and others for clinical chemistry tests. An alternative is to limit the number of clinical chemistry tests to those that are the most relevant as screening tools for major organ toxicity (e.g., urea nitrogen or creatinine, ALT or another liver-specific enzyme, total protein, albumin). Pooling of blood samples is inappropriate for clinical pathology testing.

Prestudy clinical pathology testing is not recommended for rats because of the relatively large number of animals per group, homogeneity of the population, irrelevance of prestudy data due to rapid age-related changes in values for young rodents, and risk of adversely affecting the health of young animals due to blood loss or the blood collection procedure. For repeat-dose studies in rats, testing should at least be done at study termination. Interim testing may be unnecessary for long-duration studies (e.g., a 13-week study) if testing was done in short-duration studies (e.g., a 4-week study) that used dose levels not substantially lower than those of the long-duration studies. Conversely, interim testing (e.g., a week 6 testing point during a 13-week study) can be beneficial for interpretation of subtle effects. Clinical pathology testing is not routinely recommended for rodents after 52 weeks because naturally occurring geriatric conditions

(e.g., ulceration and infection of mammary gland tumors, chronic progressive nephropathy, and pituitary tumors disrupting normal endocrine function) obscure meaningful interpretation of laboratory data.

For repeat-dose studies in large animals (e.g., rabbits, dogs, and monkeys), testing should be done before initiation (i.e., prestudy or baseline), at least once during the study, and at study termination. Animals shipped from a supplier should have several days to acclimate to the new environment before baseline testing is performed. The baseline data are important for screening out animals with potential health problems or results that notably differ from those of their peers. Findings that may signal possible subclinical health problems and eliminate animals from study consideration include low values for erythrocyte parameters (e.g., RBC count, hemoglobin concentration, hematocrit, MCV), low or high WBC and neutrophil counts, low total protein and albumin concentrations, high globulin concentration, and high liver enzyme activities. There may be a specific need to eliminate otherwise healthy animals because they have findings that might complicate interpretation of test article-related effects. The criteria for which animals to eliminate from the study may be test article-dependent. For example, if a test article is expected to cause decreases in neutrophils, an animal with unusually high or low neutrophil count may be inappropriate. Similarly, an animal with high normal triglyceride concentration might be inappropriate for a study with a test article affecting lipids. Beagles with factor VII deficiency have slightly prolonged PTs. Even though these beagles are clinically normal, it may be inappropriate to include one in a study evaluating a product that targets coagulation.

Using larger test animals with greater blood volume allows more frequent clinical pathology evaluations. This advantage is offset by the low number of animals in each treatment group (often four or fewer per sex per group) and the increased variability between animals for many tests. The quality of data interpretation is enhanced by having more than one baseline interval for studies using monkeys and dogs, regardless of the number of animals per sex per group. An additional benefit to multiple baseline intervals is that the animals become more accustomed to the blood collection procedures, and variability caused by excitement or fear is generally reduced. Some range-finding studies only use one animal per sex per group, and each animal serves as its own control. In these studies, using at least two baseline intervals affords an appreciation for normal day-to-day, intra-animal variability. Two baseline intervals are also generally desirable for animals that have been surgically manipulated (e.g., placement of indwelling intravenous catheters) to avoid using animals with iatrogenic complications.

The nature of the test article often dictates the timing and number of clinical pathology intervals for repeat-dose studies in dogs and monkeys. For studies of 6 weeks' duration or less, an interim testing interval within 7 days of initiation of dosing may be useful. The primary purpose for this early interval is to detect transient changes, such as increases in serum enzyme activities, that may be absent at later intervals.[24] This information can be very important for clinical trials. For studies with cytotoxic chemotherapeutic agents, the number and frequency of hematology intervals are often considerable because common objectives are to identify the nadir for circulating leukocyte counts and the timing of hematopoietic recovery. A single-dose study of a chemotherapeutic agent in dogs might include hematology tests twice before initiation of treatment and at days 4, 7, 10, 14, 21, and 28 and clinical chemistry tests twice before initiation of treatment and at days 7 and 28.

Urinalysis testing should be conducted at least once during a repeat-dose study. It is best to conduct the urinalysis testing at the same time as other clinical pathology tests. Although not stated in the Joint Scientific Committee's document, urinalysis testing for mice is impractical and not recommended as a routine test.

Sources and Control of Preanalytical Variation

Many study design and procedural factors affect variability of the data and impact clinical pathology evaluation and interpretation, even under the conditions of a well-controlled toxicology study. To identify test article-related effects on clinical pathology test results, preanalytical sources of variation should be reduced whenever possible within the limitations of the study. Sources of preanalytical variation can be loosely categorized as physiological, procedural, and artifactual (Table 26.2). Physiological sources of variation include differences associated with age, strain, sex, diet, fasted condition, excitement, fear, stress, and time of blood collection. Procedural sources of variation include order of sample collection (i.e., group order vs. randomized or stratified), blood collection site and technique, anesthesia, and iatrogenic blood loss. Causes of artifactual or spurious results include poor-quality specimens (e.g., partially clotted hematology samples, hemolyzed serum or plasma samples), inappropriate use of an anticoagulant, and improper sample storage.

Initiation of treatment for many regulated toxicology studies occurs when the animals are relatively young and still in a growth phase (e.g., rats 6–8 weeks old, beagles 4–6 months

TABLE 26.2
Examples of Sources of Preanalytical Variation

Physiological	Procedural	Artifact
Age	Blood collection site and technique	Poor-quality sample (e.g., hemolyzed or clotted)
Sex	Order of sample collection and analysis	Improper anticoagulant
Strain	Anesthesia	Too much anticoagulant
Diet	Other study design factors (e.g., continuous infusion, vehicle, iatrogenic blood loss)	Improper sample storage
Fasted/nonfasted		
Time of sample collection		
Excitement/fear		
Stress		

old, and juvenile monkeys). As the animals mature, the results of several clinical pathology parameters change. Typical changes in most species include increasing RBC count, hemoglobin concentration, hematocrit, absolute neutrophil count, total protein, and globulin concentration and decreasing reticulocyte count, MCV, absolute lymphocyte count, ALP activity, and inorganic phosphorus concentration. Although these and other age-related changes may be subtle, they may result in inaccurate conclusions if interpretation of posttreatment data is based solely on comparisons with pretreatment or baseline data collected more than 1–2 weeks earlier. In addition to the age-related changes in growing animals, there are also age-related changes in mature animals. These changes are generally observed in rodents, especially those greater than 1 year of age, and are often due to intercurrent disease and other geriatric changes.

Strain differences, especially for rodents, are important to consider when evaluating clinical pathology data. Differences for hematology parameters tend to be the most prominent. For example, Fischer 344 rats tend to have lower leukocyte counts than Sprague-Dawley rats but are also predisposed to developing large, granular lymphocytic leukemia.[25,26] The size of RBCs of cynomolgus monkeys (*Macaca fascicularis*) differ based on geographic origin. Cynomolgus monkeys from suppliers in China and contiguous areas such as Vietnam have much larger, but fewer, RBCs than cynomolgus monkeys from Indonesia, the Philippines, or Mauritius.[27] The differences are so great that reference intervals for these animals may not overlap for parameters such as RBC count and MCV. Interpretation of hematologic effects could be compromised if monkeys from these different geographic origins were used in the same toxicology study or perhaps different studies of the same test article.[28] For targeted biotherapeutics, it may be necessary to preselect animals for toxicology studies based on appropriate polymorphisms (e.g., CD52-positive monkeys and alemtuzumab). Although not yet reported, it is possible that other differences for clinical pathology test results exist between various populations of cynomolgus monkeys.[29]

Diet affects many clinical pathology parameters. Standardized diets are used in toxicology studies to avoid small effects that might be misinterpreted as test article-related. Data from animals fed purified or unusual diets should not be directly compared with data from animals fed standard diets (e.g., historical reference ranges). Some species, such as the rabbit and hamster, are particularly sensitive to the effects of atherogenic diets, which cause them to exhibit very high cholesterol concentrations. The amount of protein in the diet affects urea nitrogen concentration and may have subtle effects on other parameters over time. Diet can be an important factor when assessing the potential of a test article to cause gastrointestinal ulceration or hemorrhage because some diets, especially for dogs, are more prone than others to cause false-positive results for fecal occult blood.

Much has been published on the effects of fasting animals prior to blood collection for clinical pathology.[30–34] Historically, fasting has been encouraged in clinical practice as a means of reducing the variability of certain parameters, most conspicuously glucose concentration, so the physician or veterinarian can more readily compare the results from a single patient with reference intervals. In toxicology studies, because the concurrent control group is much more relevant for comparison purposes than are historical reference intervals, the key principle is to treat all of the groups the same with respect to conditions prior to blood collection. Although most laboratories routinely fast dogs and monkeys overnight prior to blood collection, procedures for rodents differ among laboratories.

Fasting of mice for longer than a few hours is discouraged because mice reduce their water consumption when fasted and rapidly become dehydrated. Not only does dehydration affect many clinical pathology parameters (e.g., RBC count, serum protein concentrations, urea nitrogen concentration), it results in more difficult blood collection and can reduce sample quality and volume. Mice are sometimes fasted for a limited time before blood collection (e.g., 4 h) because blood collection from mice is usually a terminal procedure and fasting reduces hepatocyte glycogen stores and may improve histopathologic detection of hepatocellular injury or change. Care must be taken to keep the period of fasting similar for all mice, since necropsy procedures may take several hours to complete.

Most laboratories in the United States prefer to fast rats overnight to standardize the fasted/fed conditions for all animals. If animals in the high-dose group are eating poorly, providing all animals with access to feed before blood collection can have the effect of comparing fed animals (i.e., the control animals) to fasted animals (i.e., the high-dose animals). Because several differences for clinical pathology parameters exist between fed and fasted animals, it is more difficult to determine if differences between the control and high-dose groups are due to a test article effect or simply to differences in overnight feed consumption. When compared with rats having access to feed, fasted rats tend to have lower WBC counts; lower urea nitrogen, cholesterol, triglyceride, calcium, and bilirubin concentrations; and lower ALT and ALP activities.[31,33]

Excitement, fear, and stress can have pronounced effects on clinical pathology test results.[35] Excitement and fear are associated with acute endogenous catecholamine release ("fight or flight" phenomenon), and stress is associated with endogenous glucocorticoid release. Although these processes are separated for discussion, perturbation of either system generally activates the other system to some degree. Effects of catecholamines are immediate but short lasting (e.g., generally less than 30 min). Effects of glucocorticoids tend to be more gradual and long lasting. The most obvious changes observed in very excited or frightened animals are increased erythrocyte and leukocyte counts and glucose concentrations. These changes are observed occasionally with overexcited beagles and unanesthetized monkeys that are not used to handling for blood collection. Monkeys may also react to the presence of several people in the animal room performing additional study-related procedures at the

same time as blood collections. Endogenous glucocorticoids affect leukocyte counts but somewhat differently than catecholamines (see section on Leukocytes). Whenever possible, clinical pathology testing should be delayed for at least 1 week following shipping or surgical procedures to avoid stress-related changes.

Blood samples should be collected in a manner that minimizes the possibility of temporal biases. Examples of time-related biases include differences between morning and afternoon results (circadian effects), differences caused by delayed separation of serum from clotted blood, and day-to-day differences (may be preanalytical or analytical). These biases can be minimized or eliminated by randomizing the animals for blood collection and using procedures that enable blood collection and sample processing over the shortest reasonable amount of time. An alternative to true randomization is a structured pattern of bleeding such that one animal from each group is bled in succession (round-robin or stratified collection). After samples have been collected, they should be analyzed in the same order as collected. Rearranging the samples back to group order has the potential of causing false-positive findings that are actually due to analytical drift. For example, a small drift in the analysis of chloride by ion-selective electrode (e.g., an increase of 2 mmol/L over 60 samples) may be sufficient to produce a statistically higher mean chloride concentration for the high-dose animals if the groups are sufficiently large (e.g., 15 animals per sex per group), the control animals are analyzed first, and the high-dose animals are analyzed last. If animals must be bled over 2 days because of laboratory or necropsy capacity issues, the problem of day-to-day variability can be reduced by collecting and analyzing samples from males on the first day and females the next.

Randomization of animals for blood collection is occasionally impractical (e.g., if timed collections must follow intravenous administration of the test article). When animals must be sampled by group, it is better to sample the high-dose group immediately before or after the control group (e.g., mid-dose, control, high dose, low dose) than to sample the animals in consecutive group order (i.e., control, low dose, mid-dose, high dose). If it is necessary to sample the animals in consecutive group order, then procedures must be in place to analyze or process the samples in a similar time frame. When control animals are bled 1 h or more before the high-dose animals, analysis of the hematology samples and separation of the chemistry samples should not be delayed until all groups are bled. Such a delay can result in differences between the control and high-dose groups that are due solely to time-related, in vitro changes. This is most likely to occur for rodents when blood collection is one of the terminal procedures and several hours are necessary to bleed, sacrifice, and necropsy all of the animals. If clotted blood from the control group is allowed to stand an hour or more longer than that from the high-dose group before separation of serum, the high-dose group will often have statistically significant differences for several chemistry parameters, including higher glucose concentration, lower potassium and inorganic phosphorus concentrations, and lower AST and LDH activities. These differences result from in vitro changes in the control animal samples (i.e., the consumption of glucose by erythrocytes and the release of cell constituents by erythrocytes, leukocytes, and platelets). Circadian effects are another potential source of variation when blood collection is protracted; for example, rodents bled early in the morning can have slightly higher leukocyte counts than those bled in the afternoon during their normal period of inactivity.

Collection conditions, including site, technique, and use of anesthesia, are perhaps the most commonly cited procedural influences on clinical pathology test results. Many investigators have analyzed differences in data resulting from these variables,[25,31,36–48] especially for the rat. For example, total leukocyte counts are higher in samples from the retro-orbital venous plexus or sinus than those from larger vessels such as the abdominal aorta or posterior vena cava, and glucose concentrations are higher in samples from the abdominal aorta than those from other sources. Variability may also be related to differences in bleeding technique proficiency rather than the method itself.[39] With appropriate instruction and practice, any of the commonly used techniques can generate adequate results. Use of an unfamiliar or unpracticed blood collection technique introduces unnecessary variability and spurious results. To optimize the value of clinical pathology data, a single method of collection should be used throughout a study or series of comparable studies. For example, anesthesia tends to decrease interanimal variability for clinical pathology results from monkeys, especially their cell counts and electrolyte concentrations. If pretreatment or baseline blood collection is performed on animals anesthetized for physical examinations or other baseline procedures, the relatively heterogeneous data obtained from unanesthetized animals during treatment may cause confusion or be misinterpreted. Similar problems can occur when interim blood samples from rodents are collected from the tail or retro-orbital plexus or sinus and terminal blood samples collected from the heart, aorta, or posterior vena cava.

Blood collection from dogs, monkeys, and rabbits is facilitated by relatively easy access to large vessels (e.g., jugular, cephalic, and saphenous veins for dogs; femoral, cubital, and saphenous veins for monkeys; auricular artery and jugular vein for rabbits). Blood collection from mice is complicated by size and volume limitations, and terminal procedures are often used (e.g., cardiac puncture or sampling of the posterior vena cava or abdominal aorta at necropsy). Many acceptable methods are used for blood collection from rats. Jugular blood collection is used by laboratories with sufficient study load for phlebotomists to maintain proficiency and offers many advantages for toxicology studies.[49] Several high-quality blood samples can be collected from one animal, directly from a large vessel with needle and syringe, without anesthesia or expensive equipment or risk of damaging important structures such as the eye or heart. Because no time-consuming ancillary procedures are necessary (e.g., warming the tail to dilate the tail vein or anesthesia), samples can be collected quickly, and it is possible to accurately collect timed samples

(e.g., at 1, 5, 10, and 15 min postdose) from a single animal. However, compared to other techniques, blood collection from the jugular vein of rats requires regular practice to remain proficient.

Inappropriately collected or prepared samples increase variability of the data by introducing spurious or artifactual results. Fibrin or clot formation in a hematology sample always results in a spuriously low platelet count but may also cause low erythrocyte and leukocyte counts. Small clots may form because of an insufficient amount of anticoagulant (potassium ethylenediaminetetraacetic acid [potassium EDTA] for hematology samples), inadequate mixing of blood with anticoagulant, or poor blood collection technique with exposure of blood to substances from traumatized tissue. Excess anticoagulant can cause dilutional errors for cell counts and altered cell volumes, particularly for erythrocytes. The use of an inappropriate anticoagulant can cause spurious results. Intentional or accidental exposure of clinical chemistry samples to potassium EDTA results in very high potassium concentrations, very low calcium and magnesium concentrations due to chelation, and very low activities of enzymes such as ALP and CK that use magnesium as a cofactor.[50] Trisodium citrate, the anticoagulant of choice for the coagulation assays, also chelates divalent cations and will additionally increase sodium concentration if incorrectly used for the clinical chemistry sample. Excess anticoagulant in samples for coagulation assays can cause prolonged coagulation times.

Although hemolysis can be caused by test articles, it can also be caused by poor technique for sample collection, sample transport, or serum/plasma separation. Hemolysis can result in spuriously increased or decreased test results by two principal mechanisms: release of erythrocyte constituents and interference with test methodology.[50–54] Techniques and procedures should be chosen that eliminate in vitro hemolysis to the greatest extent possible.

Handling of collected blood can affect clinical pathology results. As previously indicated, prolonged contact between serum and clotted blood causes spurious clinical chemistry changes that can be controlled by prompt separation of serum from the clot.[55] Although most analytes are relatively stable for a reasonable amount of time,[6,56,57] unnecessary delay or storage of samples before analysis should be avoided. Ideally, hematology samples should be analyzed within a few hours of collection and no later than 24 h after collection. If samples for coagulation tests cannot be run on the day of collection, the plasma should be frozen at –20°C and thawed only once before analysis.[58] If samples for clinical chemistry tests cannot be run on the day of collection, the serum or plasma (lithium heparin is the recommended anticoagulant when plasma is used for clinical chemistry) should be refrigerated or frozen overnight. If there will be a long delay before analysis or the desired analyte is relatively labile, samples should be frozen at –70°C. Samples from all animals for any given testing interval (and all intervals, if practical) should be handled the same way.

Other sources of variation for clinical pathology data are possible, but the impact of each on data interpretation in toxicology studies is generally minimized by the inclusion of age- and sex-matched control groups exposed to the same environmental conditions and undergoing the same experimental procedures. Occasionally overlooked are the effects of procedures that may differ between the control and treated groups. Because toxicology studies frequently include blood collections for analyses other than clinical pathology (e.g., toxicokinetic measurements or detection of antidrug antibody), it is imperative that control animals be bled in a manner similar to that for the treated animals. The collection procedures and volume of blood taken for these tests can have a significant effect on hematology and clinical chemistry results,[59–61] and data interpretation is seriously compromised if control animals do not undergo the same procedures. Even when control animals are bled in a like manner, iatrogenic blood loss can complicate data interpretation.[62] This is especially true for rats and small monkeys.

Analytical Variation and Quality Control

In addition to sources of variation that occur before sample analysis, the analytical procedure itself is a source of variation. Analytical variation is minimized by a robust quality-control system within the clinical pathology laboratory. Detailed discussions of quality-control systems are available in many textbooks.[2,63,64] At a minimum, the quality-control system should include initial verification that a new method satisfies the goals of the laboratory for accuracy and precision for the analyte being measured; standard operating procedures for all laboratory functions necessary for analysis and reporting of test results; documentation of routine instrument calibration procedures; documentation of routine and nonroutine instrument maintenance; documentation of appropriate personnel training; proper labeling of all reagents, controls, standards, calibrators, and other chemicals in the laboratory; routine analysis of quality-control specimens and review of quality-control data for detection of systematic errors; routine review of subject data for detection of random errors; standard procedures for responding to and documenting out-of-control situations; and participation in some form of external quality control or proficiency testing with survey samples for analysis.

Analytical variation is affected by the accuracy and precision of a test procedure. Accuracy is a measure of the extent to which the mean estimate of a quantity approaches its true value, and precision is a measure of the agreement among replicate measurements (i.e., the reproducibility of a test result). Accuracy is generally determined in a prescribed way when a new method is introduced into the laboratory, and it is continually reassessed by means of proficiency testing. Most analytical procedures exhibit a small amount of systematic bias or inaccuracy that is either constant or proportional. The mean estimate of the quantity of analyte is always in error in the same direction, either higher or lower, than the true quantity. Precision of a test can be assessed within a single run (e.g., 20 consecutive analyses of the same sample for within-run precision) and from day to

day (e.g., the same sample analyzed several days in a row for between-run precision) and is reflected by the coefficient of variation (CV) of a test. The CV expresses the error or variability of replicate test results as a percentage of the mean value: (standard deviation/mean) × 100; the lower the CV, the greater the precision or repeatability of the test.

Accuracy and precision are desirable independent qualities for a clinical pathology test. Tests can be accurate but imprecise or inaccurate but precise. In the context of most toxicology studies, where results from groups of treated animals are compared with results from concurrent control groups and their own pretreatment results, precision is more valuable than accuracy. This is in contrast to the clinical setting, where the physician or veterinarian evaluates individual patients under less controlled conditions and uses broad historical reference intervals for making decisions. In a toxicology study, an imprecise test, regardless of accuracy, is less useful in detecting small differences between the control and treated groups. If the true mean glucose concentrations for four control dogs and four treated dogs are 100 and 115 mg/dL, respectively, but the standard deviations for the groups are large because of imprecision, then it is unlikely that the observed difference between the means (i.e., 15 mg/dL) will be considered a real difference. A precise test, regardless of a systematic bias or inaccuracy, is better able to identify small test article-related differences between the groups. In the same example, if the glucose test had a positive bias but was more precise, the means of the two groups might be 110 and 125 mg/dL, respectively, with smaller standard deviations. The improved precision permits a more accurate interpretation of the same 15 mg/dL difference between group means. Currently used hematology analyzers have very high within-run precision (CVs for most parameters between 0% and 2% with slightly higher CVs for differential WBC counts); clinical chemistry analyzers have similar within-run precision (CVs of 1%–2%) and between-run precision (CVs of 1%–4%).[65]

Clinical pathology laboratories that analyze animal samples frequently use tests that are optimized for accuracy using human samples. It is likely that many of these tests have small systematic biases when used for animal specimens. In most cases, however, the excellent precision afforded by using standardized commercial reagent kits, standards, and calibrators is preferable to time-consuming, costly efforts to optimize the accuracy of a test for different animal species by using in-house formulated reagents that likely undermine precision.

Although most clinical pathology tests can be used across animal species, some tests require specific reagents. Measurement of albumin in rabbits requires the use of a rabbit-specific calibrator. Immunoassays utilizing monoclonal or polyclonal antibodies raised against human substances (such as hormones) are usually inappropriate for use on animal specimens. Hematology analyzers that enumerate and differentiate blood cells must be validated as appropriate for laboratory animal species because of differences between animal and human cell morphology.

For human hospital laboratories, federal regulations allow each laboratory to set its own policies for assaying control materials as long as at least two control samples of different concentrations (i.e., normal and abnormal levels) are assayed every 24 h. The data from each quality-control analysis are used to make decisions about the validity of the patients' data. In the setting of nonclinical toxicology studies, it may be beneficial to assay control samples with each study run if a laboratory has a small study load (e.g., only one or two studies each day). For laboratories with a high study load, the frequency of control sample testing may more closely resemble that of a hospital setting, and control samples might be assayed only at regular intervals (e.g., before every 8 h shift). If results from a control sample are found to exceed the allowable limits for one or more analytes, then steps must be taken to resolve the problem. Actions that may be taken include, but are not limited to, the following: check for problems such as reagent levels, clots, or mechanical fault; repeat the assays on control samples using fresh aliquots; repeat the assays using newly reconstituted control samples; recalibrate the instrument for the analytes in question and then reassay the control samples; change the reagents, recalibrate, and reassay the control samples; and perform maintenance, recalibrate, and reassay the control samples.

Principles of Data Interpretation

Interpretation of data from toxicology studies begins with the identification of differences between control and treated groups and ends with an assessment of toxicological or biological relevance. In simple terms, the questions to be answered are: Do real differences exist between the groups, and if so, are those differences bad? Interpretation of clinical pathology data requires an understanding of each test's characteristics, species differences, and principles of internal medicine. Factors that influence the interpretation of a potential effect include study design and conditions, clinical observations, other clinical pathology results, anatomic pathology findings, and the test article itself. Interpretations of many clinical pathology findings are interdependent, and pattern recognition is critical. With the exception of early range-finding studies, appropriate dose selection usually precludes large, dramatic effects on clinical pathology test results. Most clinical pathology effects are relatively mild and often appear secondary to small alterations in metabolic or homeostatic mechanisms. Effects on clinical pathology results are rarely the only evidence of biologically important or adverse toxic effects. Clinical observations or anatomic pathology findings usually corroborate biologically important laboratory findings.

Statistical Comparisons

Statistical analysis of clinical pathology data is commonly performed in toxicology studies and often results in identification of several statistically significant differences between control and treated groups. However, all test article effects

caused by a test article need not be statistically significant, and all statistically significant differences do not necessarily represent test article-related effects. If used, statistical tests should be viewed as a tool to help identify differences between groups and not as the principal justification for decisions concerning potential test article effects.[66,67] It is important to remember that the number of animals per group affects the power of a statistical test. Using fewer test subjects increases the likelihood that statistical tests will fail to identify a true effect. Because the number of animals per group is usually quite small for studies with dogs or monkeys (e.g., four or fewer per sex per group), it is imperative that the data for each animal at the different test intervals be examined to look for patterns of change over time among the treated animals that are absent among the control animals. As the number of animals per group increases, the frequency of identifying statistically significant differences of very small magnitude increases. In rat studies with 15 or more animals per sex per group, it is common to observe statistically significant differences that represent very small test article-related effects but are not toxicologically relevant.

TABLE 26.3
Factors to Consider for Determining Whether Differences between Control and Treated Groups Are due to the Test Article

Magnitude of the difference
Dose dependency
Consistency over time
Consistency between sexes
Correlative findings
Number of animals tested (e.g., 2/sex/group vs. 15/sex/group)
Number of animals affected (i.e., group or individual differences)
Statistical significance
Timing of the difference with respect to dosing
Known characteristics of the test article/vehicle
Potential sources of preanalytical variability (e.g., expected variability for species, age, and tests in question; effects of study-related procedures such as route of administration, toxicokinetic sampling, and anesthesia)

Is an Apparent Difference Real?

When faced with an apparent difference between control and treated groups, the first question the investigator must answer is whether or not that difference represents a true test article-related effect or an incidental finding. Many factors can influence the answer (Table 26.3). A large difference is more likely to be real than a small one. Additional factors that suggest that a difference is test article-related include dose dependency, consistency over time, consistency between sexes, correlation with clinical observations (e.g., low chloride concentration and emesis), correlation with other clinical pathology findings (e.g., low hematocrit and high reticulocyte count), correlation with anatomic pathology findings (e.g., high globulin concentration and lymph node hypercellularity), presence in a large number of animals (e.g., 15/sex/group vs. 3/sex/group), and consistency with previously identified effects of the test article or related compounds. With large animals, it may be possible to determine whether an apparent difference was present before treatment was initiated and is, therefore, incidental. The chronology of an apparent difference is also important for interpretation; for example, following a single administration of most test articles, it is more likely that a real difference will occur within a few days rather than only after 2 weeks. Following chronic repeated administration of most test articles, it is more likely that a real difference will be apparent after a few months of treatment rather than only after a longer treatment period. For example, it would be unusual for a real difference at 6 months to be completely absent at 3 months.

With regard to specific tests and test species, the amount of expected analytical, interanimal, and intra-animal variability can influence the interpretation of apparent differences. For example, because ALT activity has much greater interanimal and intra-animal variability for monkeys than for dogs, a relatively small difference for this enzyme between control and treated groups is less likely to be a true effect for monkeys. Interanimal variability increases dramatically for older rodents (e.g., >1 year of age) because of naturally occurring age-related conditions, so small differences between groups of older rodents are less likely to be true effects of a test article.

Procedural factors such as the route of test article administration, blood collection technique, animal handling, and randomization for blood collection also affect variability and must be considered. For example, continuous intravenous infusion increases interanimal variability for several tests (e.g., WBC count, hematocrit, and serum proteins), and interpretation of small differences in results of these tests is difficult. Blood collection by cardiac puncture affects variability of muscle enzymes such as CK and AST. The potential for spurious findings associated with lack of randomization for blood collection was discussed previously.

Is a Real Difference Bad?

If a difference between control and treated groups is clearly real, the next question the investigator must answer is whether or not that difference represents a bad or adverse effect. Does the finding itself (e.g., low hemoglobin concentration) or the condition that caused the finding (e.g., blood loss from gastrointestinal erosions or ulcerations) represent a toxic effect that compromises the animal's health? The answer is often ambiguous and quite subjective. Although a large difference for a given parameter is more likely to be adverse than a small difference, it is impossible to define set limits for each test that represent an adverse effect. Many other factors must be considered. The same magnitude of change for a given test can have completely different connotations depending on the mechanism for that change, correlative findings, study design and procedures, test species, animal age, and the test article itself (e.g., a clinical pathology parameter may be the target for the pharmacological activity of a drug). Urea

nitrogen may be markedly increased because of dehydration (e.g., mice that have been fasted too long before blood collection or animals that refuse to drink water containing test article) but only mildly increased in the early stages of renal toxicity. Increased ALT activity associated with histopathological evidence of hepatocellular degeneration and necrosis is likely more important toxicologically than the same level of increase for which no correlative findings exist. Because of species differences for interanimal variability, a threefold increase for ALT activity for dogs (i.e., treated vs. control group means) is more likely to represent an adverse condition than the same increase for monkeys. A 10% decrease in hemoglobin concentration is less likely to represent an adverse condition in animals bled repeatedly during a study or that received the test article by continuous intravenous infusion than in animals that were not bled repeatedly or were treated by oral gavage. Very high neutrophil counts would normally reflect an adverse condition, but if the test article was a granulocyte colony-stimulating factor, high neutrophil counts would be a desirable effect.

Some measured analytes are critical to good health (e.g., neutrophil count, hemoglobin concentration, glucose concentration, calcium concentration, potassium concentration), and correlative in-life observations (e.g., bacterial infections, lethargy, weakness, weight loss) may help determine whether an observed effect on that analyte has impacted the animals' health. Other measured analytes are markers for effects best evaluated by histopathologic examination (e.g., ALP, urea nitrogen). All clinical pathology tests can be altered by more than one process or mechanism, and some mechanisms have worse implications than others; for example, a 10% decrease in hemoglobin concentration associated with poor food consumption and reduced body weight gain is of less concern than the same decrease caused by Heinz body hemolysis (oxidative injury). When determining the biologic or toxicologic significance of an effect on a clinical pathology test result, consideration must be given to the analyte's normal function for maintaining health, correlative findings that may better define the overall impact of the test article on health, and the mechanism that brought about the change. All study data must be considered.

Reference Intervals

Reference intervals are constructed with values obtained from reference individuals. In most clinical pathology laboratories used for toxicology studies, the reference individuals are control animals from previous studies and clinically healthy animals that have not received treatment (e.g., monkeys or dogs that have clinical pathology tests performed before initiation of dosing). In human medicine, the National Committee for Clinical Laboratory Standards recommends that reference intervals be estimated by the nonparametric method and that a minimum of 120 values from reference individuals be used.[68] Test results from the reference individuals are subjected to statistical treatment such that rare values at both ends of the distribution are eliminated; for example, if the lowest and highest 2.5% of the values are eliminated, the resulting reference interval represents the central 95% of the distribution of values. If the distribution is Gaussian, the interval corresponds to the mean ± 1.96 SD. By definition, when the central 95% is used as the reference interval, 5% of the results from normal individuals (1 of 20) are outside of the reference interval for any given test. Clearly, a value outside of the reference interval does not necessarily indicate an abnormality.[69]

Like statistical comparisons, historical reference intervals can be used as a tool when assessing apparent differences between control and treated groups. The value of reference intervals for data interpretation is sometimes overestimated, and the potential for their improper use is great.[70–72] It is tempting to invoke historical reference intervals when trying to decide whether apparent differences for clinical pathology results are true or adverse effects. While historical reference intervals can be helpful for establishing some perspective concerning what is typical or expected, the conditions of every toxicology study are unique, and it is inappropriate to use a reference interval as the primary reason for dismissing an apparent difference between control and treated animals as being incidental or biologically insignificant. It can be equally inappropriate to use a reference interval as the primary reason for determining that an apparent difference is real or adverse. As detailed in the previous sections, many factors must be considered when evaluating the nature of an apparent test article effect.

The suitability of a historical reference interval for data interpretation for a given study is a function of the parameters or *partitioning factors* that define the reference population used to construct the interval. Many potential partitioning factors exist with respect to toxicology studies (Table 26.4). The most commonly used partitioning factors are species, strain, sex, and age; in other words, a typical reference population might be defined as male Wistar rats from 8 to 10 weeks of age. Many other partitioning factors, however, can influence the reference interval and make it broader, narrower, higher, or lower. These factors include animal supplier, site of blood collection, use of anesthetic, type of anesthetic, diet, fasting status, time of sample collection, housing (single or group), and sample matrix (e.g., serum or plasma). If control animals are used for the reference population, additional partitioning factors include route of administration (e.g., dietary, oral gavage, or intravenous infusion), vehicle or control article administered (e.g., sterile water or corn oil in a gavage study; sterile saline or 5% dextrose in an intravenous infusion study), and whether or not the animals were bled repeatedly for toxicokinetic analyses, multiple clinical pathology intervals, or other tests. Finally, laboratory considerations include instrumentation or techniques used to analyze the specimen and sample storage conditions (e.g., storage temperature and time interval between collection and analysis). Ideally, whenever a new instrument or reagent system is introduced, a new reference interval

TABLE 26.4
Examples of Partitioning Factors for Constructing Reference Intervals

General Factors	Factors for Control Animals	Laboratory Factors
Species	Route of administration	Instrument
Strain	Vehicle/control article	Reagents/methodology
Age	Study-related procedures prior to clinical pathology (e.g., blood collections, anesthesia, therapeutic treatments)	Sample storage (length and temperature)
Sex		
Supplier		
Site of blood collection		
Use and type of anesthesia		
Diet		
Fasted/nonfasted		
Time of sample collection		
Matrix (i.e., serum or plasma)		

is constructed. In practice, laboratories often rely on evidence that demonstrates relative consistency between the old and new analytical methods to avoid complete replacement of old intervals.

The number of partitioning factors used to define a reference interval has a direct impact on the number of reference individuals available, and obtaining enough data for meaningful reference intervals, applicable to a given study, can be difficult or impossible. Because of this, laboratories often ignore many partitioning factors and lump data together from control animals of dissimilar types of studies or from samples that have been handled differently. The result is broader reference intervals with less relevance to data interpretation.

Even when reference intervals are appropriate for a given study (i.e., the partitioning factors match the study animals, conditions, and procedures), it is wrong to assume that a difference between control and treated groups represents a true or adverse effect simply because values for the treated group fall outside of the reference interval. Concurrent control animal data for a given study are never a perfect reflection of the historical reference data; they do not exhibit the same means and distribution. The mean for the control group may be higher or lower than the mean for the reference interval, and the distribution of results for the control group is almost always narrower than that of the reference interval. Depending on the location of the control group data within the reference interval, a very small, incidental difference for the treated group may fall outside of the reference interval, and a relatively large, adverse test article-induced difference may fall within the reference interval. A small difference, within the reference interval, may represent a very significant adverse effect; for example, dehydration can result in normal RBC counts and protein concentrations in anemic, hypoproteinemic animals. Loss of an entire subpopulation of lymphocytes is not necessarily enough to cause the absolute total lymphocyte count to fall below the reference interval. When reference intervals are broad, as occurs when few partitioning factors are used or normal interanimal variability is great (e.g., ALP activity for monkeys), significant toxicity can occur without individual test results exceeding the limits of the intervals. Investigators must understand the limitations of reference intervals with respect to data interpretation. By themselves, reference intervals do not determine whether or not an apparent difference is real or adverse. They are simply an adjunct to sound scientific judgment.

Regardless of the many pitfalls affecting their use for data interpretation, historical reference intervals do have other important functions. They serve as a nonspecific measure of quality control that can detect changes over time in assays, study conditions, or animal characteristics. For example, it may be noticed that liver enzyme activity in mice has increased over time, and the cause of this finding might be traced to changes in handling practices or animal supplier. Reference intervals also serve as a nonspecific measure of analyte variability. The cause of seemingly excessive variability might be inadequate assay precision, nonstandardized preanalytical procedures, or true interanimal variability. Finally, reference intervals can be valuable when only treated animals are tested at an unscheduled interval because of signs of toxicity or when very few animals are used for small investigational studies with no concurrent control group. Reference intervals may help to determine that animals acquired for a small study have preexisting problems that could confound data interpretation.

Hematology Tests and Interpretation

Hematology tests most often performed for toxicology studies evaluate erythrocytes, leukocytes, platelets, and coagulation. Technological advances by manufacturers of hematology and coagulation analyzers have increased the availability of highly sophisticated instruments to perform tests on animal blood with precision and accuracy suitable for nonclinical safety assessment studies. Automated hematology methods are a necessity in toxicology because manual methods are unacceptably imprecise, labor intensive, and slow. Regardless of the method of analysis, however, absolute WBC and reticulocyte counts (cells/unit of volume)

should always be reported rather than relative counts (percent of total). Relative counts are irrelevant for interpretation of effects.

Hematology and Coagulation Analyzers

Many currently marketed hematology instruments are capable of measuring or calculating all of the routinely recommended tests using less than 300 μL of anticoagulated whole blood. The instruments typically use a combination of two or more principles including electrical aperture impedance, laser light scatter, and differential staining characteristics to determine cell number (RBC, WBC, and platelet counts), cell type (WBC differential count and reticulocyte count), and cell size and size distribution (MCV, mean platelet volume [MPV], and RDW). Hemoglobin concentration is measured directly. From the previously mentioned parameters, hematocrit, MCH, and MCHC are calculated, and a few less common parameters may also be determined. Because the blood cells of laboratory animal species differ morphologically from human blood cells, only instruments with software validated for animal samples should be used.[73]

Coagulation analyzers measure the time required for fibrin clot formation after the reagent is added to a plasma sample. Clot detection can be accomplished by evaluating changes in conductivity, physical resistance, light scatter, or optical density. Most instruments can be programmed to permit detection of clot formation at times appropriate for animal specimens; these times can be much shorter (e.g., PT for dogs) or longer (e.g., PT for guinea pigs) than those for human samples.

Erythrocytes

Effects on erythrocyte parameters typically reflect a change in the balance between RBC production and RBC loss or destruction. Changes in plasma volume (e.g., dehydration or volume expansion) can also indirectly affect erythrocyte parameters. Although mechanisms for effects on erythrocyte parameters may be obvious, such as hemorrhage from gastric ulceration, they are often relatively obscure. Even when the exact mechanism is unclear, the effects can usually be described in terms of broad mechanistic categories and impact on health. RBC count, hemoglobin concentration, and hematocrit are measures of red cell mass. These parameters may or may not change proportionally, depending on the cause of the change and whether or not cell size and hemoglobin content are affected.

Decreased Red Cell Mass

Decreases in red cell mass must be differentiated from anemia. Anemia is defined clinically as a condition characterized by a hemoglobin concentration below the lower reference limit but can also be defined functionally as a decrease in red cell mass sufficient to cause decreased oxygen delivery to peripheral tissues. Decreased oxygen delivery may result in clinical signs such as pallor, weakness, exercise intolerance, tachycardia, or tachypnea. Humans generally have no clinical signs of anemia until hemoglobin concentration decreases below 8 g/dL.[74]

In many toxicology studies, animals in a treated group have lower RBC counts, hemoglobin concentrations, or hematocrits than those for the control animals, but the differences are less than those necessary to cause clinical signs or affect tissue oxygenation and are, therefore, not indicative of anemia. Unless the differences for RBC count, hemoglobin concentration, and hematocrit are quite large, it is preferable to simply discuss the magnitude of the differences between the control and treated groups and avoid using the term *anemia*. For example, reductions from control values for these parameters up to approximately 10% are relatively mild and probably do not have an adverse effect on the health of the animal. Reductions from approximately 10% to 30% may be considered a moderate effect and may or may not be clinically adverse. Reductions of more than 30% are generally marked and clearly represent a clinically adverse anemic condition. Although a 5% reduction in hemoglobin concentration may be insufficient to adversely affect health, the cause of the reduction (e.g., liver toxicity, gastric ulceration, or immune-mediated red cell destruction) may be an adverse process.

An animal's response to decreased red cell mass can be broadly categorized as regenerative or nonregenerative. A regenerative response is characterized by an appropriate increase in RBC production (erythropoiesis), and a nonregenerative response is characterized by the absence of an appropriate increase. The most important parameter for determining whether the response is regenerative or nonregenerative is the absolute reticulocyte count. For proper interpretation, the absolute reticulocyte count (i.e., reticulocytes/μL) should always be determined by multiplying the relative reticulocyte count (i.e., percent of erythrocytes) by the RBC count. If an animal is severely anemic, its relative reticulocyte count may be increased, yet its absolute reticulocyte count (most relevant because it measures the erythropoietic response) may be decreased.

Conditions Characterized by a Regenerative Response

Regenerative responses result from two general causes of reduced red cell mass: blood loss (hemorrhage) and accelerated erythrocyte destruction (hemolysis). Following acute blood loss or hemolysis, new erythrocytes (i.e., reticulocytes) begin to increase in number in peripheral blood within 3–4 days. Reticulocytes are larger and contain less hemoglobin per volume than do mature RBCs. During a strong regenerative response, the increased number of reticulocytes usually increases MCV and decreases MCHC. In practice, increased MCV is more commonly observed than decreased MCHC, especially for dogs and monkeys. In addition, the presence of variable cell size (anisocytosis) often results in increased RDW. Reticulocytes stain slightly more basophilic than mature erythrocytes with Romanowsky-type stains typically used for assessing RBC morphology and doing manual WBC differential counts (e.g., Wright or Giemsa stain). During a strong regenerative response, erythrocyte morphology

evaluated microscopically is described by the terms *anisocytosis* (variable size) and *polychromasia* (variable color) because of the increased numbers of reticulocytes. Greater numbers of nucleated RBCs, also called *metarubricytes*, and Howell–Jolly bodies (small pieces of nuclear material not cleared from the erythrocyte) may also be observed microscopically during a regenerative response.

During a strong regenerative response, erythrocyte production, as measured by reticulocyte count, can increase as much as sixfold to eightfold over normal levels. As long as the accelerated erythropoiesis is able to match erythrocyte loss or destruction, red cell mass parameters will remain relatively stable. If the cause of loss or destruction is removed or if erythropoiesis exceeds loss or destruction, the red cell mass will return to normal. When recovery periods are included in toxicology studies with compounds causing regenerative responses, affected animals occasionally exhibit transiently increased red cell mass parameters, during or following recovery, compared with control animals. Although the impetus for increased erythropoiesis is removed at the beginning of the recovery period, committed erythrocyte precursors continue to mature into RBCs, resulting in this rebound effect.

Blood Loss

Blood loss occurs secondary to a variety of conditions or study-related procedures and should always be considered when a decrease in red cell mass is accompanied by a similar decrease in serum protein concentration. The source of the blood loss may be identified by clinical observations (e.g., dermal ulceration, melena, epistaxis), necropsy findings (e.g., gastrointestinal ulceration or cystitis), or other laboratory tests (e.g., fecal or urine occult blood tests). Blood loss associated with serial blood collection for pharmacokinetic investigations, clinical pathology tests, or other study-specific requirements may also cause effects on erythrocyte parameters. Blood loss typically results in increased reticulocyte count, MCV, and polychromasia unless the loss is very recent (i.e., within the previous 2 or 3 days) or complicating factors affect erythropoiesis (see Indirect Causes of Nonregenerative Conditions).

Hemolysis

Hemolysis is characterized by premature elimination of RBCs from the circulation and is classified as either intravascular or extravascular. Hemolysis is considered intravascular when RBCs are destroyed directly within circulation and extravascular when RBCs are phagocytized, most commonly in the spleen and liver, by cells of the mononuclear phagocyte system (i.e., macrophages). Of the two, extravascular hemolysis is more commonly encountered in toxicology studies and usually results from oxidative or immune-mediated injury to RBCs.[75] Regardless of the underlying mechanism, extravascular hemolysis is usually accompanied by splenomegaly, bone marrow hypercellularity, and/or extramedullary hematopoiesis (more pronounced in rodents). Histologically, increased pigment is generally observed in the spleen and sometimes other organs, indicating increased red cell turnover and processing of hemoglobin. Increased serum bilirubin (bilirubinemia) and urine bilirubin (bilirubinuria) are less common correlative findings. Intravascular hemolysis is characterized by the presence of free hemoglobin in plasma or urine. Processes resulting in intravascular hemolysis generally occur rapidly and may cause rapid and profound changes in red cell mass. Some hemolytic processes have both extravascular and intravascular components.

Extravascular hemolysis secondary to oxidative injury is sometimes identified by the presence of Heinz bodies. Heinz bodies are particles of irreversibly denatured hemoglobin attached to the inner surface of the RBC membrane. They result when test articles with oxidative properties cause disulfide bonds to form between sulfhydryl groups of hemoglobin. RBCs containing Heinz bodies may be removed from circulation by macrophages and/or become morphologically distinct (e.g., ghost and blister cells) following selective removal of the Heinz bodies. Although Heinz bodies can be difficult to detect with the Romanowsky-type stains, they stain prominently with supravital stains (e.g., methylene blue) used for manual reticulocyte counts. The number of cells containing Heinz bodies can be determined in the same manner as that for manual reticulocyte counts. The size and number of Heinz bodies are dependent on the causative agent, dose, and time after exposure. Acute exposure to a potent oxidative agent typically causes acute anemia characterized by the observation of many RBCs containing a single, large Heinz body or, less frequently, multiple, small Heinz bodies. Ghost cells, blister cells, and other morphologic abnormalities may be present. Increased reticulocyte counts (reticulocytosis) develop within 3–4 days. Chronic, low-level exposure to an oxidizing agent usually does not cause a significant reduction in red cell mass but may cause increased reticulocytes. Under these conditions, the number of RBCs containing Heinz bodies is generally low, and the condition may go unrecognized if not looked for specifically. Identification of test articles that cause Heinz body formation is particularly important clinically because a deficiency of the erythrocyte enzyme glucose-6-phosphate dehydrogenase is relatively common in humans and results in greater susceptibility to oxidant-induced hemolysis.[76]

Oxidant test articles that cause Heinz body formation may also cause methemoglobinemia and vice versa.[77] Methemoglobin is hemoglobin in which the iron has been reversibly oxidized from the ferrous state (Fe^{2+}) to the ferric state (Fe^{3+}). Cells containing increased methemoglobin concentrations are not more susceptible to hemolysis but transport less oxygen than normal. The clinical signs of significant methemoglobinemia, therefore, are those of hypoxia.[78] Mucous membranes become cyanotic at methemoglobin concentrations above 10%. Lethargy and weakness occur at concentrations of approximately 30% or more. At greater than 80%, methemoglobinemia may be fatal. Blood containing a high concentration of methemoglobin appears brown. Accurate and efficient measurement of methemoglobin concentration can be accomplished with instruments

called *hemoximeters* or *cooximeters*. These multiwavelength, microprocessor-controlled photometers are designed to measure hemoglobin pigments such as carboxyhemoglobin and methemoglobin as well as the percentage of hemoglobin oxygenation. Samples collected for methemoglobin determination should be analyzed quickly (e.g., within 30–60 min) because methemoglobin is normally reduced by the erythrocyte enzyme methemoglobin reductase. The combination of a sensitive, precise instrument and a well-executed toxicology study allows detection of test article-induced methemoglobin formation at concentrations well below those necessary to cause clinical signs.[79] Among laboratory animals, the mouse is a poor model for studying the potential of test articles to cause methemoglobinemia because their erythrocytes have very high methemoglobin reductase activity.[80] Test article-induced methemoglobin in mice is quickly reduced to hemoglobin and, therefore, is more difficult to detect than in other species.

Immune-mediated RBC destruction has been associated with many drugs[81] but is largely a nondose-related idiosyncratic phenomenon and is detected infrequently in nonclinical toxicology studies. When it is observed, only one or two animals are typically affected, and they may or may not be in the high-dose group. In contrast to hemolytic conditions that occur soon after exposure to the test article, immune-mediated hemolysis is typically not observed until the test article has been administered for at least 1 week. Three general mechanisms exist for immune-mediated hemolysis: (1) the test article acts as a hapten bound to the RBC membrane; (2) the test article elicits an antibody response, and the resulting antigen–antibody complex binds to the RBC; and (3) the test article causes the immune system to mistakenly recognize normal RBC membrane antigens as foreign, and true autoantibodies are produced. Cells of the mononuclear phagocyte system, especially in the spleen, recognize antibody-coated RBCs and either phagocytize the entire cell or remove a portion of its membrane, creating morphologically distinct cells called *spherocytes*. On rare occasions, immune-mediated hemolysis is complement-mediated and occurs intravascularly. In a blood film, spherocytes appear smaller and denser than other RBCs; they are round and lack central pallor. Although small numbers of spherocytes can be observed with other conditions, they are the predominant morphologic feature of immune-mediated hemolysis. RBC autoagglutination is occasionally observed in the blood film, especially if the antibody response is primarily immunoglobulin M (IgM). Alternatively, autoagglutination may be detected in a wet mount of fresh blood diluted with saline or by special evaluation of automated hematology analyzer parameters. Direct antiglobulin tests may be used to confirm the presence of antibody or complement on RBCs, but species-specific anti-immunoglobulin or anticomplement must be used.[82] Animals with test article-induced immune-mediated hemolytic anemia can become severely anemic with repeated test article administration; however, they usually exhibit a strong regenerative response and will generally recover if test article administration is stopped. Confirmation that the anemia was test article induced can be accomplished by rechallenging the animal following recovery. Upon rechallenge, hemolysis should be evident within 1–2 days. Immune-mediated hemolytic anemia, unrelated to test article administration, is an unusual finding in all species used in toxicology studies, with the exception of Fischer 344 rats; immune-mediated hemolytic anemia often occurs as a sequela to large granular lymphocyte leukemia.[26]

Additional mechanisms associated with extravascular hemolysis include processes that result in RBC structural changes recognized as abnormal by the mononuclear phagocyte system. These include effects on RBC membrane lipids and proteins, intercalation of test article into the cell membrane bilayer, and effects on RBC metabolic processes. In addition, numerous biotherapeutics have been shown to cause activation of the mononuclear phagocyte system, resulting in extravascular hemolysis.[83] Direct damage to the RBC membrane is most commonly caused by test articles administered intravenously. The lipid bilayer of the cell membrane is sensitive to test articles with detergent-like properties, and intravascular hemolysis can occur rapidly when RBCs are exposed to high concentrations of the test article at the site of intravenous infusion (e.g., the tip of the catheter or needle). Administration of a very hypotonic solution, as might occur if sterile water is inappropriately chosen as the vehicle for a low-concentration solution, is another potential cause of acute intravascular hemolysis. Water passively enters erythrocytes because of the ionic concentration gradient, and the cells swell and rupture. Intravascular administration of hypertonic solutions typically does not cause hemolysis. If the amount of released hemoglobin exceeds the carrying capacity of circulating haptoglobin, unbound hemoglobin passes through the glomerulus and is excreted in the urine. Visible hemoglobinuria (red-tinged urine) may be observed within a few hours of treatment, and a regenerative response is detectable within 3 or 4 days of treatment. Intravenously administered test articles that cause extensive intravascular hemolysis are usually associated with local damage to endothelium. The effect of the vascular damage may be observed grossly (e.g., tail lesions in rodents treated via the tail vein) or histologically. Additional histopathologic evidence of intravascular hemolysis is the presence of hemoglobin pigment within renal tubular epithelial cells and hemoglobinuric nephrosis. Compounds administered by other routes rarely cause significant intravascular hemolysis.

Whenever regenerative anemia is identified in a nonhuman primate study, the role of the hemotropic parasite *Plasmodium* must be considered as a possible contributing cause. Many imported monkeys, even though they are captive bred, harbor subclinical infections with this organism that causes malaria.[84,85] The readily identifiable intracellular organism is frequently observed in blood films from healthy animals that have no signs of anemia.[86] Parasitemia is inconsistent or cyclical, however, and multiple blood samples may be examined from an animal before the organism is identified. On rare occasions, test article administration or the stress of shipment and study-related procedures precipitates a hemolytic crisis. When this occurs, the parasitemia is usually quite obvious in the blood film.

Another infrequent cause of hemolysis in toxicology studies is mechanical fragmentation or microangiopathic hemolysis. This form of hemolysis typically occurs when RBCs are forced to pass through small, fibrin-obstructed vessels in highly vascular tissue. The resulting fragmented RBCs are called *schizocytes* (helmet cells) and are easily identified microscopically. Disseminated intravascular coagulation (DIC) is the best example of a condition causing microangiopathic hemolysis, but widespread vascular injury in tissues such as lung, liver, or intestine also causes some degree of fragmentation. These conditions, especially DIC, are generally so severe that an animal may fail to mount a significant regenerative response before it dies or is humanely euthanized. Test articles that target endothelial growth factors and/or cause less severe vasculopathies may also result in RBC fragmentation.

Nonregenerative Conditions

Nonregenerative conditions are characterized by the absence of appropriately increased erythropoiesis in the presence of declining red cell mass. This can result in progressively decreased red cell mass over time because red cells that are normally destroyed or lost are no longer replenished. Erythrocyte morphology in nonregenerative conditions is characterized by the absence of changes observed with regeneration (e.g., anisocytosis, polychromasia, increased MCV, decreased MCHC). Most often, erythrocytes appear normocytic (normal size) and normochromic (normal color), and the MCV is unchanged (large animals) or decreased (rodents). Absolute reticulocyte count is unchanged or decreased, depending on whether the problem is simply an inability to adequately respond to increased needs or, more significantly, failure of erythropoiesis. Nonregenerative conditions result from indirect or direct causes.

Indirect Causes

A common finding in nonclinical toxicology studies is mildly decreased red cell mass (i.e., RBC count, hemoglobin concentration, and hematocrit) without a regenerative response and without an obvious mechanism for the effect. Red cell mass is usually no more than about 10% lower than that of the respective control group (e.g., mean control group hematocrit = 44%; mean high-dose group hematocrit = 40%). Slightly lower MCV is sometimes observed, especially in rodents. The animals may or may not exhibit clinical signs of poor health (e.g., poor grooming habits or decreased activity) or reductions in body weight, body weight gain, or, less frequently, feed consumption. In some cases, no clinical signs are observed. Potential concurrent effects on other clinical pathology test results include mildly decreased serum total protein and albumin. These relatively mild, nonspecific findings for red cell mass are identified most frequently in rat studies where the number of animals per group is high, the dose levels used are typically higher than those for dog or monkey studies, and the normal interanimal variability of hematology data is relatively low. In addition, because the circulating life spans of mouse and rat erythrocytes (approximately 25–40 and 45–65 days, respectively) are shorter than those for dogs (approximately 100–120 days) and nonhuman primates (varies with species; for rhesus monkeys, approximately 85–100 days),[4] reductions in RBC production of similar magnitude will become apparent more quickly in rodents. In many cases, specific mechanisms for these changes are not identified; however, they suggest a generalized reduction of anabolic processes, including those of hematopoietic tissues. Anything that affects the normally brisk pace of RBC production (in humans, approximately 100 billion new cells per day) will ultimately be reflected in the test results. It is also possible that decreased physical activity and correspondingly decreased tissue oxygen demand may contribute to reduced erythropoiesis.

Indirect effects on erythropoiesis are relatively common in toxicology studies and result from toxic effects on other tissues or organ systems. Collectively, they are sometimes referred to as *anemia of chronic disease*. Chronic inflammatory diseases[87–89] and significant kidney,[90] liver,[91] and endocrine dysfunction (e.g., hypothyroidism and hypoadrenocorticism)[92] negatively affect erythropoiesis and erythrocyte survival, and all of these conditions can be associated with this type of mild to moderate reduction in red cell mass. With chronic inflammatory conditions, the causes of reduced erythropoiesis are thought to be decreased availability or transfer of iron to developing erythrocytes, decreased responsiveness of the bone marrow to erythropoietin, and decreased red cell life span. With renal disease, reduced erythropoiesis is attributed to decreased renal production of erythropoietin and the effects of uremic toxins. Liver disease is sometimes associated with acanthocytosis, a morphologic abnormality of erythrocytes characterized by several blunt cytoplasmic projections resembling pseudopodia. The acanthocytes are thought to result from the accumulation of free, nonesterified cholesterol in the RBC membrane. Acanthocytes are relatively inflexible and removed prematurely from circulation by cells of the mononuclear phagocyte system, indicating that extravascular hemolysis plays a role in some decreases in red cell mass due to chronic conditions. Small reductions in circulating red cell mass are observed with hypothyroidism and may result from reduced basal metabolic rate and cellular requirements for oxygen. Changes in thyroid metabolism may play a role in the mild erythrocyte effects observed in some animals with reduced food consumption because caloric malnutrition can result in decreased T_3 and decreased responsiveness to T_3, which may, in turn, lead to reduced production of erythropoietin. These indirect effects on the erythron tend to be relatively mild and are not as toxicologically important as the direct effects on the primary target tissue.

Chronic iron deficiency, most commonly associated with chronic blood loss or inadequate dietary iron, is relatively rare in toxicology studies and is characterized by microcytic (low MCV), hypochromic (low MCHC) erythrocytes.

Direct Causes

Sustained direct toxic effects on the development and maturation of erythrocytes result in severe anemia because

senescent erythrocytes are not replaced. Rodents become anemic faster than dogs or monkeys because of the short circulating life span of their erythrocytes. In contrast to most toxicities that indirectly affect erythropoiesis, direct toxic effects markedly reduce absolute reticulocyte count.

Direct injury to pluripotent hematopoietic stem cells or their stromal microenvironment causes failure of blood cell production, resulting in the condition called *aplastic anemia*.[93,94] Aplastic anemia is characterized by varying degrees of pancytopenia—decreased erythrocytes, leukocytes (primarily neutrophils), and platelets—and hypocellular bone marrow. Because leukocytes are affected, decreased resistance to bacterial infections usually causes severe illness or death before the anemia becomes life-threatening. Irradiation is a classic model of stem cell injury and is used therapeutically as part of the process for bone marrow transplantation. In addition to irradiation, several chemicals and drugs are known to cause aplastic anemia in humans. These include benzene, toluene, lindane, pentachlorophenol, chloramphenicol, phenylbutazone, penicillamine, gold salts, and acetazolamide. Chemotherapeutic drugs such as alkylating agents (e.g., busulfan and cyclophosphamide), antimetabolites (e.g., fluorouracil and methotrexate), and cytotoxic antibiotics (e.g., doxorubicin and daunorubicin) have the potential to cause aplastic anemia because of their pharmacologic activity. Normally, however, their effects are reversible following completion of each treatment cycle.

In toxicology studies with test articles such as these, decreased WBC and platelet counts are typically recognized earlier in the study (generally 5–10 days after initiation of treatment) than decreased RBC counts because the circulating life spans for neutrophils (12–24 h) and platelets (7–10 days) are much shorter than that for erythrocytes. Conversely, absolute reticulocyte count can be a sensitive indicator of hematopoietic injury and may be better than WBC or platelet counts for identifying the onset of toxic effect and subsequent recovery. This is most apparent in rodent studies because the normally high absolute reticulocyte counts for young rodents facilitate identification of decreases, while their normally low neutrophil counts make neutropenia more difficult to recognize. Furthermore, reticulocyte counts are not compromised by poor blood collection technique that may cause increased interanimal variability for platelet count.

The timing of sample collection following administration of a test article that temporarily interrupts hematopoiesis will dictate the findings in peripheral blood and can impact interpretation. During the peak effect, reticulocytes, neutrophils, monocytes, eosinophils, platelets, and often lymphocytes are decreased in number, and histopathologic examination of the bone marrow reveals hypocellularity. Once the effect ends, hematopoietic tissue usually mounts a strong recovery, often called a *rebound effect*, that is characterized peripherally by increased counts for reticulocytes, platelets, and sometimes neutrophils. Increased extramedullary hematopoiesis in the spleen, especially for rodents, is often apparent before the bone marrow repopulates. Because the time to peak effect and the duration of effect vary for different test articles, it is necessary to perform hematology tests at multiple intervals for proper interpretation.

Pure red cell aplasia, a condition in which only the production of red cells fails, is rarely observed in toxicology studies, even though several drugs are known to cause the disorder in humans.[95] Because most drug-induced pure red cell aplasias are idiosyncratic and may be immune mediated, recognition of this toxic effect is low in animal studies using a limited number of test subjects. Furthermore, it would be very difficult to prove that unexplained nonregenerative anemia in a single animal was a direct test article effect. Pure red cell aplasia has been observed in animals dosed with recombinant human erythropoietin because the animals produced neutralizing antibody that cross-reacted with and neutralized endogenous erythropoietin. The lack of erythropoietic stimulation caused red cell aplasia.

Megaloblastic anemia is a nonregenerative reduction in red cell mass characterized by macrocytic erythrocytes (increased MCV), asynchronous maturation of cytoplasm and nucleus in hematopoietic precursors, and hypersegmented or giant neutrophils.[96] In humans, it is associated with a variety of disorders that cause folate or vitamin B_{12} deficiency (e.g., sprue, alcoholic cirrhosis, and pernicious anemia) and drugs (e.g., methotrexate) that impair DNA synthesis. Macrocytosis results because developing erythrocytes undergo fewer divisions before maturation. Megaloblastic conditions are rarely identified in laboratory animals, perhaps due to differences in uptake and metabolism of folate and vitamin B_{12}. On the other hand, nonhuman primates have been used frequently as animal models for folate and vitamin B_{12} deficiency.[97] Some antiviral drugs impair cellular DNA synthesis and cause mildly increased MCV that may or may not be associated with decreased red cell mass.

Finally, nonregenerative anemia is a feature of some hematopoietic tumors and leukemias. Nonregenerative anemias caused by neoplastic processes are due to a combination of factors, including blunted response to erythropoietin, excessive cytokine release (IL-1, IL-6, and TNFα) suppressing erythropoiesis, hemolysis, nutritional deficiencies, and competition for bone marrow space.[98,99] It is not unusual in carcinogenicity studies to observe severe anemia secondary to naturally occurring leukemia.

Leukocytes

Leukocytes are evaluated by counting the concentration of WBCs in peripheral blood and differentiating them into specific cell types: neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Hematology analyzers capable of automated differentials provide more accurate data than manual counts and enable the detection of changes in even minor leukocyte types (e.g., decreased eosinophils). When interpreting and reporting differential WBC count results, it is essential to evaluate the absolute cell counts (i.e., cells/μL). Relative or percent counts are simply a means for determining the absolute counts and have little or no inherent value for assessing an animal's condition. For example, a

50% neutrophil count in a dog could be normal, increased, or decreased, depending on the total WBC count. As with changes in red cell mass, small changes in leukocyte counts should generally not be described using diagnostic terminology. When changes are pronounced, diagnostic terminology for increases and decreases in leukocytes may be used. These clinical terms include *leukocytosis* and *leukopenia*, *neutrophilia* and *neutropenia*, *lymphocytosis* and *lymphopenia*, *monocytosis* and *monocytopenia*, *eosinophilia* and *eosinopenia*, and *basophilia*. Neutrophils and lymphocytes are the most numerous WBC types in peripheral blood and are usually the cells affected when peripheral blood leukocyte counts change due to toxicity. Indirect effects on these two cell lines are often observed in response to study-related procedures or test article effects on other tissues. Direct test article effects on these two cell lines can occur but are less common than indirect effects.[100]

Physiological Leukocytosis

Physiological leukocytosis occurs in excited or frightened animals secondary to endogenous catecholamine release. Increased heart rate, blood pressure, and muscular activity shift leukocytes from the marginal pool (i.e., cells that adhere to the endothelium of small vessels or are sequestered in vascular beds of tissues such as the spleen) to the circulating leukocyte pool. Physiologic leukocytosis may cause the total WBC count to double. The specific cell type predominantly responsible for the increase varies because of species-dependent differences in the normal distribution of leukocyte types. Increased neutrophil count is the most prominent change for dogs, while increased lymphocyte count is most conspicuous for rats. Physiological leukocytosis of nonhuman primates generally results in a relatively similar increase in neutrophils and lymphocytes. Because physiological leukocytosis is most frequently observed in animals not accustomed to handling or blood collection, it is common for a few animals to have notably high WBC counts only at the first blood collection interval for a study. Recognition of this phenomenon is critical in studies using few animals (usually dogs or monkeys) and only one pretreatment interval. Overinterpretation of data from a study using this design might lead to the false conclusion that a test article has a myelosuppressive effect because posttreatment WBC counts, determined when the animal is more accustomed to being handled, may be much lower than pretreatment counts. For this reason, strong consideration should be given to acquiring at least two pretreatment hematology samples to facilitate proper data interpretation, especially if peripheral leukocyte counts are critical endpoints.

Steroid- or Stress-Induced Leukocyte Response

This leukocyte pattern occurs following exogenous glucocorticoid administration or when stressful conditions result in increased production of endogenous glucocorticoids. It is characterized primarily by increased neutrophil count (immature neutrophils, such as bands, are absent) and decreased lymphocyte and eosinophil counts. Increased monocyte counts may or may not be present. Differentials measured by an automated instrument are generally necessary to detect decreases in eosinophil count. Even though study-related procedures or test articles appear to create conditions generally considered stressful, this pattern is typically exhibited by individual animals and not entire groups. The changes are common in animals exhibiting significant toxicity. Leukocyte changes consistent with stress can be integrated with in-life and other clinical and anatomic pathology endpoints to develop a weight-of-evidence approach to determine that animals experienced stress.[35]

Increased and Decreased Neutrophil Counts

Increased neutrophil counts are usually caused by increased production or decreased margination but can also be caused by increased circulating life span. Increased production is generally the result of signals prominent in inflammatory conditions, with or without the involvement of an infectious agent. The signal for increased neutrophil production may be an inherent property of the test article (e.g., cytokines or hematopoietic growth factors) or may be secondary to a toxicity (e.g., intravascular hemolysis or tissue necrosis) or study-related procedures (e.g., chronic catheterization or repeated injections). Various cytokines and hematopoietic growth factors developed as therapeutic agents directly affect neutrophil kinetics and may result in mild to marked neutrophilic leukocytosis. The term *left shift* refers to an increased number of immature neutrophils (e.g., band neutrophils) in circulation, usually in response to an inflammatory lesion with a significant demand for neutrophils or administration of inflammatory cytokines. Inflammatory lesions resulting in left shifts or marked neutrophilia are usually identified either by physical examination, other diagnostic tests, or at necropsy. The term *degenerative left shift* describes the combination of a normal or decreased absolute neutrophil count with more immature than mature neutrophils. This pattern indicates that the production of neutrophils is insufficient to meet the peripheral need for neutrophils. It generally reflects a very severe infection as might occur with aspiration pneumonia, gastrointestinal perforation, or septicemia associated with bacterial contamination of an indwelling intravenous catheter. In conditions such as these, *toxic neutrophils* may be observed. These neutrophils have distinct morphological characteristics including cytoplasmic basophilia, vacuolation, and granulation and the presence of Döhle bodies (small, bluish-gray cytoplasmic inclusions made of aggregated rough endoplasmic reticulum). The term *toxic neutrophils* is a misnomer in that these cells only indicate greatly accelerated neutrophil production, regardless of cause; for example, toxic neutrophils may be observed following the therapeutic administration of hematopoietic growth factors. Increased neutrophil count due to decreased egress may occur with glucocorticoid therapy or test articles that inhibit the normal processes of egress from the vasculature.

Decreased neutrophil counts can be due to decreased production, increased margination, or increased egress from circulation. Leukocyte effects of cytotoxic or antiproliferative

agents are typically observed in most or all of the animals in groups receiving toxic dose levels. Because mice and young rats normally have very low neutrophil counts (e.g., less than 1000/μL), recognition of neutropenia is more difficult for these species than for others. In addition, although a neutrophil count below 500/μL is generally considered an indicator of risk for bacterial infection in humans, monkeys, and dogs, low neutrophil counts in rodents are generally not associated with increased incidence of infection. For noncytotoxic drugs, the observation of severe neutropenia, with or without a left shift or toxic neutrophils, is generally limited to one or two individuals that have severe complications secondary to test article toxicity or study-related procedures.

Single or short-term administration of a potent cytotoxic agent is usually characterized by neutropenia within a few days to a week of treatment, followed by a recovery that may include the presence of immature neutrophils for a short time and may result in a rebound neutrophilia. Detection of these changes is dependent on the timing and frequency of hematology testing. Similarly, the appearance of the bone marrow, whether hypocellular or hypercellular, is also dependent on the timing of sample collection. Hematology findings for test articles that directly injure pluripotent stem cells or rapidly dividing committed precursor cells are usually characterized by decreased numbers of reticulocytes, platelets, neutrophils, monocytes, eosinophils, and, possibly, lymphocytes. Selective damage of granulocyte precursors without affecting erythropoiesis or thrombopoiesis is unusual.

Immune-mediated reductions in neutrophil counts are relatively rare but can occur.[101–103] Like immune-mediated hemolytic conditions, however, they are often idiosyncratic and difficult to identify in nonclinical safety studies using limited numbers of test subjects.

Increased and Decreased Lymphocyte Counts

Lymphocytes are responsible for a wide variety of immune system functions. Lymphocytes are relatively long-lived compared with other leukocytes and have the ability to leave the vascular system through venules in lymph nodes and eventually reenter the blood via the thoracic duct. Automated hematology analyzers and light microscopy only determine total lymphocyte counts; subpopulations of lymphocytes must be determined by other methods such as flow cytometry. Increased lymphocyte counts can be caused by increased production, decreased margination, or redistribution. Physiological leukocytosis (i.e., excitement causing decreased margination) should be considered whenever increased lymphocyte count is present in only a few animals. Cynomolgus monkeys occasionally exhibit markedly increased lymphocyte counts (e.g., >60,000/μL) of unknown etiology; these are generally transient but can obfuscate test article-related findings. Increased lymphocyte count is an uncommon test article-related effect, although it may be observed in conjunction with compounds that cause chronic inflammatory lesions, especially in rodents, or with administration of test articles that are antigenic and elicit an immune response by the test animals. Increased lymphocyte count may be a pharmacodynamic effect of immunomodulatory drugs designed to alter lymphocyte trafficking.

Decreased lymphocyte counts can be caused by decreased production, increased margination, or redistribution. Decreased lymphocyte count is most frequently observed as part of the stress- or glucocorticoid-induced leukocyte response. Marked lymphopenia is commonly observed in moribund animals. Cytotoxic test articles often cause decreased absolute lymphocyte counts, but the magnitude of the decrease is usually less prominent than that for neutrophils in nonrodent species. In rodents, it is generally easier to detect effects on lymphocytes because of the normally high number of lymphocytes compared with neutrophils. During recovery from effects of cytotoxic test articles, lymphocyte counts may remain decreased longer than neutrophils and typically do not exhibit a prominent rebound response. Because of the many subpopulations of lymphocytes, it is difficult to gauge the biological importance of small decreases in absolute lymphocyte count. Selective reduction or elimination of a subpopulation may occur (e.g., selective decreases in B lymphocytes by belimumab, a BLyS antagonist) without greatly affecting the total lymphocyte count.[28] Evaluation of corollary information (e.g., overall health and findings that might suggest immunosuppression) is essential to putting small changes in lymphocyte count into context.

Monocytes, Eosinophils, Basophils, and Large Unstained Cells

Although absolute counts for these cell types are generally quite low (e.g., <1000/μL), test article-induced effects can often be detected using analyzers that do automated differential counts. As with neutrophils and lymphocytes, increases in circulating numbers of these cells are generally secondary phenomena unless the test article is a hematopoietic growth factor or other cytokine that directly stimulates cell production (e.g., interleukin-5 increases eosinophil count).

Monocytes phagocytize and digest particulate matter such as senescent cells and necrotic cell debris. They also serve as precursors to cells of the mononuclear phagocyte system and as a source of macrophages in inflamed tissues. Monocytes participate in modulation of the inflammatory response through cytokine production and antigen processing and presentation to lymphocytes. Increased monocyte counts may occur secondarily to any condition with substantial tissue destruction, such as widespread inflammation, tumor-associated necrosis, or hemolytic anemia. Decreased monocyte counts may be identified following administration of a cytotoxic chemotherapeutic agent.

Eosinophils are part of the body's defense against helminthic parasite infections, and eosinophilia is occasionally observed in nonhuman primates with a heavy parasite load. Increased eosinophil count is sometimes observed secondarily to hypersensitivity reactions. In addition, platelet clumps may be erroneously counted as eosinophils by some hematology analyzers.[104] Decreased eosinophil count may be identified as part of the stress-induced leukocyte pattern or following administration of cytotoxic chemotherapeutic agents.

Basophils may play a role in some hypersensitivity reactions. Effects on basophil counts under the condition of a toxicology study are rare. Large unstained cells are counted by automated hematology analyzers manufactured by Siemens Corporation (previously Bayer). These cells cannot be classified by the instrument into one of the five major cell types but generally are thought to represent lymphocytes, monocytes, or some immature cells. Changes in large unstained cell counts are typically observed with changes in the other cell types.

Leukemia

In most 2-year carcinogenicity studies using rodents, some of the animals will develop leukemia, a neoplastic proliferation of a hematopoietic cell line. The diagnosis of leukemia is best made by histopathologic examination of tissues infiltrated with the neoplastic cells. The odds of correctly identifying animals with leukemia are much greater by doing routine histopathology than by doing periodic hematologic examinations at regularly scheduled intervals. Although leukemias are sometimes characterized by markedly elevated WBC counts and the presence of neoplastic cells (e.g., blasts) in circulation, many animals with leukemia exhibit neither of these characteristics. Even when neoplastic cells are present in peripheral blood, it is often difficult to determine from which cell line they were derived (e.g., granulocytic, lymphocytic, myelomonocytic) because immature, anaplastic, or blast-stage cells from different cell lines can be indistinguishable by light microscopy using standard staining techniques.

Lymphocytic leukemias are the most commonly observed leukemias in laboratory rats and are occasionally observed as an incidental finding in 90-day toxicology studies.[17] Fischer 344 rats may develop large granular lymphocyte leukemia (also called *mononuclear cell leukemia*) at incidences of 30%–40% in the second year of a carcinogenicity study.[26] This neoplasm appears to arise in the spleen and commonly infiltrates other tissues, particularly the liver. Affected rats develop an immune-mediated hemolytic anemia and often exhibit hyperbilirubinemia and elevated liver enzyme activities in the serum. Neoplastic cells in peripheral blood appear as large, immature lymphocytes and may contain prominent azurophilic granules. Erythrophagocytosis by the neoplastic cells is occasionally observed.

Platelets

Platelets play a key role in primary hemostasis. When blood vessels are damaged, platelets quickly adhere to the subendothelium, undergo a shape change, and begin to aggregate, forming a primary platelet plug that is sufficient to control bleeding from minor injuries to small vessels. These activated platelets secrete a variety of substances that stimulate vasoconstriction and promote fibrin formation. Fibrin serves to cement the aggregated platelets into a stable hemostatic plug. Healthy endothelial cells in close proximity to the damaged vessel release inhibitors of platelet aggregation and fibrin formation in order to limit the clot size.

Increased Platelet Count

Increased platelet counts are caused by increased production by megakaryocytes or release of sequestered platelets. Extremely high platelet counts have the potential to increase the risk of thrombosis in humans, but increases in platelet count are generally asymptomatic in laboratory animals. Increased platelet counts in toxicology studies are usually indirect or secondary responses, but they can occur as a direct test article effect.

Increased platelet count as an indirect effect occurs in conjunction with generalized bone marrow stimulation as observed with hemolysis, blood loss, and many types of acute and chronic inflammation. These increases are generally of small magnitude and unlikely to have any biological significance; they have been termed *reactive* or *secondary thrombocytosis*. Release of hematopoietic growth factors such as erythropoietin and cytokines such as interleukin-6 and interleukin-11 may be at least partially responsible for the increased platelet production in some of these conditions.[105,106] Acute but transient increases in platelets may occur in association with physiological leukocytosis because catecholamine-induced splenic contraction releases platelets sequestered within the sinusoids of the spleen. A rebound thrombocytosis often follows recovery from significant thrombocytopenia caused by test articles such as chemotherapeutic agents that reversibly inhibit platelet production.

A few test articles directly stimulate platelet production and the release of platelets from megakaryocytes. These test articles include hematopoietic growth factors such as thrombopoietin and erythropoietin and small molecules such as vincristine.

Decreased Platelet Count

Decreased platelet count can be caused by decreased production or increased consumption/sequestration. Clinical signs associated with decreased platelet count include petechial and ecchymotic hemorrhages (most commonly observed in mucous membranes or at mucocutaneous sites), epistaxis, melena, menorrhagia, and prolonged bleeding from small wounds such as venipuncture sites. These signs typically do not occur spontaneously unless the platelet count is very low (e.g., less than 20,000/μL) or there is some type of hemostatic challenge (e.g., surgery).[107] Because of their protected environment, animals in toxicology studies are less prone to exhibit signs of hemorrhage when their platelet counts are extremely low.[108]

Decreased platelet counts are relatively common spurious findings associated with difficult venipuncture or inadequate anticoagulation of blood samples and subsequent in vitro platelet aggregation. In vitro aggregation can generally be confirmed by the observation of platelet clumps at the feathered edge of the blood film or by the characteristic scattergram produced by some automated hematology analyzers. Although typically observed for individual mice and rats, platelet clumps and spuriously low platelet counts can sometimes appear group-related because animals receiving

the test article may be more difficult to bleed as a result of poor health, dehydration, or small size relative to the control animals.

Decreased platelet counts due to decreased production can be caused by chemotherapeutic agents. These drugs reduce erythroid and myeloid cell production and frequently inhibit production of platelets by megakaryocytes as well. Moderately to markedly reduced platelet counts tend to occur a few days after reductions in neutrophil and reticulocyte counts because the circulating life span of platelets is longer, about 5–10 days.

Decreased platelet counts due to increased consumption of platelets can occur secondarily to acute lesions of highly vascular tissues (e.g., the gastrointestinal tract) or result from extensive hemorrhage, especially from multiple sites. If lesions affecting blood vessels are severe and widespread, DIC may develop, and platelet counts will be markedly decreased. Test articles may also stimulate immune reactions against platelets. Immune-mediated thrombocytopenia, like immune-mediated hemolysis, has been associated with many drugs[109] but is usually an idiosyncratic phenomenon and is, therefore, detected infrequently in nonclinical toxicology studies. When observed, only one or two animals are typically affected, and these animals may or may not be in the high-dose group. Immune-mediated thrombocytopenia and immune-mediated hemolysis may occur together. Antiplatelet antibody can be detected using flow cytometry; however, the best evidence that thrombocytopenia is immune mediated may come from a rechallenge exposure with the test article following cessation of treatment and recovery. Upon rechallenge, platelet count should drop acutely if the mechanism is an immune-mediated destruction. Compounds may also result in activation of platelets, which leads to their premature removal from circulation.[108] Activated platelets can be detected in some species by flow cytometric evaluation of activation markers.

Like immature red cells, young platelets have residual RNA that stains with nucleic acid dyes. Using methods analogous to those used for erythrocytes, these reticulated platelets can be counted using flow cytometry. Decreased platelet production is associated with inappropriately decreased concentrations of reticulated platelets, while platelet consumption is usually associated with increased reticulated platelet counts. Young platelets also tend to be relatively large, and MPV often increases during times of increased platelet production in response to platelet consumption.

Platelet Function

Test articles that affect platelet function have the potential to cause the same clinical signs as marked thrombocytopenia, but the tendency to do so is much less because of the complexity of platelet function and the presence of alternative or redundant pathways in vivo for the various platelet functions. Platelet function tests such as bleeding time and platelet aggregation may be beneficial when evaluating safety of therapeutic agents related to coagulation and platelet function, but it is important to recognize the considerable analytical and interanimal variability for these tests. Group results may be less meaningful than assessment of results from individual animals before and after test article administration. In addition, an effect noted in vitro may have no in vivo relevance.

Bone Marrow Smear Evaluation

The most important aspect of bone marrow smear evaluation is understanding when it should and should not be performed.[13] Although preparation of smears is advisable for most repeat-dose toxicology studies, evaluation of those smears is usually unnecessary. The standard hematology tests provide considerable information concerning bone marrow function, and if results from these tests are unaffected, it is extremely unlikely that bone marrow evaluation will provide any additional knowledge concerning potential significant test article effects on the hematopoietic system. Likewise, if the effects observed in peripheral blood are relatively small, it is unlikely that bone marrow smear evaluation will be beneficial.

Even when hematology test results are affected by test article administration, bone marrow evaluation has no benefit if mechanisms for the hematology findings are clear from the peripheral blood data and other findings. For example, decreased red cell mass parameters associated with increased absolute reticulocyte counts indicate a normal regenerative erythropoietic response to hemorrhage or hemolysis. In this example, bone marrow evaluation would simply confirm the presence of increased erythroid cellularity/production and provide no new information. Likewise, increased absolute neutrophil count in response to an inflammatory condition is normal, and bone marrow evaluation would only confirm increased granulocytic cell cellularity/production. Bone marrow smear evaluation is also not warranted following administration of a cytotoxic chemotherapeutic drug that predictably causes myelosuppression and can be monitored by peripheral blood tests.

The main indications for bone marrow smear evaluation in toxicology studies are moderate to marked nonregenerative anemia, leukopenia, or thrombocytopenia (or any combination of the three) of unknown etiology or significant morphological abnormalities of peripheral blood cells. The primary objective of the bone marrow smear evaluation is to assess the relative numbers and maturation of precursor cells to explain peripheral blood changes. Miscellaneous observations, including increased iron stores, plasma cell hyperplasia, and excessive cytophagia may also be recognized. Because bone marrow smears are relatively poor indicators of the actual cellularity of the bone marrow, it is necessary to consider the histopathologic findings for sections of sternum, rib, or femur. Although histologic sections generally are inadequate for evaluating individual cell types and abnormal cell morphology, they provide a good assessment of overall cellularity and are useful in providing estimations of cell density (e.g., for megakaryocytes or mast cells).

There are three major approaches to bone marrow smear evaluation. Regardless of the approach taken, the results of

the bone marrow evaluation (both bone marrow smear and histologic evaluation) must be interpreted in conjunction with peripheral blood test results. The most simplistic and least informative bone marrow evaluation is to determine the M/E ratio by counting a certain number of nucleated cells (usually between 200 and 500 cells) and classifying cells as either granulocytic or erythroid. The results generally are not useful in interpretation of peripheral blood changes and generally do not help to understand the underlying problem. For example, increased M:E ratio is consistent with increased granulocytic cellularity/production, decreased erythroid cellularity/production, or a combination of both. If peripheral blood data indicate a high neutrophil count and normal hematocrit, then an increased M:E ratio likely indicates increased granulocytic cellularity/production. If peripheral blood data indicate a normal neutrophil count and a nonregenerative anemia, then an increased M:E ratio likely indicates decreased erythroid cellularity/production. In both cases, the outcome of the M:E ratio could have been predicted from the peripheral blood results, and bone marrow evaluation provided no additional information. If an animal is neutropenic and has a nonregenerative anemia, an increased M:E ratio only indicates that there are relatively more granulocytic cells than erythroid cells and has not increased understanding of the process.

The most time-consuming and labor-intensive bone marrow evaluation is to perform a bone marrow cell differential count by differentiating the cell type and stage of development of at least 500 cells. When completed, an M:E ratio can be calculated from the results if desired. This thorough evaluation yields more information but at a very high cost. Differential counts provide numeric information concerning the relative numbers of different precursor cells and whether a cell line is maturing normally. Unusual or abnormal morphologic characteristics of the cells must be described separately. Techniques for conducting bone marrow differentials using flow cytometry have been described and have the potential to rapidly provide more accurate information than manual 500-cell differential counts.[13,110]

The most cost-effective and informative approach to bone marrow evaluation is the subjective cytological examination. In this approach, the bone marrow smear is examined in much the same manner as a morphologic pathologist examines a histologic section of liver, and a diagnosis or interpretation is recorded. The person performing the examination, usually a veterinary anatomic or clinical pathologist, assesses the quality and cellularity of the smear, the presence and relative number of precursors for each of the three major cell lines (erythrocytes, granulocytes, and platelets), and the maturation of each of the cell lines. Abnormal morphology is noted, as well as unusual numbers or characteristics of other cell types such as lymphocytes, plasma cells, monocytes, macrophages, and mast cells. A diagnosis or interpretation is rendered based on the examination of the smear and the peripheral blood tests results.

Regardless of the method used for evaluation of bone marrow smears, the end goal of the evaluation is to assess any negative impact of the test article on the number or maturation of hematopoietic cell precursors.[13,111,112] Conclusions from the bone marrow evaluation should address the relationship between the bone marrow findings (from both smears and histologic sections) and peripheral blood changes. For example, if a test article causes decreased platelet count, the absence of megakaryocytes in bone marrow smears and sections indicates that the decrease is due to failure of platelet production rather than increased platelet consumption peripherally. Likewise, if bone marrow evaluations find increased numbers of morphologically normal megakaryocytes, the results indicate that thrombocytopenia is due to a consumptive process.

Coagulation

The clotting mechanism or cascade has traditionally been divided into two pathways. In vivo, the intrinsic pathway begins with the activation of zymogen factor XII following exposure to negatively charged subendothelial components such as collagen. Factors XI, IX, and VIII are also part of the intrinsic pathway. The extrinsic pathway begins with the activation of zymogen factor VII following exposure to tissue factor (also called *tissue thromboplastin*) expressed by cells deep in the vessel wall. Both pathways share the same terminal sequence of events including the activation of factor X, conversion of prothrombin to thrombin, and conversion of fibrinogen to fibrin. It is thought that the extrinsic pathway is the primary initiator of coagulation in vivo.[113] The extrinsic and intrinsic pathways are routinely evaluated by the one-stage prothrombin time (OSPT; generally referred to as PT) and APTT, respectively. An alternative test, the activated coagulation time (ACT) test, is a simple, rapid measure of the intrinsic pathway that does not require a coagulation analyzer.[114–116] These three coagulation assays are relatively insensitive and nonspecific. Activity of a single clotting factor must be reduced to approximately 30% of normal before noticeably prolonged times are detected for an individual animal. When the results from groups of animals are compared, statistically significant differences are occasionally observed that are smaller than what would generally be considered an important change for an individual animal (e.g., less than 2 s of difference between the means for the control and high-dose groups). The toxicologic significance of differences such as these is sometimes difficult to determine. Although they clearly do not represent an effect likely associated with a bleeding diathesis for individual animals, they may be an indication of an important change in coagulation homeostasis. It may be valuable to design a longer study or increase the dose level to see if the effect is repeatable, dose related, and associated with clinical signs.

Under the conditions of most toxicology studies, where animals are exposed to high concentrations of a test article for a prolonged period of time, any major effect on the production of a clotting factor will likely result in a clinically obvious, bleeding diathesis. The administration of vitamin K antagonists such as dicumarol or the ingestion of synthetic or poorly absorbed fat substitutes is associated with bleeding

and prolonged PT and APTT because the fat-soluble vitamin K is required by the liver for production of functional forms of factors II, VII, IX, and X. In theory, PT will be affected before APTT because factor VII has the shortest half-life of the clotting factors. Although the liver synthesizes nearly all of the clotting factors, PT and APTT are insensitive measures of liver function. Because of the liver's large functional reserve, liver injury must be relatively severe before coagulation times are noticeably affected. DIC is characterized by depletion of all clotting factors, including fibrinogen, and moderately to markedly prolonged coagulation times. In many cases of DIC, the plasma samples fail to clot during the coagulation assays. These changes are observed in conjunction with decreased platelets (discussed previously).

Similarly to platelet count, coagulation times can be spuriously prolonged by difficult blood collection or poor collection technique. The combination of low platelet count, prolonged coagulation times, and/or low fibrinogen concentration, in an otherwise healthy animal, is an indication of poor sample quality. Inherited factor VII deficiency affects a small number of laboratory beagles.[58] These animals can usually be distinguished during pretreatment screening by PTs that are 2 or 3 s longer than those of the other animals acquired for the study. Although the deficiency rarely causes a clinical problem, it would be inappropriate to use these animals if the test article is known or suspected to affect coagulation. PT and APTT can both be artifactually prolonged because of excessive sodium citrate anticoagulant in the plasma sample.[117,118] This can occur if insufficient blood volume is added to standardized collection tubes or if the animal's hematocrit is elevated because of hemoconcentration (e.g., dehydration) or drug-induced polycythemia. Normal coagulation times vary from one laboratory animal species to another. Among the notable differences are the relatively fast PTs for dogs (e.g., 6–8 s) and slow PTs for guinea pigs (e.g., 30–40 s). It is not unusual for normal coagulation times to change slightly when a new reagent lot is introduced.

Although fibrinogen is occasionally measured along with PT and APTT as a coagulation assay, its primary value is as an acute-phase protein produced in response to inflammation. Increasing fibrinogen concentration almost always indicates an inflammatory process.

Clinical Chemistry Tests and Interpretation

Routinely performed clinical chemistry tests provide information concerning hepatocellular and biliary integrity and function, renal function, carbohydrate, lipid and protein metabolism, and mineral and electrolyte balance. Modern clinical chemistry analyzers require very small sample volumes; less than 250 μL of serum is needed to perform as many as 20 tests. It is possible, therefore, to obtain complete biochemical profiles from rats at multiple time points during a study without excessive blood collection. Most of the common clinical chemistry assays developed for human testing are applicable, without modification, to animal clinical chemistry testing.

Hepatocellular and Hepatobiliary Integrity and Function

Many routine clinical chemistry tests can be affected by liver toxicity because of the critical metabolic, synthetic, and excretory functions of the liver and the abundant enzymatic machinery required to perform these functions.[119,120] Conversely, a significant loss of liver tissue with little or no detectable change in routine tests is possible because of the liver's large functional reserve. No single test is superior for detecting all of the various types of liver toxicity, but the pattern of abnormal findings in a battery of tests may help characterize the location and severity of liver lesions.[121]

Liver Enzymes

Serum activities of liver enzymes are used primarily to identify hepatocellular injury and cholestasis, with or without hepatobiliary injury. Although sometimes referred to as liver function tests (or LFTs), they do not provide specific information about liver function. The liver can be severely dysfunctional in the absence of effects on serum liver enzyme activities. Animals with end-stage liver cirrhosis, for example, can exhibit normal serum enzyme activities. Conversely, focal lesions causing marked elevation of certain liver enzyme activities may have no appreciable effect on overall hepatic function because of the large functional reserve of the liver.

The specificity, sensitivity, and predictive value of liver enzyme tests are largely dependent on the models of hepatotoxicity used to make those determinations. This fact is at least partially responsible for the practice of including multiple liver enzymes in the clinical chemistry test panels for toxicology studies. The apparent absence of changes in liver enzyme activities does not necessarily rule out the possibility of hepatotoxicity. Possible reasons for this include suboptimal timing of clinical pathology testing and excessive variability of control animal results, especially for mice and monkeys.

Serum activities of many enzymes present within hepatocytes are increased following hepatocellular injury (i.e., degeneration or necrosis). The utility of a particular enzyme for the identification of hepatocellular injury depends on factors such as relative specificity to liver, intrahepatic location, intracellular location, the concentration gradient between the hepatocyte and serum, serum half-life, in vitro stability, and economy of measurement.[122–124] The most frequently used enzymes to assess hepatocellular injury are ALT, formerly serum glutamic pyruvic transaminase (SGPT), and AST, formerly serum glutamic oxaloacetic transaminase (SGOT).[18] SDH, GDH, and LDH are measured less frequently.

ALT is the most useful enzyme for the detection of hepatocellular injury in the majority of laboratory animal species. Although the enzyme is present in many tissues, its greatest concentration in most species is within hepatocytes, and significant elevations of serum ALT activity usually indicate release of ALT by hepatocytes. Species for which ALT is less useful because of relatively low hepatocyte concentrations include the guinea pig[125] and large domestic animals

such as pigs (including minipigs), goats, sheep, cows, and horses.[36,123,126,127] Because ALT is primarily cytosolic, and its concentration within the cell is up to 10,000 times greater than that in the serum, ALT may enter the serum in any condition that sufficiently alters cell membrane integrity. In addition to release from degenerating or necrotic cells, there may be other mechanisms (e.g., blebbing) for movement of the enzyme across the cell membrane[128] because high serum activities of ALT are occasionally observed with no evidence of cell death. The magnitude of serum activity elevation is generally proportional to the number of affected hepatocytes and is not indicative of the reversibility of the lesion. As a general guideline, test article-related ALT increases in excess of 200 IU/L are usually accompanied by histopathologic evidence of hepatocellular injury, while activities below this level may or may not have correlative findings.

Following an acute but reversible hepatotoxic event, serum ALT activity increases relatively rapidly, peaks within 1 or 2 days, and then declines over the next few days. Significant hepatotoxicity can go undetected if clinical pathology tests are delayed for 1 or 2 weeks following a single administration of the test article. Prolonged elevations following a single insult may reflect increased concentrations of ALT in regenerative liver tissue or continued loss of ALT from cells in close proximity to the primary lesion that undergo degenerative changes as a result of the altered microenvironment.

Increased serum ALT activity does not always indicate primary hepatocellular injury. Biliary disease or toxicity and bile duct obstruction may cause increased serum ALT activity at least in part due to the effect of retained bile salts on the cell membranes of neighboring hepatocytes. Muscle damage, when severe and extensive, can increase serum ALT activity in the absence of hepatic injury.[129,130] Increased intracellular activity of ALT will cause serum ALT activity to increase proportionately. Some studies have shown an association between increased serum ALT activity and certain drugs (e.g., glucocorticoids and anticonvulsants) that may be due, at least in part, to increased intracellular activity.[128]

Assessment of hepatotoxicity using serum ALT activity in monkeys can be complicated by the presence of subclinical, enzootic hepatitis A infection.[131] Transiently increased serum ALT activity occurs concurrently with seroconversion to the virus and periportal inflammation. Because animals entering a facility may not have been exposed to the virus previously, it is possible to observe transiently increased ALT activities (e.g., up to approximately 300 IU/L) for a few individual monkeys during the course of a toxicology study. Some facilities choose to bank serum collected from monkeys before a study is initiated for possible serologic testing to help clarify ambiguous ALT results. Interpretation of serum ALT activities for mice can be complicated by considerable interanimal variability. In toxicology studies using mice, it is relatively common for a few animals, including control animals, to have much higher serum ALT activities than those of the majority (e.g., 200 vs. 40 IU/L). One cause of these high activities is thought to be physical damage to the liver or possibly muscle, especially when mice are handled by grasping the body.[132] Restraint of rats, dogs, and nonhuman primates can also result in increased ALT, generally with concurrent AST.

Serum SDH and GDH activities have been recommended as good indicators of hepatotoxicity in laboratory animal species[12,121,133,134] because increased serum activities are liver specific and relatively sensitive. SDH is a cytosolic enzyme, and GDH is located in mitochondria. Elevations in serum SDH activity generally return to baseline levels faster than for other liver enzymes because of a short serum half-life. The addition of either of these tests to a standard clinical chemistry profile is a good choice if potential liver toxicity is of particular interest. SDH is routinely used in large animal veterinary practices, and GDH enjoys popularity in Europe. Because neither enzyme is used in human medicine in the United States, assay test kits are less available and may be difficult to obtain.

Serum AST and LDH activities tend to parallel serum ALT activity with respect to liver damage but are much less liver specific because of high concentrations in muscle and other tissues. Compared with larger animals, rodents tend to exhibit more interanimal variability for these enzymes. The variability may be due to contamination with muscle tissue during blood collection procedures such as cardiac puncture and rupture of the retro-orbital plexus or sinus. There is little advantage to determining both AST and LDH. Generally, only AST is determined, as LDH has greater interanimal variability and does not improve identification of hepatocellular injury. Elevations in serum AST activity caused by hepatotoxicity are usually less pronounced than concurrent elevations in serum ALT activity. Because a portion of intracellular AST is located in mitochondria, a more severe injury may be necessary for the release of like quantities of AST. As with ALT, drugs such as corticosteroids and anticonvulsants may increase intracellular activity of AST.

Decreased serum activities of ALT and AST are occasionally observed in toxicology studies. Among the potential causes for these findings are decreased hepatocellular synthesis or release of the enzymes, inhibition or reduction of enzyme activity, and assay interference. The most widely recognized of these causes involves an effect on pyridoxal 5′-phosphate (vitamin B_6), a coenzyme cofactor required for full catalytic activity of the aminotransferases. If a test article negatively affects this cofactor, directly or indirectly, serum aminotransferase activities may decrease.[135–137] This phenomenon is occasionally observed in monkeys that develop chronic watery diarrhea and may result from loss of the water-soluble cofactor. Because the aminotransferase assays can be run with or without additional pyridoxal 5′-phosphate in the reagent system, a test article-related effect on pyridoxal 5′-phosphate can be identified by analyzing for enzyme activity with and without excess cofactor. Regardless of the mechanism involved, decreased serum activities of the aminotransferases are generally not associated with toxicologically significant effects on the liver.

Several enzymes that originate from hepatocytes and biliary epithelial cells are increased in serum as a result of increased synthesis or release following intrahepatic or

extrahepatic cholestasis or in conjunction with biliary hyperplasia. These include serum ALP, GGT, and 5′-N. Of these, the most commonly measured are ALP and GGT.

A variety of related enzymes contribute to total serum ALP activity. In humans, at least four genes have been identified that code for different ALP isoenzymes: tissue nonspecific (found in liver, bone, and kidney), intestinal, placental, and germ cell. In most laboratory animals, only two ALP genes have been identified; these code for the tissue-nonspecific and intestinal isoenzymes. Tissue-nonspecific ALP enzymes originating in the liver, bone, and kidney are the product of the same gene and are, therefore, isoforms rather than isoenzymes. The isoforms can be distinguished because of differences in degree of posttranslational glycosylation and tissue of origin.[138,139]

The contribution of each isoenzyme and isoform to total serum ALP activity is dictated by tissue production and serum half-life. Because bone ALP is produced by osteoblasts, bone ALP activity is highest in young, growing animals, regardless of species, and decreases as animals mature. In puppies, the bone isoform may be responsible for up to 95% of total serum ALP activity. In adult dogs, the liver isoform is most prevalent. Bone ALP is responsible for approximately 60% of serum ALP activity in 6-week-old rats.[138]

In rats, serum activity of the intestinal ALP isoenzyme increases after feeding and is reduced with fasting.[140] Dog intestinal ALP is rarely identified in serum due to its short half-life. Diseases of the intestine are not typically associated with increased serum ALP. Kidney ALP may be found in urine but is not released into blood to any significant extent and has a short serum half-life. In response to glucocorticoids (either administered or endogenous), dogs can produce a unique hyperglycosylated form of the intestinal ALP isoenzyme (corticosteroid-induced ALP isoenzyme) from the liver. Corticosteroid-induced ALP activity is absent from the serum of most dogs.

In spite of the different ALP isoenzymes and isoforms, serum ALP activity is most often a measure of cholestasis. It is a sensitive indicator of cholestasis in the dog and usually increases well before other markers such as GGT and total bilirubin. Primary hepatocellular toxicities of mild to moderate severity often cause enough intrahepatic cholestasis to elevate serum ALP activity because of cell swelling and pressure obstruction of small bile ductules. Periportal effects result in higher activities than do centrilobular effects. Extrahepatic cholestasis, as might occur with pancreatitis, biliary calculi, or complications of bile duct cannulation, results in higher serum ALP activity than intrahepatic cholestasis. The value of serum ALP activity for identifying cholestatic lesions is less in monkeys than dogs or rats because of marked interanimal variability.

Alterations in serum ALP activity can be the first indication of an effect on bone formation. Elevations of serum ALP activity due to increased osteoblast activity tend to be less pronounced than those due to cholestasis (e.g., generally no greater than twofold to threefold higher than control animals). If the test article is administered for sufficient duration, the effect on ALP activity is usually accompanied by clinical or histopathologic evidence of bone changes. If a test article has concurrent liver and bone effects, bone-specific ALP measurement may be indicated.

Drugs such as anticonvulsants and glucocorticoids can induce synthesis of liver ALP, with or without evidence of hepatobiliary disease. Following corticosteroid administration to dogs, serum activity of liver ALP increases within a few days, while that for corticosteroid-induced ALP does not increase noticeably for about 10 days.[141] Increased corticosteroid-induced ALP activity has been observed in dogs with a variety of chronic disease conditions[73] and may be related to increased endogenous corticosteroid release, but this dog-specific isoenzyme has not been closely evaluated in toxicology studies.

Measurement of serum GGT activity gained popularity because it is more specific than ALP and was shown to be effective in certain models of biliary toxicity in the rat.[142] Although the highest tissue concentrations of this membrane-localized enzyme are in the kidney and pancreas, serum elevations have been reported only with hepatobiliary toxicity and following induction by drugs that stimulate microsomal enzyme production.[119,143] Unlike ALP, GGT is unaffected by bone growth or disease, and its serum activity is less likely to increase secondary to primary hepatocellular toxicity or intrahepatic cholestasis due to hepatocellular swelling. Serum GGT activity is often undetectable in rodents, and even small increases may be significant.

Serum 5′-N activity has been investigated as an alternative to ALP and GGT but has not found general acceptance.[121,144]

Bilirubin

In contrast to serum liver enzyme activities, serum total bilirubin concentration is primarily a function test. In the absence of hemolysis, increased serum bilirubin concentration indicates an effect on uptake, conjugation, secretion, or excretion of bilirubin by the liver. However, serum total bilirubin concentration is a relatively insensitive marker of hepatotoxicity because of the large functional reserve of the liver.

Bilirubin results from the breakdown of heme by cells of the mononuclear phagocyte system. Hemoglobin from senescent erythrocytes accounts for approximately 85% of all serum bilirubin. When macrophages release bilirubin into circulation, it is known as free, unconjugated, prehepatic, or indirect bilirubin. It is water insoluble and circulates bound to albumin. Hepatocytes efficiently remove unconjugated bilirubin from plasma and prepare it for elimination from the body by a four-step process that includes uptake, conjugation, secretion, and excretion. Secretion of conjugated bilirubin across the canalicular membrane is the rate-limiting step, and small amounts of conjugated or direct bilirubin escape into plasma. Conjugated bilirubin is not bound to albumin and is freely filtered through the glomerulus. In most species, conjugated bilirubin is completely reabsorbed by renal tubular epithelial cells unless the amount of filtered bilirubin is excessive. In the dog, the renal threshold is low and traces of bilirubin are normal in concentrated urine.

Conjugated hyperbilirubinemia occurs as a result of impaired secretion of bilirubin, cholestasis, or both. Because bilirubin secretion is the rate-limiting step, any disease that damages enough hepatocytes can potentially increase serum conjugated bilirubin concentration. Periportal lesions cause higher serum bilirubin concentrations than do centrilobular lesions, and extrahepatic cholestasis causes higher serum bilirubin concentration than does intrahepatic cholestasis. When increased bilirubin concentration results from cholestasis, serum ALP activity is generally elevated, particularly in the dog.

Unconjugated hyperbilirubinemia usually occurs as a result of relatively severe, acute hemolysis. If hepatocytes cannot process the large amount of unconjugated bilirubin produced by macrophages during a hemolytic episode, serum bilirubin concentration increases. A hemolytic event sufficient to overload a normal liver always produces other findings indicative of hemolysis. It is possible, however, for relatively modest hemolysis to cause unconjugated hyperbilirubinemia if hepatic function is already compromised. Although a number of nonhemolytic, unconjugated hyperbilirubinemia syndromes are known, these syndromes are usually due to hereditary defects in the uptake and conjugation of free bilirubin.

Unconjugated (or indirect) bilirubin can be differentiated from conjugated (or direct) bilirubin by the Van den Bergh test. The test is used clinically to help distinguish prehepatic causes of hyperbilirubinemia, such as hemolysis, from hepatic or posthepatic causes such as hepatitis or biliary obstruction. In well-designed toxicology studies, the combination of clinical observations, other laboratory data (e.g., hematocrit or liver enzyme activities), and anatomic pathology findings (e.g., hemosiderin accumulations in splenic macrophages or periportal hepatocellular necrosis) is usually more than sufficient to determine the primary mechanism for any observed hyperbilirubinemia. In addition, the correlation between bilirubin conjugation state and the underlying process is often inconsistent for very small increases observed in some nonclinical toxicology studies. For these reasons, laboratory determination of direct and indirect bilirubin is not recommended as part of the routine panel of tests performed in toxicology studies. Bilirubin fractionation may be useful if total bilirubin concentration is greatly increased in the absence of correlative findings because test article inhibition of uridine diphosphate glucuronosyltransferase, the bilirubin-conjugating enzyme, can substantially increase serum unconjugated bilirubin in the absence of other liver effects.[18,145]

Increased bilirubin can occasionally result from interference by the test article or its metabolites with the enzymatic assay generally used to measure bilirubin concentration. In these cases, use of an alternate assay such as the vanadate assay can be useful to assess true bilirubin concentrations.[146]

Decreased serum bilirubin concentration is occasionally associated with administration of test articles that induce microsomal enzyme production.[143] Human patients receiving phenobarbital therapy have lower serum bilirubin levels than the general population as a whole.[147] Enzyme induction apparently enhances the metabolism and excretion of bilirubin and could potentially mask an otherwise elevated bilirubin level.

Other Liver-Related Parameters

Like serum bilirubin concentration, total serum bile acid concentration is a measure of hepatic function. Bile acids are synthesized from cholesterol by hepatocytes, conjugated to an amino acid, secreted into the biliary system, and excreted into the intestine, where they facilitate fat absorption. Bile acids undergo efficient enterohepatic circulation, with most reabsorption occurring at the level of the ileum. Portal blood conveys the bile acids to the liver for uptake, reconjugation, and resecretion. Liver toxicity has the potential to alter bile acid metabolism at any of multiple steps and cause increased serum bile acid concentration. Unfortunately, although it is considered a sensitive and specific test for hepatobiliary disease in clinical veterinary medicine,[148] measurement of total serum bile acids has not proven effective at increasing the identification of hepatotoxicity beyond that of the standard battery of tests routinely performed in toxicology studies. Like total bilirubin, increased serum bile acid concentration does not discriminate between different types of hepatic lesions. In addition, serum bile acid concentrations tend to exhibit relatively high inter- and intra-animal variability, further decreasing their utility.

The liver is wholly or partially responsible for the synthesis of many substances including glucose, cholesterol, urea, and a variety of proteins. Severe hepatocellular dysfunction can cause decreased serum urea nitrogen concentration, hypoglycemia, hypocholesterolemia, hypoproteinemia (especially hypoalbuminemia), and prolonged coagulation times. Liver disease can also result in hypercholesterolemia and hyperglobulinemia. The patterns of change in clinical pathology tests caused by different types of liver toxicity, whether primary (e.g., chloroform-induced hepatic necrosis) or secondary (e.g., hypoxia-induced centrilobular necrosis), are often overlapping. Examination of the entire biochemical profile, along with other clinical pathology and anatomic pathology findings, is necessary to properly evaluate data for potential liver toxicity.

Renal Function

Serum urea nitrogen (or urea) and creatinine concentrations, in conjunction with measures of urine concentration (urine specific gravity or osmolality) and volume, are the most common tests used to evaluate renal function.[149–153] These tests are easy and inexpensive to perform but are relatively insensitive to small effects on the kidney and can be affected by nonrenal factors.

Urea is synthesized by the liver from ammonia that is absorbed from the intestine or produced by endogenous protein catabolism. Urea is freely filtered through the glomerulus and excreted in urine. Some urea is reabsorbed passively with water in the proximal tubule; the amount reabsorbed is

inversely related to the rate of urine flow through the tubule. Serum urea nitrogen concentration is, therefore, affected by rate of urea production, glomerular filtration rate (GFR), and flow rate of urine through the renal tubule. Mechanisms for increased urea nitrogen (also called azotemia) are categorized as prerenal, renal, or postrenal.

Prerenal causes for increased urea nitrogen are increased urea synthesis and decreased renal blood flow. Increased urea synthesis results from consumption of high-protein diets or conditions that increase protein catabolism such as starvation, fever, infection, tissue necrosis, or high gastrointestinal hemorrhage. Decreased renal blood flow decreases GFR and may be caused by conditions such as dehydration (the most common cause of increased urea nitrogen in toxicology studies), cardiovascular disease, or shock. Changes in urea nitrogen concentration caused by increased urea synthesis are typically small. Changes caused by decreased renal perfusion may also be small, but if GFR is severely affected, the increase in urea nitrogen is indistinguishable from that which would occur due to primary renal failure. When increased urea nitrogen is due to prerenal causes, renal concentrating ability is typically maintained. If the prerenal condition is dehydration, urine volume is reduced and urine concentration is increased as kidneys attempt to conserve water.

Increased urea nitrogen due to renal causes results from diseases or toxicity of the renal parenchyma. Like the liver, kidneys have a large functional reserve capacity. In clinical practice, it is commonly said that serum urea nitrogen concentration does not increase notably until approximately 75% of the kidneys' nephrons are nonfunctional. In nonclinical toxicology studies, however, it is likely that differences between control and treated animals can be detected prior to that degree of impairment. When the cause of increased urea nitrogen is primary renal disease or toxicity, renal concentrating ability may be impaired, and urine specific gravity may be isosthenuric (i.e., the same as the glomerular filtrate; approximately 1.008–1.012). Increased urea nitrogen due to renal causes is generally accompanied by histopathologic evidence of renal damage (e.g., proximal tubular nephrosis or chronic progressive nephropathy), and animals may exhibit signs of poor health such as inappetence, weight loss, or inactivity.

Postrenal causes of increased urea nitrogen reduce GFR by obstructing outflow of urine. Obstruction by naturally occurring urinary calculi is occasionally an incidental finding in rodent studies, but test articles that promote urinary calculi formation can also be responsible for this condition.

Creatinine is a nonprotein nitrogenous waste product formed at a relatively constant rate by the nonenzymatic breakdown of creatine. Creatine is a breakdown product of phosphocreatine, a molecule that stores energy in muscle. Serum creatinine concentration is, therefore, influenced by muscle mass and conditioning, but it is relatively independent of dietary influences and protein catabolism. Creatinine is freely filtered by the glomerulus, but unlike urea, it is not reabsorbed by the tubules. Following alterations in renal blood flow, renal function, or urine outflow, changes in serum creatinine concentration tend to parallel those for serum urea nitrogen concentration. The timing and magnitude of the changes for serum creatinine may lag behind those for serum urea nitrogen. This usually occurs as a result of the tubular reabsorption of urea, especially when urine flow is slow or when there is increased formation of urea. Serum creatinine is usually a better reflection of glomerular filtration than serum urea nitrogen because it is influenced by fewer factors. However, the most commonly used method for determining serum creatinine concentration, the Jaffe reaction, is nonspecific, and interfering compounds called *noncreatinine chromagens* can affect its accuracy. Although noncreatinine chromagens are typically of insufficient quantity to complicate data interpretation, other analytical methods for creatinine (e.g., enzymatic) can be used to investigate the possibility of interferences when serum creatinine concentrations are increased in the absence of correlative effects on serum urea nitrogen or renal histopathology. Endogenous creatinine clearance is sometimes used as a noninvasive measure of GFR because blood levels of creatinine are relatively stable over short intervals, creatinine is freely filtered, and creatinine is not significantly secreted or reabsorbed.[149,154] Unfortunately, this test is better suited for individual patients because it requires procedures (e.g., emptying the bladder before and at the end of the urine collection period) that are difficult in a toxicology study. Serum cystatin C concentration is being used more commonly in human medicine as an indicator of GFR because it is affected by fewer extrarenal factors than urea nitrogen or creatinine,[155,156] and it may see increased use in toxicology studies as more is learned about its performance in animals.

Other clinical chemistry findings sometimes observed when renal function is significantly impaired include increased serum inorganic phosphorus concentration and decreased serum sodium and chloride concentrations. Whereas increased inorganic phosphorus is primarily due to reduced filtration, decreased sodium and chloride result from loss of tubular function and normal reabsorption.

Proteins, Carbohydrates, and Lipids

Serum Proteins

Serum total protein concentration is a measure of all plasma proteins with the exception of those consumed in clot formation such as fibrinogen and other clotting factors. Serum total protein concentration is, therefore, about 0.3–0.5 g/dL lower than plasma total protein concentration. Albumin is the most abundant serum protein and is largely responsible for maintaining intravascular osmotic pressure. Albumin serves as a storage reservoir of amino acids and as a transport protein for plasma constituents that do not have a specific transport protein. Many test articles bind to and are transported by albumin. Globulins are a heterogeneous population of proteins that include specific transport proteins (e.g., transferrin for iron, lipoproteins for lipids, haptoglobin for hemoglobin, and thyroxine-binding globulin for thyroxine), mediators of inflammation (e.g., complement), acute-phase

proteins, clotting factors, enzymes, and immunoglobulins. Globulins are loosely categorized by their electrophoretic migration pattern as α, β, and γ globulins. Several different globulin proteins are present in each region of the electrophoretic pattern.[157] The regions can be further subdivided (e.g., most species have two α regions), but serum protein electrophoresis cannot distinguish specific globulin proteins. Immunoglobulins are generally thought of as γ globulins, but some, particularly IgM, extend into the β regions of the electrophoretogram.

The liver synthesizes albumin and most of the globulin proteins, while lymphocytes and plasma cells synthesize immunoglobulins. Serum total protein and albumin concentrations are measured directly, and serum globulin concentration is calculated by subtracting albumin from total protein. Hydration status must be considered for proper interpretation of changes in serum protein concentrations. Low serum protein concentrations, like low red cell mass, can be masked by dehydration.

In toxicology studies, the most frequent reason for increased serum total protein concentration is reduced hydration of treated animals relative to control animals. Serum albumin and globulin concentrations increase proportionately when simple dehydration occurs. The effect on hydration status of treated animals may or may not be detectable as clinical dehydration. Possible correlative clinical observations include gastrointestinal fluid losses (e.g., vomiting, diarrhea, excessive salivation), polyuria, and reduced water consumption. Because water consumption in rodents is closely associated with food consumption, any cause of decreased food consumption in rodents has the potential to cause dehydration. In short-term dietary studies, for example, palatability problems may result in higher serum protein and urea nitrogen concentrations for treated animals because of differences in hydration; however, if decreased food consumption is protracted and body weights or body weight gains are affected, serum protein concentrations will typically decrease over time.

Other common causes for increased serum total protein concentration in toxicology studies are inflammatory conditions that stimulate production of globulins called *acute-phase proteins* (e.g., fibrinogen, haptoglobin, α_2-macroglobulin, C-reactive protein) and/or immunoglobulins.[158–160] A concurrent decrease in serum albumin concentration often occurs because albumin is a negative acute-phase protein. The opposite direction of changes in albumin and globulin concentration may result in the absence of a change in total protein concentration. However, albumin-to-globulin ratio will be reduced. This serum protein pattern is commonly observed in animals affected by complications of long-term intravenous catheterization.

Decreased serum protein concentrations result from either decreased protein synthesis or increased protein loss. Protein synthesis can be negatively affected by decreased food consumption, maldigestion or malabsorption, and hepatic dysfunction. Although the functional reserve capacity of the liver is quite substantial and hepatic injury must be relatively severe before protein synthesis is notably diminished for individual patients, small differences between control and treated groups in large studies may be apparent with relatively modest hepatotoxicity. Loss of both albumin and globulin occurs with hemorrhage, exudative lesions such as burns or severe dermal toxicity, and, occasionally, severe diarrhea. Albumin may be the principal protein lost as a result of protein-losing enteropathies and glomerulopathies because of its relatively small size. Globulin concentrations may increase secondarily to enteropathies because of inflammation and increased systemic exposure to gastrointestinal toxins and bacterial flora. Decreased globulin concentrations, without concurrent or proportional decreases in albumin concentration, may be indicative of decreased synthesis of immunoglobulins. Histopathologic evidence of effects on lymphoid tissue strengthens this interpretation, and peripheral blood absolute lymphocyte counts are sometimes concurrently affected. Reduced serum globulin concentrations have been observed following prolonged administration of antibiotics (e.g., 4 weeks) to young animals, perhaps as a result of inhibition of normal bacterial flora and subsequently reduced antigenic stimulation.

A small decrease in serum albumin concentration is one of the most frequent findings in animals given poorly tolerated test articles. Like small decreases for other parameters (e.g., hematocrit, glucose concentration, cholesterol concentration, body weight, and body weight gain) that may occur concurrently, decreased albumin due to nonspecific causes is usually considered an indication of the animals' poor overall condition. Decreases in serum albumin concentration are more commonly observed for rodents than for larger species. Although this may be due simply to higher numbers of animals per group, faster turnover of albumin in smaller species may also be a factor[157]; for example, the half-life of albumin is approximately 2 days for mice, 8 days for dogs, and 16 days for baboons.

Serum Glucose

Serum glucose concentration is a reflection of intestinal glucose absorption, hepatic glucose production, and tissue uptake of glucose. The balance between hepatic production and tissue uptake is influenced by many hormones, including insulin, glucagon, glucocorticoids, adrenocorticotropic hormone, growth hormone, and catecholamines. In oversimplified terms, insulin promotes uptake of glucose by tissues, glucocorticoids and glucagon stimulate hepatic gluconeogenesis, and catecholamines and glucagon stimulate glycogenolysis.

The most frequently encountered causes of increased serum glucose concentration in toxicology studies are failure to fast an animal and catecholamine release secondary to excitement or fear. Moribund animals occasionally exhibit marked hyperglycemia, probably as a result of both glucocorticoid and catecholamine release. Glucose concentration decreases at a rate of approximately 7–10 mg/dL/h when serum is not separated from the blood clot. If blood collection is performed in group order (starting with the control group)

and samples are not processed promptly, the high-dose group may appear to have higher serum glucose concentrations than those of the control group because of greater glucose consumption by erythrocytes in the control group's samples. Infrequent causes of increased serum glucose concentration in toxicology studies include some relatively common clinical conditions such as diabetes mellitus, pancreatitis, hyperadrenocorticism, and steroid therapy. Test articles can also cause increased glucose by affecting the sensitivity of cells to insulin (insulin resistance).

In toxicology studies, test article-related decreases in serum glucose concentration are most commonly observed in animals that fail to thrive and gain body weight, with or without a concurrent decrease in food consumption. When this occurs, the difference for serum glucose concentration between control and treated animals is usually no more than 10 or 15 mg/dL. Although a precise mechanism for decreased glucose is typically undetermined, the difference may reflect the overall process that has caused the animals to do poorly and is frequently accompanied by small decreases in circulating red cell mass and serum protein concentrations. Clinical conditions that cause decreased serum glucose include intestinal disease with malabsorption, severe hepatic disease, endotoxemia, and some tumors, in particular insulinomas and hepatomas. Test articles can cause decreased glucose as a primary or secondary effect; frequent monitoring of large animals using glucometers and supplementation of the diet may be necessary to avoid severe hypoglycemia with some glucose-lowering test articles.

Serum Lipids

The two major serum lipids are cholesterol and triglycerides. Cholesterol is utilized for synthesis of cellular membranes, bile acids, corticosteroids, and some sex steroid hormones, and triglycerides are an important source of energy. Serum cholesterol and triglycerides are derived from dietary intake and endogenous synthesis, primarily by the liver. Cholesterol and triglycerides circulate as components of chylomicrons and lipoprotein particles: high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL).[161] Chylomicrons are produced by intestinal cells after ingestion of a fatty meal and are rich in triglycerides. Hepatocytes synthesize VLDLs, particles with less triglyceride but more cholesterol than chylomicrons. Triglycerides in chylomicrons and VLDLs are broken down to free fatty acids and monoglycerides by lipoprotein lipase attached to the surface of endothelial cells, especially in capillaries of adipose tissue and muscle. Adipocytes tend to reesterify fatty acids for storage as triglycerides. Myocytes tend to oxidize fatty acids for energy. Loss of triglyceride transforms VLDL to LDL. In humans, LDLs transport about two-thirds of serum cholesterol. In contrast, HDL is responsible for a significant majority of cholesterol transport in most laboratory animal species. For example, cholesterol of rats and mice is carried almost exclusively by HDL particles. Clinical chemistry analyzers can determine the amount of cholesterol associated with HDL and LDL particles (i.e., HDL cholesterol and LDL cholesterol). Species differences in lipid metabolism cause difficulties in correlating lipid effects in animal models with potential effects in humans.[162] In addition, some of the commonly used HDL and LDL cholesterol assays for clinical chemistry analyzers have species-specific biases, depending on the underlying methodology (Wescott, Everds, unpublished observations). Hamsters, some rabbit strains, and genetically engineered mice have been used as animal models for lipid research.

Small changes in serum cholesterol and triglyceride concentrations, both increases and decreases, are relatively frequent findings in toxicology studies. The changes are generally believed to represent minor alterations in lipid metabolism that do not adversely affect the animals' health. Unfortunately, exact mechanisms for the changes are rarely identified. Factors to consider include alterations in food consumption and assimilation, body weight and composition, liver function, and hormones.

Serum triglyceride concentration is elevated postprandially, while serum cholesterol concentration is relatively stable. When fat is mobilized to meet energy requirements because of significant anorexia, starvation, malabsorption, or maldigestion, serum triglycerides are usually increased, sometimes markedly. Cholesterol levels, however, are variable. Clinical conditions that often increase both serum cholesterol and triglyceride concentrations include hypothyroidism and diabetes mellitus. Lipoprotein lipase activity is reduced in both conditions. In most species, hypercholesterolemia is more prominent in hypothyroidism, and hypertriglyceridemia is more prominent in diabetes mellitus. Cholestasis and other forms of liver disease can increase serum cholesterol concentration because the liver is the major excretory pathway for cholesterol. Conversely, liver disease may also be associated with hypocholesterolemia. Hypercholesterolemia, increased urinary protein excretion (due to glomerular disease), and hypoalbuminemia are characteristics of nephrotic syndrome. Young rats (e.g., 7–20 weeks of age) usually exhibit less interanimal variability for cholesterol and triglyceride concentrations than dogs and monkeys. It is, therefore, more common to detect subtle effects on serum lipid concentrations in short-term rat studies versus large animal studies, especially because the number of animals per group is typically much higher for rat studies. Serum cholesterol and triglyceride concentrations are extremely variable for older rats because of a number of naturally occurring diseases.

Minerals and Electrolytes

Serum Calcium and Inorganic Phosphorus

Serum calcium concentration is controlled primarily by parathyroid hormone, calcitonin, and vitamin D and reflects a balance among intestinal absorption, bone formation and reabsorption, and urinary excretion.[163] Serum inorganic phosphorus concentration is affected by the same hormones but is more sensitive to changes in dietary intake and urinary excretion. Approximately 50% of serum calcium is in its biologically active, ionized form. Ionized calcium is critical

for neuromuscular activity, bone formation, coagulation, and other biochemical processes. Approximately 40% of serum calcium is bound to albumin, and the remainder is complexed to anions such as phosphate and citrate.

Although many disease conditions are associated with hypercalcemia,[163] increased serum calcium concentration is relatively uncommon in toxicology studies unless the test article specifically targets bone or calcium metabolism or has properties of either parathyroid hormone or vitamin D. Because approximately 40% of serum calcium is bound to albumin, mildly increased serum calcium concentration is occasionally observed when serum albumin concentration is increased. This change is physiologically appropriate, not adverse. Rare causes of increased serum calcium in toxicology studies include primary hyperparathyroidism, pseudohyperparathyroidism (a paraneoplastic syndrome), hypervitaminosis D, and renal disease.

Mildly decreased serum calcium concentration, as a result of decreased serum albumin concentration, is a frequent finding in toxicology studies. Signs of toxicity secondary to decreased calcium, such as neurological and neuromuscular abnormalities, are absent because ionized calcium is relatively unaffected. Rare causes of decreased serum calcium in toxicology studies include hypoparathyroidism, nutritional hyperparathyroidism, acute pancreatitis, bone effects, and renal disease.

Young, rapidly growing animals have high serum inorganic phosphorus concentrations (e.g., greater than or equal to serum calcium concentration) that decrease with maturity. Serum inorganic phosphorus concentration is affected by GFR, and increased concentrations parallel changes in serum urea nitrogen and creatinine and should be interpreted in conjunction with those parameters. Rare causes of increased serum inorganic phosphorus in toxicology studies include hypoparathyroidism, nutritional hyperparathyroidism due to excess dietary phosphorus, and hypervitaminosis D. Decreased serum inorganic phosphorus concentration observed in toxicology studies is most commonly associated with reduced food consumption.

Serum Sodium, Potassium, and Chloride

Sodium, the major extracellular cation in plasma, is the principal determinant of extracellular fluid volume. Chloride, the major extracellular anion in plasma, supports fluid homeostasis and balances cation secretion. Potassium is the major intracellular cation and has a critical role in neuromuscular and cardiac excitation. Reference intervals for electrolyte concentrations for animals on toxicity studies tend to be much narrower than the range of values obtained clinically. For this reason, it is common to observe very small, but statistically significant, differences between control and treated groups with no apparent mechanism or effect on animal health. Many of these differences are likely incidental, but others may represent subtle homeostatic effects or uncontrolled preanalytical influences. Changes in serum sodium and chloride concentrations tend to parallel each other when they are associated with relative water content, but serum chloride concentrations are disproportionately affected in disorders affecting acid–base balance.

Significant increases in serum sodium are rare in toxicology studies. Increases in serum chloride are occasionally observed secondarily to metabolic acidosis resulting from diarrhea. In this condition, renal tubular reabsorption of chloride is increased because of decreased availability of bicarbonate. Approximately proportional decreases in serum sodium and chloride concentrations can occur with gastrointestinal losses (e.g., vomiting or diarrhea), renal losses (e.g., renal failure), diuretic effects, and hypoadrenocorticism (rare in toxicology studies). Vomiting may cause decreased serum chloride concentration without affecting sodium because stomach secretions are rich in chloride.

Increased serum potassium concentration may be observed with a variety of conditions causing acidosis because extracellular hydrogen ions are exchanged for intracellular potassium ions. Severe tissue necrosis and anuric or oliguric renal failure are infrequent causes of increased serum potassium. Serum potassium may be falsely elevated because of hemolysis (either technique- or test article-related) in species that have high intraerythrocytic potassium (e.g., nonhuman primates). Marked thrombocytosis and thrombocytopenia can be associated with increased and decreased serum potassium, respectively, because potassium is released from platelets during clot formation. Serum potassium is very sensitive to potassium intake, and decreased concentrations are often associated with anorexia. Similar to effects on sodium and chloride, decreased serum potassium is sometimes associated with gastrointestinal losses and polyuric renal losses. Disorders resulting in alkalosis (e.g., persistent vomiting) may cause decreased serum potassium because intracellular hydrogen ions are exchanged for extracellular potassium ions.

Miscellaneous Serum Chemistry Tests

Serum CK activity is measured primarily as a marker for skeletal muscle toxicity. Test article-related increases in CK activity can be detected only if complicating factors or study-related procedures do not obscure results. For example, intramuscular injections, surgical procedures, and poor venipuncture technique can all give rise to marked elevations in CK that preclude identification of meaningful differences between control and treated animals. Serum aldolase activity is a less frequently used marker for skeletal muscle injury. The utility of additional markers of skeletal injury is currently under investigation.

Several tests have been used with varying degrees of success in human medicine as markers of acute myocardial injury,[164] and cardiac troponins I and T have emerged as cardiotoxicity markers of choice for toxicology studies designed specifically to evaluate myocardial injury.[165–179] Cardiac troponin I is the more readily available assay, and there appears to be no need to measure both cardiac troponins in a given toxicology study. Although cardiac troponins are best used to assess myocardial injury within a day or two of toxic insult,

they are sensitive enough to detect subclinical myocardial toxicity in long-term studies.

Amylase and lipase activities are measured clinically to diagnose diseases causing acute pancreatic necrosis. These enzymes have limited value in toxicology studies, especially repeat-dose studies, because toxin-induced pancreatitis will cause severe illness and have prominent, unmistakable morphologic consequences. Amylase and lipase are not sufficiently sensitive to be markers for test articles with specific action against pancreatic islet cells.

URINALYSIS AND URINE CHEMISTRY TESTS AND INTERPRETATION

Urinalysis

Urinalysis has traditionally been considered part of the minimum laboratory database for evaluating patients. Although urinalysis provides specific information about the urogenital tract and general information regarding some systemic conditions, it is not particularly well suited for most toxicology studies. The cost-to-benefit ratio is poor because sample collection and analysis are labor intensive and relatively few toxicities produce detectable effects on urinalysis parameters. Technical difficulties associated with collecting a large number of urine specimens from small laboratory animals or uncooperative large animals can impact test results. If a test article is known or suspected to affect the urinary system, then measures can be taken to provide appropriate specimens for urinalysis (e.g., by catheterization, cystocentesis, or carefully collected fresh voided samples). Usually, however, when a large number of animals are being administered a test article of unknown toxic potential, the most efficient method of urine collection (in a collection vessel at the bottom of a metabolic cage) can produce artifacts that affect test results and complicate interpretation. Preservatives and methods for keeping the urine chilled may help limit artifactual changes, but these procedures are not without cost and other issues.

The standard urinalysis includes measurement of physicochemical properties and microscopic evaluation of urine sediment. The physicochemical properties include volume (for timed collections), color, clarity, specific gravity or osmolality, and reagent strip tests (pH, protein, glucose, ketones, bilirubin, urobilinogen, and blood). Some reagent strips have additional tests for nitrite (indicates presence of nitrite-producing bacteria) and leukocyte esterase, but these are screening tests normally used to decide whether to examine urine sediment or culture urine from human patients and are not particularly valuable for animal specimens, especially those collected overnight.[180] Urinary sediment evaluation is a semiquantitative microscopic measure of the presence of cells (urogenital epithelial cells, WBCs, and RBCs), casts, bacteria, crystals, and other formed elements. This is the most expensive component of the urinalysis and, in most instances, the least informative or necessary.[181] In general, disorders that increase the number of cells or casts in urine sediment will be better characterized by histopathologic examination of the kidneys and bladder. Occasionally, however, test article-specific crystals that might otherwise go undetected are identified by urine sediment examination.

Urine Volume and Concentration

Timed urine volume and a measure of urine concentration (urine specific gravity or osmolality) can be valuable in assessing renal function because they demonstrate concentrating ability. Loss of urine-concentrating ability usually precedes development of azotemia as a consequence of chronic renal disease. Timed urine volume (e.g., 16 or 24 h) and a measure of urine concentration are probably the most beneficial of the routine urinalysis tests. Urine concentration generally varies inversely with urine volume and is most commonly assessed by measuring urine specific gravity. Urine specific gravity determined by refractometry is an approximation of urine solute concentration. Although urine osmolality (usually determined by freezing-point depression osmometry) is a more accurate estimation of urine solute concentration, it is more expensive and labor intensive and measured infrequently. Reagent strip specific gravity measurements are not sufficiently accurate for use in nonclinical toxicology studies and should not be used. Animals with impaired ability to concentrate urine due to renal disease have decreased urine specific gravity or osmolality and increased urine volume. With advanced renal disease, urine specific gravity may become fixed in a range from approximately 1.008 to 1.012 (termed *isosthenuria*). Isosthenuria and hyposthenuria (i.e., urine specific gravity below 1.008) are particularly meaningful when serum urea nitrogen concentration is elevated. This combination usually indicates primary renal dysfunction. Test articles with diuretic activity or that cause increased water consumption also produce dilute urine and increased urine volume, but serum urea nitrogen is usually unaffected. Increased urine concentration (e.g., urine specific gravity greater than 1.030 in dogs) indicates that the kidneys have functional concentrating ability. Very high urine concentrations may be observed in dehydrated animals because kidneys attempt to conserve water. In toxicology studies, urine specific gravity is sometimes high in treated animals, particularly rodents, that are anorectic and consequently not drinking normally. Problems with watering systems can create interpretive issues because faulty sipper tubes or animals that habitually play with their water source may result in very dilute, water-contaminated urine, while dysfunctional systems that limit water availability may result in dehydrated animals.

Reagent Strip Tests

Reagent strip tests provide a semiquantitative biochemical evaluation of urine. When one of the following tests indicates a test article effect, a more quantitative determination of that parameter may prove valuable. Because urine reagent strip reactions are usually measured by reagent strip readers that use a grayscale system, highly concentrated (dark) or abnormally colored urine may interfere with test results. In these cases, reagent strips can be manually evaluated, and some parameters can be measured by alternate methods.

Urine pH can be affected by diet, test article pH, and sample handling. Animals consuming high-protein meat diets tend to produce urine of lower pH than do animals consuming cereal or vegetable diets. If administered in large enough quantities, an acid or alkaline test article can affect urine pH. Urine pH is often artifactually elevated in samples collected overnight for two reasons: urease-producing bacteria cause ammonia formation, and carbon dioxide is lost from open containers. Urine pH is generally not a good indicator of acid–base balance.

A low concentration of protein is a normal finding in the urine of most animals, especially if the urine is concentrated. A high concentration of protein is not generally observed in younger animals, especially in dilute urine. Increased urine protein concentration may result from glomerular injury, defective tubular reabsorption, hemorrhage, inflammation, or proteinaceous secretions from the lower urogenital tract in voided specimens. Sediment examination may help identify the cause of proteinuria. Older rats, particularly males, may develop marked proteinuria as a consequence of chronic progressive nephropathy, a common, naturally occurring disease of rats. Highly alkaline urine and quaternary ammonium disinfectants can cause false-positive findings for urine protein measured by reagent strips. Urine protein can be determined quantitatively by automated clinical chemistry analyzers.

The finding of glucose in urine is abnormal. Under normal conditions, renal tubules reabsorb all glucose filtered through the glomerulus. If the glucose load is excessive as a result of markedly increased serum glucose (e.g., >180 mg/dL for the dog), glucose spills into urine. Urine glucose may be observed with renal toxins that target proximal tubular epithelial cells because of failure to adequately reabsorb filtered glucose. Drugs developed for diabetes mellitus that specifically target tubular reabsorption of glucose are typically associated with marked glucosuria and secondary osmotic diuresis that may negatively affect reabsorption of other urinary constituents. If urine glucose is considered an important endpoint for a study, precautions should be taken to avoid false-negative findings resulting from a proliferation of bacteria that consume glucose. Diabetes mellitus is the most frequent clinical disease associated with glucosuria, but it is rarely observed in toxicology studies.

Ketones are normally not present in urine of most animals, but fasted monkeys occasionally and male rats usually have trace to 1+ urine ketones. Ketonuria can also be observed in anorectic animals and animals that have been fasted for a prolonged period of time. Ketonuria usually indicates that energy metabolism has shifted from gluconeogenesis to incomplete oxidation of fatty acids. False-negative findings for urine ketones occur as a result of bacterial degradation and loss of volatile ketones from open containers.

Bilirubin is normally absent in the urine of most laboratory animals. A small amount, however, can often be measured in the urine of male dogs, especially in concentrated urine. Increased urine bilirubin results from the same conditions that cause increased serum bilirubin and may precede the change in serum. False-negative findings for urine bilirubin occur from prolonged exposure of urine to light, causing oxidation of bilirubin to biliverdin.

Urobilinogen is normally present in the urine of animals. Theoretically, urine urobilinogen tests for patency of the bile duct. Intestinal bacteria convert conjugated bilirubin to urobilinogen, a portion of which is reabsorbed by the intestine. Because a small amount of the reabsorbed urobilinogen is normally excreted in the urine, a negative urine urobilinogen is purported to indicate obstruction of the bile duct, an extremely rare occurrence in toxicology studies. The test has little value and is generally determined only because it exists on the same reagent strip as the other tests.

Blood is occasionally present in the urine of normal animals. Although the origin of the blood is usually unknown, estrus is a common source in female dogs and monkeys. Reagent strips do not effectively discriminate among erythrocytes, hemoglobin, and myoglobin, but examination of the urine sediment may help differentiate hematuria from hemoglobinuria. Hematuria may result from bleeding disorders or inflammation, trauma, or neoplasia of the urogenital tract. Hemoglobinuria as a result of significant intravascular hemolysis (typically associated with test articles administered intravenously) is usually associated with concurrent hematologic evidence of hemolysis, and hemoglobinuric nephropathy may be detected microscopically.

Urine Sediment Evaluation

Cells, casts, and crystals are poorly preserved in urine during overnight or 24 h collections; for example, it is common to find no erythrocytes in the urine sediment even though the reagent strip blood test is positive and hemolysis has been ruled out. If sediment detail is deemed an important endpoint for a study, other means of urine collection (e.g., cystocentesis or free catch) should be considered.

Small numbers of erythrocytes, leukocytes, and epithelial cells are normal findings in urine sediment obtained from voided urine specimens. Large numbers of these cells may or may not be abnormal, and gross or histopathologic correlates are necessary to determine their origin. Increased numbers of large epithelial cells (i.e., squamous and transitional cells) generally do not indicate a significant abnormality, but increased numbers of small epithelial cells (i.e., renal tubular cells), especially in conjunction with granular or cellular casts, are indicative of kidney disease.

Urinary casts are infrequently observed in the urine of normal animals. A cast is the cylindrical mold of a segment of renal tubule formed by protein alone or protein and cells. Casts are generally classified as hyaline, cellular, waxy, or broad. Hyaline casts are made of protein alone, and increased numbers are sometimes observed with glomerular disorders that cause excessive proteinuria. An occasional hyaline cast is normal. Cellular casts (erythrocyte, leukocyte, or epithelial) usually indicate renal tubular lesions but are rarely observed in animal urine. If not moved rapidly into the urine, cellular casts become granular casts as the cells degenerate and take on a granular appearance. Waxy casts represent the final stage of degeneration of the cellular cast and indicate

prolonged intrarenal urine stasis. Granular and waxy casts, therefore, may also be an indication of renal tubular disease. Broad casts are identified by their width and represent casts formed in collecting ducts or pathologically dilated portions of the nephron. Broad casts also indicate intrarenal urine stasis. Although the origin of individual cells in urine cannot always be determined, increased numbers of casts (cylindruria) indicate that renal tubular injury has occurred proximal to the renal pelvis.

Bacteria are a consistent finding in the urine of normal animals, given the routine methods used to collect urine during toxicology studies. If the test article is an antibiotic, a test article-related decrease in the number of bacteria may be observed.

Crystals are common in the urine of laboratory animals. The type of crystals observed is dependent on urine pH and the test article. Crystals observed frequently in alkaline urine include triple phosphate, amorphous phosphate, calcium carbonate, and ammonium urate crystals. Urate and oxalate crystals are associated with acid urine. Although rarely observed, ammonium biurate crystals are associated with liver failure. In addition to the previously mentioned crystals that occur naturally, test article-specific crystals will occasionally form when a test article or metabolite is highly concentrated in the urine. These crystals may be pathologically significant if they obstruct renal tubules or lead to the development of calculi. Urine crystals may be important in the establishment of rodent-specific mechanisms of bladder carcinogenesis. Mechanistic studies with specific urine collection techniques may be designed to evaluate urine crystals for this purpose.[182]

Quantitative Urine Chemistry Tests

Because serum urea nitrogen and creatinine and standard urinalysis tests are relatively insensitive markers of renal injury, several urine chemistry tests have been proposed as better methods for identifying and quantifying early renal injury or dysfunction. For the most part, these tests are impractical as part of the general screen in routine, regulated toxicology studies; however, they may be valuable as tools for assessing early renal toxicity at dose levels below those that cause histopathologic lesions and for determining the intrarenal location of the earliest toxic insult. Some of these tests are also well suited for acute screening studies to determine the relative nephrotoxicity of different analogues of a compound with known nephrotoxic action for lead candidate selection.

Urinary Enzyme Activity

Many urinary enzymes have been evaluated for use as early markers of nephrotoxicity,[20,23] and several have been proven effective in specific models of nephrotoxicity.[22,183–187] Perhaps the two most frequently measured urinary enzymes are GGT and *N*-acetyl-β-D-glucosaminidase (NAG) because they are relatively stable at room temperature and have somewhat different cellular locations; GGT is located in the brush border of proximal tubular epithelial cells, and NAG is a lysosomal enzyme with apparently greater distribution along the nephron. Other enzymes that have been evaluated as indicators of nephrotoxicity include ALP (another brush-border enzyme), LDH, ALT, and AST (all primarily cytosolic enzymes). Urinary enzyme activities should be corrected for variations in urine concentration by calculating the total activity excreted per unit time or the ratio of urinary enzyme activity to urinary creatinine concentration. Urinary enzyme activities are most effective for assessing acute renal injury. They appear to have much less utility for assessing chronic conditions (e.g., repeat-dose studies of several weeks' duration) and, like liver enzymes, do not provide information concerning renal function.

Urinary Proteins

Quantitative measurement of urinary protein excretion has historically been used to evaluate protein-losing nephropathies. A variety of chronic renal diseases can result in significant proteinuria.[188–191] Nephrotoxins that produce readily identifiable proteinuria typically exhibit correlative histopathologic findings for glomeruli, tubules, or both. As with urinary enzyme activities, urine protein excretion should be corrected for urine concentration by calculating the total amount excreted/unit time (e.g., mg/16 h) or the ratio of urinary protein concentration to urinary creatinine concentration. Although glomerular disorders (increased protein filtration) tend to exhibit greater proteinuria than tubular disorders (decreased protein reabsorption), the exact source of the protein loss cannot be determined by simply quantifying total urinary protein.

β_2-Microglobulin is a low-molecular-weight plasma protein that is freely filtered through the glomeruli and almost completely reabsorbed (>99%) by proximal tubular epithelial cells. Immunoassays for urinary β_2-microglobulin have been developed and used to differentiate glomerular from tubular protein loss and assess tubular function.[192–194] Unfortunately, antibodies specific for animal β_2-microglobulin are not commercially available, and the structure of the protein appears to be highly species specific.[151] The use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis has been proposed as a means of classifying renal injury by the molecular weight pattern of excreted urinary proteins.[21] An increase in high-molecular-mass proteins (e.g., >69,000 Da) is associated with glomerular injury, and an increase in low-molecular-mass proteins (e.g., 12,000–60,000 Da) is associated with tubular injury.

Numerous biomarkers have been proposed for monitoring renal toxicity in nonclinical studies. Several of these have undergone extensive qualification under defined conditions in rats and are being used more frequently to detect injury.[195–204]

Urinary Electrolytes

Urinary electrolyte concentrations (sodium, potassium, and chloride) from timed urine collections are the most commonly performed quantitative urine chemistry tests for regulated toxicology studies because they have been listed in the study guidelines for nonclinical evaluation of new pharmaceutical

products by Japan's MHLW.[11] Unfortunately, these tests have a very poor cost-to-benefit ratio as screening tools for nephrotoxicity. If only concentrations are assessed, they offer little advantage or information beyond that obtained from urine specific gravity or osmolality. Additional information can be obtained by calculating the total amount of each electrolyte excreted per unit time (e.g., mmol/16 h) or the fractional clearance of each electrolyte. In contrast to effects on urinary enzyme activities or protein concentrations, effects on urinary electrolyte excretion are most often a reflection of the normal homeostatic mechanisms required to maintain electrolyte and fluid balance in the face of changes in intake (e.g., anorexia) and output (e.g., gastrointestinal losses from diarrhea) rather than specific measures of renal injury or dysfunction. Of course, increased excretion of urinary electrolytes can occur secondarily to administration of many nephrotoxins and compounds with diuretic activity, but routine tests are typically more sensitive and cost-effective for detecting test article-related changes. One reason for the relative insensitivity of urinary electrolyte measurements is that they tend to exhibit considerable interanimal variability.

With respect to the effect of diuretic agents on urinary electrolyte excretion, the period of time for which the urine is collected has a major impact on the results obtained. For example, if a diuretic is administered in the morning, fluid and electrolyte excretion may be quite high during the first several hours postdose. But, if the urine sample collection is performed overnight, after the effect of the diuretic has subsided, electrolyte excretion may appear decreased because of compensatory electrolyte reabsorption to counteract the loss of fluid during the day.

QUESTIONS

26.1 Time-related biases are sources of preanalytical variation. Give at least three examples of time-related bias, and describe how these biases might affect interpretation of several different clinical pathology test results.

26.2 Describe how reference intervals are constructed, and compare their uses with those of concurrent control groups and correlative findings for a given toxicology study.

26.3 Differentiate regenerative from nonregenerative conditions as they relate to decreased red cell mass and give examples of each.

26.4 Describe how the common tests of hepatocellular and hepatobiliary integrity and function are used to characterize hepatotoxicity.

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27 Dermatotoxicology

Benjamin B. Hayes, Esther Patrick, and Howard J. Maibach

CONTENTS

Human skin, a dynamic multilayered organ comprising approximately 10% of the normal adult body, has numerous functions essential for terrestrial life, including thermoregulation, sensory perception, nutrient storage, vitamin synthesis, and barrier protection [226,227,270,301]. Resiliency and tensile strength protect against physical injury, pigmentation protects against ultraviolet light, barrier properties protect against the entry of environmental chemicals into the body, and the growth pattern and surface characteristics protect against microbial colonization and invasion. The regenerative capacity following wounding and the numerous processes by which skin can deal with environmental insults provide strong evidence of the importance of healthy skin to the organism. The psychological value of healthy skin has led in part to the development of multibillion-dollar cosmetics and personal care industries.

The policies of agencies such as the Occupational Safety and Health Administration, Department of Transportation (DOT), Consumer Product Safety Commission (CPSC), and Food and Drug Administration (FDA) in the United States and the Organization for Economic Cooperation and Development (OECD) and European Economic Community (EEC) internationally indicate that the identification of chemicals hazardous to the skin and the protection of society from exposure to those chemicals should be given high priority. These agencies mandate specific assays to evaluate the effects of skin exposure prior to registration, transport, and marketing of chemicals of formulated products. Adverse skin responses associated with repetitive low-dose exposure to industrial chemicals and consumer products all too often are not accurately predicted by the required assays. The need to market products with low risk of producing dermal and systemic injury to increase consumer satisfaction has led to the development of numerous assays to rank chemicals for their ability to injure the skin. Although these assays are not mandated by regulatory agencies, the frequency with which they are conducted and their utility warrant attention.

The discipline of dermatotoxicology evaluates the toxic effects of chemicals on the skin and includes both the pharmacokinetics of epicutaneously applied chemicals and assays that evaluate the development of neoplasms, trigger an immune response, directly destroy the skin (corrosion), irritate the skin, produce urticaria (hives), and produce noninflammatory painful sensations. The inflammatory responses of skin are the most common chemically induced diseases in humans.

SKIN STRUCTURE AND FUNCTION

To understand the variety of adverse responses to skin and the basis for the predictive assays for skin injury, some understanding of skin anatomy and physiology is necessary. Approximately 2 m^2 (20,000–23,000 cm^2) of skin cover the body of an adult human. Skin is heterogeneous. Its characteristics (e.g., sweat glands, hair follicles, sebaceous glands, and the thickness of skin) vary by body region [138,331];

TABLE 27.1
Regional Variation of Skin Thickness in Humans

Region	Thickness (μm)[a]	Reference
Stratum corneum of abdomen	8.2	Holbrook and Odland [139]
Abdomen	46.6	Whitton and Ewell [333]
Stratum corneum of back	9.4	Holbrook and Odland [139]
Back	43.2	Bergstressor et al. [20]
Stratum corneum of thigh	10.9	Holbrook and Odland [139]
Thigh	54.3	Bergstressor et al. [20]
Stratum corneum of forearm	15.0	Holbrook and Odland [139]
Forearm	60.9	Bergstressor et al. [20]
Cheek	38.8	Whitton and Ewell [333]
Forehead	50.3	Whitton and Ewell [333]
Back of hand	84.5	Whitton and Ewell [333]
Fingertip	369.0	Whitton and Ewell [333]

[a] Values are for full-thickness skin unless stratum corneum is specified.

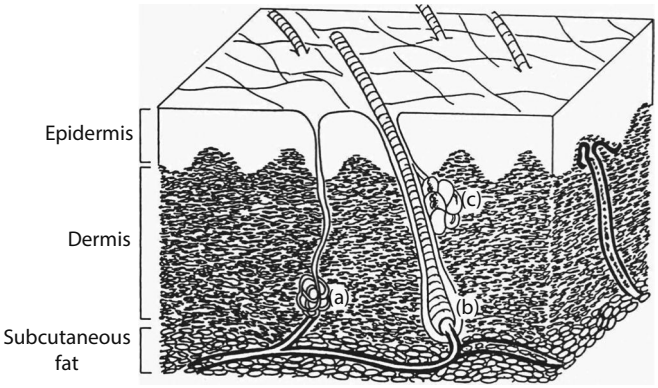

FIGURE 27.1 Schematic of human skin. (a) Eccrine sweat glands are located in the dermis; a duct transports sweat through the epidermis to the surface. (b) Hair follicles are located deep in the dermis; each hair extends through the skin via an epithelized channel. (c) Contents of sebaceous glands are released into the follicular channel as the sebocytes die. Each skin appendage has its own blood supply. Plexuses formed in the upper dermis (shown in the drawing to the far right) supply nutrients to the upper epidermis and upper dermis.

for example, the thickness of the skin of the eyelid is approximately 0.51 mm, whereas that of the palm and sole is approximately 4.1 mm (see Table 27.1).

A film composed of triglycerides, phospholipids, esterified cholesterol, and other materials released by holocrine sebaceous glands, as well as salts and water released by eccrine sweat glands, normally covers the outer surface [234]. This surface film has been referred to as an *acid mantle*; the pH of the skin normally varies between 4.2 and 5.6. Micrococcinae and *Corynebacterium* species normally colonize the skin surface [192,226]. Changes in surface film composition (e.g., changes caused by inflammatory conditions or occlusion) may result in a 1000-fold increase in the absolute number of microorganisms colonizing the area and a shift in flora present [28,29]. The surface film penetrates the outermost cellular layers of the skin.

Based on the structure and embryonic origin, the cellular skin layers are divided into two distinct regions (Figure 27.1): epidermis and dermis. The outer region, the epidermis, develops from embryonic ectoderm and covers the connective tissue; the dermis is derived from the mesoderm [137,139,220]. The epidermis accounts for approximately 5% of full-thickness skin [191,227]. For descriptive purposes, the epidermis is subdivided into five to six layers based on cellular characteristics (Figure 27.2). Layers of keratinocytes are formed by the ordered differentiation of cells from one layer of mitotic basal cells. The number of distinguishable layers varies by anatomical site.

Basal layer keratinocytes are metabolically active cells with the capacity to divide. Some daughter cells of the basal layer move upward and differentiate. Cells adjacent to the basal layer contain large mitochondria; the Golgi apparatus and rough endoplasmic reticulum (RER) are well developed. These cells produce lamellar granules (intracellular organelles) that later fuse to the cell membrane to release neutral lipids believed to form a barrier to penetration through the epidermis [82,83,293,326]. Microscopically, the desmosomes and bridges connecting adjacent cells resemble spines, and the three- to four-cell thick layer of cells above the basal layer is referred to as the *stratum spinosum*. The spines connecting adjacent cells are temporary structures; keratinocytes dissociate from neighboring cells and form new associations as they move upward, individually, in the epidermis [227]. Cells of the third subdivision of the epidermis, the stratum granulosum, are characterized by the presence of keratohyalin granules, polyribosomes, large Golgi apparatus, and RER. Cells of the granular layer are the uppermost viable cells of the epidermis. Here, the lamellar granules are released at the cell surface. An intermediate zone of cells separates the cornified layers of the outer epidermis from the viable granulosum. In the palms and soles, the stratum lucidum, or clear cell layer, lies above the stratum granulosum. This layer is indistinguishable in skin sections from other areas. Cells of the intermediate zone may contain enzymes capable of metabolizing exogenous chemicals but have lost the ability to synthesize proteins. The outermost cornified layer, the stratum corneum, consists of cells that have lost their nucleus and all capacity for metabolic activity. The dominant constituent of these cells is keratin, a scleroprotein with chains linked by both disulfide and hydrogen bonds that are synthesized and stored in the deep epidermal layers. The intracellular attachments between these cells gradually break, and the outermost cells are sloughed.

In addition to the visible intercellular and metabolic changes observed during keratinocyte differentiation, their size and shape have also changed. Cells derived from basal cuboidal cells approximately 5 μm in diameter have elongated and flattened to approximately 30 μm [191]. Four

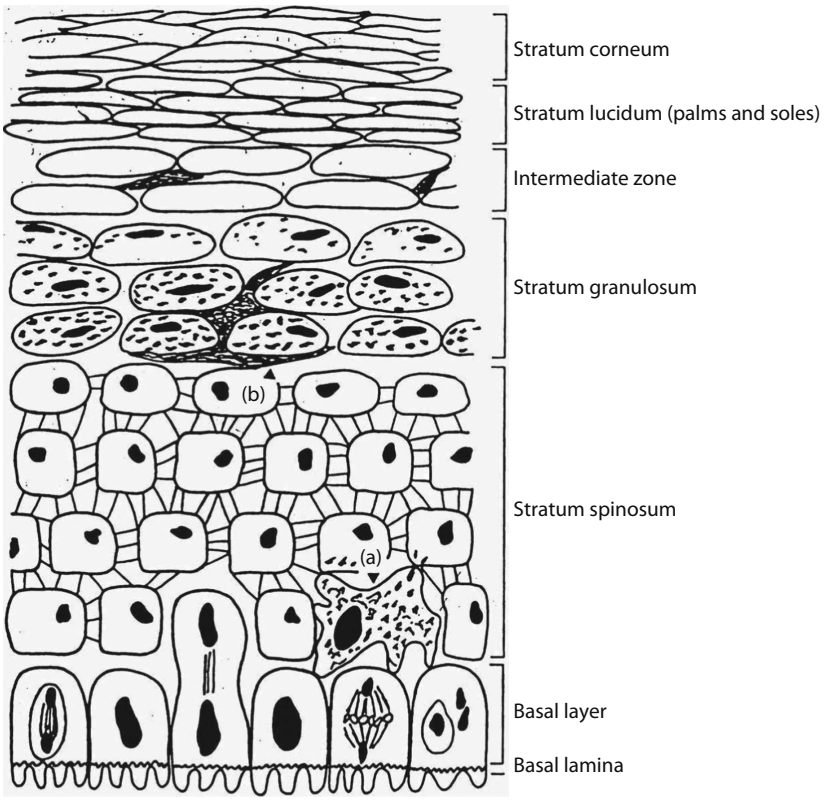

FIGURE 27.2 The epidermis, showing all possible cell layers and locations of the two dendritic cell types: (a) melanocytes and (b) Langerhans cells.

differentiated cornified cells of the stratum corneum (2 × 2) cover the same area as 100 basal keratinocytes (10 × 10). Each basal cell has the capacity to cover itself many times with modest mitotic rates. The pattern of papillae (ridges and grooves) of the basal layer formed by accessory structured from the dermis to the skin surface increases the area of germative layer relative to surface area. This provides a large reserve in capacity to cover the area. Estimates of normal turnover rate for keratinocytes vary considerably [73]. Early investigators [256] estimated normal turnover to be 28 days, with considerable increases in disease states. Since then, the turnover rate has been calculated to be between 17 and 71 days [90]. Turnover varies by anatomical site (e.g., 32–36 days for the human palm vs. 58 days for the anterior surface of the forearm). Recent studies utilizing deuterated H_2O support these observations [359].

The epidermis also contains two dendritic cell types: melanocytes and Langerhans cells. Between 460 and 1000 melanocytes and Langerhans cells per square millimeter of glabrous nonspecified skin is normal [191,236]. Melanocytes, derived from embryonic neural crest cells, lie directly adjacent to the basal layer. Melanocytes produce melanin, the principal pigment of human skin, which is then transferred to basal layer keratinocytes in granules. The dendrites of the melanocyte allow one cell to supply melanin to many basal cells. Langerhans cells express Ia (immune recognition) antigen and receptors for IgG and C3 on their surface. Like cells of the monocyte/macrophage lineage that bear these markers, Langerhans cells are derived from the bone marrow mesenchyme. They process low-molecular-weight haptens during induction of immune responses [134,290]. Although this function has been questioned, Langerhans cells take up small molecules (nonlipid) and increase in number in areas that have developed allergic reactions [289]. Note that Langerhans cells lie in epidermal layers containing enzymes that can metabolize exogenous chemicals. In some cases, metabolites of the agent applied to the skin cause allergic contact dermatitis.

The dermis and epidermis are separated by a basal lamina. The dermis is attached to this membrane by fine fibers of connective tissue. Cells of the basal layer are anchored to the lamina by anchoring proteins. This area of attachment, called the *marginal layer*, is identified histologically by periodic acid Schiff reaction. There are occasional breaks in the attachments. Large breaks are observed in exfoliative skin conditions [37,191]. The dermal connective tissue enclosed by the epidermal papilla is referred to as the *papillary dermis*, and the area below the papilla is the *stratum subpapillare* or *reticular dermis*. Fibers of the papillary dermis are finer than those of the reticular dermis. The reticular dermis contains thick collagen bundles, especially in areas adjacent to blood vessels and skin appendages. Connective tissue fibers are separated by the ground substance, an amorphous material consisting of proteins and glycosaminoglycans, such as chondroitin A sulfate and hyaluronic acid. Constituents of ground substance are derived from both fibrocytes and

blood plasma. The physical behavior of the dermis, including elasticity, is determined by the fiber bundles and ground substance. Variations in plasma content of ground substance may alter physical properties substantially.

The dermis contains all tissue types, except cartilage and bone. Skin appendages originate in the subpapillary dermis. Eccrine sweat glands, sebaceous glands, and hair follicles with their erector muscles are found in the skin of most anatomical sites; however, the number of each varies significantly by site [272,291]. Sebaceous glands normally are adjacent to hair follicles, and the hair shaft serves as an excretory duct. The axillae, anogenital region, eyelid, and external ear contain apocrine sweat glands [265,286]. These glands develop at puberty and form odorless secretions that are decomposed by bacteria to produce characteristic odors. The dermis also contains nerve cells with highly specialized sensory endings in some areas, fat lobules, migratory white blood cells, and mast cells. Mast cells are indistinguishable from fibroblasts in size or appearance; however, they contain granules that stain metachromically with agents of a thiazine group. Mast cells are most numerous in areas adjacent to blood vessels, skin appendages, and nerves. The precise function of mast cells is unknown; however, they appear to be involved in the pathogenesis of some inflammatory conditions [296,320]. Their granules contain histamine, heparin, and other vasoactive agents that may be released upon stimulation of the cell surface by IgE cross-linking 48/80, activated serum compounds, and some enzymes. Release of these mediators is accompanied by formation of other agents, such as the metabolites of arachidonic acid [296], which are inflammatory mediators in some conditions.

The dermis and fascia of muscles are separated by the subcutis, a layer of fatty tissue. The extent and development of subcutis depend on sex, age, diet, and body region. Blood vessels supplying the skin arise from the subcutis. Vascular plexuses are formed in the transition zone of the subcutis and dermis adjacent to coils of the eccrine sweat gland. Arteries extend upward to mid dermis, forming anastomoses there. Similar, but independent, plexuses form at the base of the hair shaft and sebaceous glands. A third vascular network is formed from arteries branching off from vessels at the level of eccrine sweat glands that branch into finer vessels that form plexuses in the papillary dermis. Plexuses of the papillary dermis supply the upper dermis, including the upper hair shaft, and the epidermis with nutrients. The adjacent but separate vascular units in the dermis sometimes react differently in pathological processes (i.e., follicular rash).

A simple visual comparison allows one to conclude that the skin of humans and animals varies considerably. The most obvious difference is hair coat covering the skin. In lower mammals, each hair shaft may contain several follicles, a large follicle arising from the subpapillary dermis and several accessory follicles arising from the papillary dermis. In humans, sebaceous gland density varies from 100 to 900 glands per square centimeter; in other mammals, sebaceous glands are more evenly distributed [286]. Human sweat is produced by eccrine sweat glands. Apocrine sweat glands are the dominant sweat gland of animals. Eccrine sweat glands open directly to the skin surface, whereas apocrine glands empty into the hair shaft. Apocrine sweat is less acidic than eccrine sweat, and the pH of the skin surface of animals usually is somewhat higher than that of humans [180]. The thickness of skin also varies extensively by species and body site. Differences in the content of granules of mast cells from different species have been reported [320], as have differences in sensitivity to various inflammatory mediators applied to the skin. These differences undoubtedly contribute to the lack of correlation between the results of some animal and human predictive assays [252]. The lack of correlation justifies predictive skin testing in humans after preliminary screening in animals if the risks to subjects are minimal.

Pharmacokinetics Following Application of Chemicals to the Skin

Until the beginning of the twentieth century, skin was considered a relatively inert barrier to chemicals that might enter the body [274]. We now know that this view is incorrect. Although the barrier properties of the skin are impressive, many chemicals penetrate the skin, and the skin can metabolize exogenous compounds. Because of its large surface area, skin may be a major route of entry into the body for some exposure situations. Delivery of drugs through the skin to treat systemic conditions has become almost commonplace. Interest in cutaneous pharmacokinetics has increased as the skin has been reconsidered to be a route for systemic administration of drugs and chemicals, as well as a route of entry for toxins. A variety of assays, both in vivo and in vitro, for measuring absorption through the skin have been developed [9,10,328,329], and many factors that govern absorption through the skin have been determined (Table 27.2).

The stratum corneum is a major diffusion barrier of the skin [129,271]. Removal of the stratum corneum by tape stripping increases the rate of absorption of some chemicals [29]. Absorption of chemicals through shunts, openings of skin appendages, and gaps in the stratum corneum associated with these structures has been considered [121,273,300,332]. Because of the relative surface area of these shunts (0.1%–1.0% of the total area), they may not play a decisive role in absorption [273]; however, they may be important initially after the application of the penetrant [275], and sebaceous glands may act as a drug reservoir for some materials [129,141,271]. The stratum corneum is not viable and has no capacity for active transport processes; therefore, absorption can be described as passive diffusion across this membrane: $J = K_m \times C_v \times D_m \times y$ [77], where J is the rate of absorption, K_m is the partition coefficient between the vehicle and stratum corneum, C_v is the concentration, D_m is the diffusion constant of the penetrant in the stratum corneum, and y is the thickness of the stratum corneum. Skin from different animals or sites of different thickness from the same animal will vary in barrier properties to absorption.

The concentration term is concentration at the skin surface. Application of suspensions of penetrant with slow dissolution

TABLE 27.2
Factors Determining Percutaneous Absorption

Release from vehicle
Varies with solubility in vehicle
Varies with concentration
Varies with pH
Kinetics of skin penetration
Influenced by anatomical site
Influenced by degree of occlusion
Influenced by intrinsic skin condition
Influenced by animal age
Influenced by concentration of dosing solution
Influenced by surface area dosed
Influenced by frequency of dosing
Tissue distribution
Excretion kinetics
Substantivity to the skin
Volatility
Wash and rub resistance
Binding to skin components
Cutaneous metabolism
Anatomic pathways

Source: Adapted from Wester, R.C. and Maibach, H.I., *Drug Metab. Rev*., 14, 169, 1983; Ngo, M. and Maibach, H.I., 15 Factors of percutaneous penetration of pesticides parameters for pesticide QSAR and PBPK/PD models for human risk assessment, January 1, 2012 Chapter 6, pp. 67–86.

rates, emulsions, or penetrants in vehicles in which the diffusion rate is slow will alter the surface concentration and may control the rate of penetration [41,49,56,136,344,345]. This principle has been used in designing slow-release transdermal delivery devices. Other factors that affect thermodynamic activity of the solution at the skin surface, such as pH and temperature, may alter the absorption rate [274,275]. The influence of the vehicle cannot be overstated for a specific concentration of drug; thermodynamic activity may vary by 1000-fold from one vehicle to another. Some vehicles may promote penetration by altering the characteristics of the stratum corneum [174]. Other factors that affect percutaneous absorption include the condition of the skin [95], age [132,204,268], surface area to which the material is applied [329], penetrant volatility, temperature and humidity [104], substantivity, wash and rub resistance to removal from the skin, and binding to the skin [245]. Skin may become saturated by a penetrant and thus resist penetration from subsequent applications.

In an intuitive sense, it has been assumed that multiple applications would provide significantly greater mass transfer; however, dermatopharmacokinetics studies demonstrate that, in any one given day, multiple applications may or may not add little transfer. This may explain why many dermatologic drugs can be successfully dosed on a once-daily basis. Over many days of topical application, however, this unique dermatopharmacokinetic phenomenon does not appear to continue [69,327].

When a chemical has gained access to the viable epidermal layers, it may initiate a local effect, may be absorbed into the circulation and produce an effect, or may produce no local or systemic effects. Viable epidermis contains many enzymes capable of metabolizing exogenous chemicals [21,22,212,235,352], including cytochrome P450 isoenzymes, mixed-function oxidases, and glucuronyl transferases. Enzymes have been identified in three compartments [8,61]. Skin enzymes are inducible by systemic phenobarbital, rifampin, and 3-methyl cholanthrene [313] and by topical 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [257], 3-methyl cholanthrene, and Aroclor® 1254 [68].

Early studies indicated that enzymatic activity in skin was only a fraction of the activity of the liver. Those studies were conducted in vitro using whole skin; the enzymatic activity is in the epidermis, which makes up less than 5% of whole skin [235]. When enzymatic activities of the epidermis were calculated, activities ranged from 80% to 240% of those in liver. In some cases, different metabolites are formed in liver and skin from the same parent compound.

The list of enzymes isolated from skin continues to grow, but the skin does not have the capacity to metabolize all chemicals; for example, topically applied hexachlorophene does not appear to be metabolized. At present, it is not possible to predict metabolic pathways or rates following topical application; these must be determined experimentally. Comprehensive reviews of the metabolic capability of skin have been published [8,158].

In Vivo Percutaneous Absorption Assays

Percutaneous absorption can be determined by applying a known amount of chemical to a specified surface area and then measuring the level of the chemical in the urine or feces. To correct for excretion of the material through the lungs, sweat levels (of retention in the body) measured following topical administration usually are expressed as a percentage of levels following parenteral administration of the chemical [39,328]. Because the analytical techniques to measure the chemical are not always available, and because some chemicals may be metabolized, radioactive-labeled chemicals, usually carbon-14 or tritium, are customarily used in these assays. Although studies with radiolabeled compounds accurately reflect absorption, they may not provide accurate estimates of bioavailability; for example, comparison of bioavailability from the nitroglycerin (unmetabolized drug) level and the level of radioactive tracer indicates that use of the tracer overestimates available drug by as much as 20%. This corresponds to the metabolism of the drug to an inactive form.

In vivo studies have been conducted in humans and in a number of species [9,10]. Comparison of the absorption rates of a number of compounds shows that absorption rates in the rat and rabbit tend to be higher than in humans and that the skin permeability of monkeys and swine more closely resembles that of humans (Table 27.3). Although these differences are not predicted by any single factor, such as epidermal thickness, they are not unexpected in light of differences in skin characteristics. There are interspecies differences in

TABLE 27.3
Species Differences in In Vivo Absorption (% Dose Absorbed)

Compound	Rat	Rabbit	Pig	Squirrel Monkey	Human	Reference
Haloprogin	95.8	113.0	19.7	—	11.0	Bartek et al. [9]
Acetylcysteine	3.5	2.0	6.0	—	2.4	Bartek et al. [9]
Cortisone	24.7	30.3	4.1	—	3.4	Bartek et al. [9]
Caffeine	53.1	69.2	32.4	—	47.6	Bartek et al. [9]
Butter yellow	48.2	100.0	41.9	—	21.6	Bartek et al. [9]
Testosterone	47.4	69.6	29.4	—	13.2	Bartek et al. [9]
DDT	46.3	—	43.4	1.5	10.4	Bartek and LaBudde [10]
Lindane	51.2	—	37.6	16.0	9.3	Bartek and LaBudde [10]
Parathion	97.5	—	14.5	30.3	9.7	Bartek and LaBudde [10]
Malathion	64.6	—	15.5	19.3	8.2	Bartek and LaBudde [10]

routes of excretion of some chemicals as well. This may be due in part to metabolism of the chemical, and the metabolic capabilities of the species should be considered when selecting an animal model and designing the experiment. Ingestion of the test material by the animal must be prevented, and this may require restraint of the animal or design of specialized protective apparatus for the site of application. Because urine and feces are collected for analysis, specialized cages are also required.

The difficulties in conducting these types of pharmacokinetic assays, such as collecting excrement for relatively long periods (24 h), requirements for specialized cages and protective apparatus, and the increased space requirements for housing animals individually, have led to the use of other in vivo assays and to the development of in vitro models. Loss of radioactive material from the skin surface has been used to estimate in vivo percutaneous absorption [207]. The difference in applied dose and residue on the skin is assumed to be absorbed. The characteristics of the radioisotope, penetrant, and vehicle may limit the usefulness of this procedure. Volatile materials leave the surface without penetrating, and it is difficult to recover all materials from the skin surface. In addition, skin may retain a reservoir of the penetrant that has not entered the circulation.

A clear relationship exists between the mass of a chemical residing in the stratum corneum that has been washed 30 min after application and the eventual penetration that can be measured in urine or blood. This principle has led to a facile method for estimating percutaneous absorption in animals and humans. The approach of determining the stratum corneum content via cellophane tape sampling is also used for bioequivalence determination [39].

In vivo biological responses, such as vasoconstriction assays to estimate the absorption of corticosteroids [218] and changes in blood flow being used to study penetration through various types of skin and under diverse conditions [127,128], have been used to estimate penetration rates. These endpoints are complicated biological processes and may vary with the ability of the tissue to produce the response. For example, application of histamine produces increased blood flow; however, the degree of change would depend not only on the rate of penetration but also on the reactivity of receptors at that time. Most exogenous chemicals produce their vascular effects by triggering the formation and release of endogenous mediators; thus, the usefulness of penetration studies using biological endpoints is limited to comparisons between closely related chemical structures that can be assumed to trigger the same process.

In Vitro Percutaneous Absorption Assays

The excised skin of humans or animals can be used to measure penetration of chemicals. In vitro assays utilizing excised skin use specially designed diffusion cells [10,40,102]. The skin is stretched over the opening of a collecting receptacle, epidermal side up. The chemical to be studied is applied to the epidermis, and fluid from the receptacle is assayed to measure the penetration of the chemical. Chemicals usually are radioactively labeled. Some investigators have used diffusion cells in which the epidermis was covered with fluid containing the chemical; however, the preferred method for toxicological relevance is a one-chambered cell in which the stratum corneum is exposed to the air and the underside of the skin is bathed in saline or other receptacle fluid. Because diffusion through a membrane depends on relative concentrations on each side, some chambers have been designed to allow periodic replacement of the receptacle fluid. Fluid in the receptacle base usually is constantly stirred and maintained at a physiological temperature. Either full-thickness skin or epidermis alone may be used in in vitro assays. With relatively hairless skin, epidermis can be separated from the dermis by heat treatment.

This type of in vitro assay offers advantages over in vivo assays. Highly toxic compounds can be studied in human skin. Several cells can be run simultaneously. Diffusion through the membrane, eliminating other pharmacokinetic factors, can be studied. In addition, these assays may be less expensive and easier to conduct; however, these assays do not mimic human exposure in some important areas. Because excised skin must often be stored prior to use, it cannot be assumed that the skin will retain full enzymatic activity. This may alter the metabolic profile of compounds entering the receptacle. In intact skin, chemical penetrating the epidermis would

enter the circulation through vessels and lymphatics located just below the epidermis. In excised full-thickness skin, the dermis is also involved in the absorptive process. The influence of the dermis can be minimized by using heat-separated epidermis or by removal of the skin with a dermatome at the level of the upper dermis. In the intact animal, the chemical enters the peripheral circulation in plasma; the collecting fluid of diffusion chambers is usually saline or water. The relative solubility of hydrophobic and hydrophilic chemicals in these collecting fluids may alter the rate at which they leave the skin. Surface conditions of excised skin may vary from normal skin; changes in the surface emulsion occurring during storage have not been studied. Storage conditions and procedures for preparing the tissue may affect skin absorption and metabolism. The suitability of each specimen of excised skin should be verified by measurement of penetration of a standard tritiated water through the tissue prior to its use to study the penetration of other chemicals. Comparisons of penetration rates obtained from in vitro and in vivo assays have been made [10,40]. Often a good correlation between the two methods has been obtained; however, with some compounds, correlation of the methods is poor. Differences between in vivo and in vitro results for some compounds can be explained because of solubility in the receptacle fluid and blood. Differences observed for other compounds cannot be explained.

In vitro penetration rates through skin of various species have also been compared (Table 27.3). Skin of the weanling pig and miniature swine appears to serve as good in vitro models for most compounds [9]. The skin of monkeys seems to be a good model, as well [328]. For most compounds, mouse and hairless mouse skin appears more permeable than the skin of other species. Rat skin appears to be a good model for some compounds; however, when differences have been noted, they have been large.

A few investigators have estimated percutaneous absorption using *model* membranes, including excised stratum corneum, and physiochemical data have been used to predict absorption. Lipid/water partition coefficients have been correlated with skin permeability. Smaller molecules (molecular weight ~400) are more readily absorbed than large molecules. Molecules with polar groups, in general, do not penetrate as well as nonpolar molecules [270]. The addition of hydroxyl groups also lowers the permeability. Substitutions that increase lipid solubility may increase penetration, depending on the vehicle in which the chemicals are applied [274]. Electrolytes do not penetrate the skin well [275]; shunt diffusion through skin appendages for these molecules may be important.

The presumed simplicity of in vitro penetration assays has led to their universal acceptance for preclinical and other screening purposes. This acceptance and wide-scale use have resulted in many variations in how studies have been conducted. It is not surprising that confusion regarding the interpretation of data generated by variations of this method occurs. Bronaugh [40] collated the experimental variables leading to discrepancies, and his text provides a catalog of variables that, properly considered, can lead to experimental designs that may have in vivo relevance, especially for hydrophilic materials.

Government agencies and individual investigators utilize quantitative structure–activity relationships (QSARs) based largely on in vitro datasets to estimate penetration when actual flux data are not available. Faramind and Maiback[360] compared several such models to in vivo human data and noted significant under- and overflux estimations.

Neoplastic Response of Skin

The skin is the most common site of cancer in humans. Both benign and malignant tumors may be derived from viable keratinocytes and melanocytes of the epidermis, and rarely from skin appendages, blood vessels, peripheral nerves, and lymphoid tissue of the dermis [191,192]. Historically, basal cell and squamous cell carcinomas, which develop from keratinocytes, account for 60% and 30%, respectively, of all skin cancer. The remainder includes malignant melanoma and the rare tumors developing from other cell types; however, the incidence of malignant melanomas appears to be increasing. Melanomas often metastasize, and the prognosis for patients with this disease is poor. Only 4%–5% of squamous cell carcinomas are metastatic, and basal cell tumors rarely metastasize. The relatively noninvasive nature of the common forms of skin cancer accounts for a cure rate of over 95%. Skin cancer accounts for less than 0.3% of all cancer deaths.

The association between exposure to environmental carcinogens and the development of basal cell and squamous cell carcinomas is strong [284]. Epidemiological studies demonstrate a strong correlation between exposure to ultraviolet radiation and development of skin cancer [86]. Clinical experience leaves little doubt that radiographs can also produce cancer of the skin. Both forms of radiation have induced tumors in experimental animals [38]. The association between environmental chemicals and skin cancer was first demonstrated by Sir Percival Potts in 1775. Following Potts's association between skin cancer of the scrotum and soot exposure, experimental studies in animals revealed that polycyclic aromatic hydrocarbons such as benzo(*a*)pyrene are the carcinogens in soot, coal tar, pitch, and various cutting oils [93]. The same types of experiments demonstrated that skin cancer development is a multistage process [37,38].

Despite abundant experimental evidence that chemicals can produce skin cancer, few chemicals have been associated with increased incidences of skin cancer in humans (http://www.iarc.fr/). Epidemiological studies have demonstrated associations between polycyclic aromatic hydrocarbons and arsenic and the increased incidence of benign precancerous lesions and basal cell and squamous cell carcinomas [148]. The ability to establish relationships between chemical exposure and the development of skin cancer by epidemiology is minimized by many confounding factors, including exposure to ultraviolet light, high background incidence rates, long latency periods for the development of cancer [93], and incomplete reporting due to the nonlethal nature of the disease. Even without strong epidemiological evidence, the

experimental evidence that exposure of the skin can lead to tumor development and the degree of dermal exposure to chemicals in the workplace justifies the practice of evaluating carcinogenic potential by dermal exposure. Furthermore, it is likely that certain internal tumors (bladder cancer from occupational aniline exposure) result from chemicals absorbed through the skin.

In vivo skin carcinogenesis studies are generally conducted using Sprague–Dawley rats, but mice have also been used frequently. Differences in species sensitivity to various agents have been demonstrated [38,247], and a review of the data suggests that when differences in species were noted, rats tended to be more sensitive. The design of carcinogenesis assays has been reviewed in other texts [100,101,146,194,244] and is described in Chapter 25. The method, in brief, is daily application of the test material to the clipped skin of up to six groups of animals (untreated, vehicle control, and up to four dose groups) for 104 weeks. The dose is generally administered at a constant volume of 1.0 mL/kg body weight to 5%–10% of the animal's body surface. Typically, 70 male and 70 female animals are included in each group, with additional groups dosed on the same schedule used for periodic blood analysis and for evaluating the toxicokinetics of the test material. Two variations of standardized skin carcinogenicity studies have also been reported. In the first, the skin is treated with the chemical of interest. Then, a promoting agent, such as tetradecanoyl phorbol acetate (TPA), may be applied to reduce the latency period. In the second, the skin is treated with a noncarcinogenic dose of a carcinogen, such as dimethyl benzanthracene, followed by repeated doses of the agent under study. These approaches may be helpful in elucidating the mechanisms of action of the chemicals.

Numerous factors may influence the outcome of dermal carcinogenic assays, and the choice of what to test is crucial in such assays. Although it is tempting to evaluate pure chemicals, it should be remembered that other agents in mixtures could act as promoting agents; for example, coal tar and pitch may contain catechol and pyrogallol, which are promoters of the carcinogen benzo(*a*)pyrene found in these mixtures. Wounding increases the number of tumors that spontaneously develop, and severe inflammatory responses may cause tissue destruction. Care should be taken in selecting an appropriate nonirritant dose.

Epidemiology and experimental studies have shown that sunlight (ultraviolet light) is a skin carcinogen [88,89]. Various investigators [305] have shown that exposure to some chemicals in combination with a dose of ultraviolet light increases the number of tumors or decreases the latency period for tumor development following exposure to the chemical or ultraviolet (UV) light alone. No standard protocol has yet been put forth by any regulatory agency, and typically regulatory agencies request to review specific protocols prior to study conduct [100,101,146]. In general, the studies have involved repeated intercurrent exposures to simulated sunlight and the test article. The endpoint is the time required for UV radiation (UVR) to produce skin cancer. Two doses of UVR are used as a control: a high dose that will result in a short latency period for tumor development and a lesser dose that would result in a lower tumor yield and longer latency period. The interaction of the test article and the lesser dose of UVR is compared to each control and to a vehicle control group irradiated with the lower dose of UVR. Complete details of a typical protocol have been published by Forbes et al. [101,102].

The FDA has expressed concern that some topical drugs and cosmetic ingredients may promote neoplastic changes in the skin induced by other agents, such as sunlight. Although, to date, there is no epidemiological evidence for such an occurrence, the exploration of this possibility will require developing new approaches to conventional assays for carcinogenicity.

A number of in vitro assays for studying chemical carcinogenesis have been developed [147]. Of particular interest for dermal carcinogenesis is the ability to cultivate epidermal keratinocytes of rats, mice, and humans [181]. Cultured human keratinocytes can metabolize polycyclic aromatic hydrocarbons, and chemical transformation using human fibroblasts has been achieved [181]. The establishment of human epidermal lines for in vitro carcinogenesis testing will provide an important new predictive tool.

Taken together, the clinical and public health implications of positive animal assays remain an incompletely understood science. Many dermatologies (systemic, topical, and over-the-counter [OTC]) are tumorigenic in animals. New approaches and insight into hazard and risk management regarding such dermatologies based on such data are needed.

Skin Allergy (Delayed-Type Hypersensitivity)

Since the turn of the twentieth century, certain forms of eczema have been recognized as allergic in nature. Jadassohn [150,151] demonstrated that in some patients, dermatitis was due to increased sensitivity following repeated contact with a substance, not the toxic (irritant) properties of the material [148,149]. By 1930, a procedure for producing this hypersensitivity to chemicals in guinea pigs had been developed [30]. The pioneering work of Landsteiner and his colleagues demonstrated that low-molecular-weight chemicals conjugate with proteins to form an antigen that stimulates the immune system to form a hyperreactive state [190]. They demonstrated that immunogenicity is related to chemical structure [189] and that two types of immunologic response exist, one transferable by serum and another transferred by suspensions of white blood cells [188].

Most cases of allergic contact dermatitis are of the cell-mediated type, transferable by lymphocytes. This type of skin response is often referred to as *delayed-contact hypersensitivity* (DCH) because of the relatively long period (approximately 24 plus hours) required for the development of the inflammation following exposure.

Some understanding of the processes by which this hypersensitivity develops is helpful in selecting and interpreting results of predictive sensitization tests. During ontogenesis, stem cells from the yolk sac, fetal liver, and bone marrow

migrate to the central lymphoid organs, the thymus, and bone marrow in mammals. After birth, stem cells derive from bone marrow. In the central lymphoid organs, stem cells differentiate into immunocompetent lymphocytes. This results in two classes of lymphocytes: thymus-processed T lymphocytes and B lymphocytes processed in bone marrow. B lymphocytes are precursors of antibody-producing cells responsible for immune responses transferable by serum. T lymphocytes are responsible for producing delayed-type hypersensitivity (DTH) and for the regulation of the immune system. This regulation is accomplished by subsets of T cells (i.e., T helper and T suppressor cells). Lymphocytes leaving the lymphoid organs are programmed to recognize a specific chemical structure via receptor molecules. If, during circulation through body tissues, a cell encounters the structure it is programmed to recognize, an immune response may be induced. The ability to develop and express a hypersensitivity response is determined by the relative activities of the T helper and T suppressor cell types [258].

To stimulate an immune response, a chemical must be presented to lymphocytes in an appropriate form [189,190]. Chemicals usually are haptens that must conjugate with proteins in the skin or other tissues to be recognized by the immune system. Haptens conjugate with multiple proteins to form antigens that may stimulate an allergic response by stimulating T lymphocytes with different recognition capabilities [259].

Hapten–protein conjugates are processed by macrophages, Langerhans cells, or other cells expressing immune-response Ia proteins on their surface. Although the exact nature of this process is not completely understood, it is known that physical contact between macrophage and T cells is required [60,304], suggesting that receptor interactions are necessary. Physical interaction is accompanied by the release of interleukins, a family of soluble regulatory proteins that stimulate cell division, act as growth factors, and increase expression of immune proteins on the surface of some cells [94,143,224,228].

Following antigen stimulation in the skin, lymphocytes enter the lymphatic system and migrate to the draining lymph nodes. Disruption of lymphatic drainage prevents sensitization of an animal [103]. Stimulated cells settle in the paracortical regions of the lymph nodes, and T lymphocytes differentiate into immunoblasts. This differentiation involves interaction with other cell types. Immunoblasts eventually give rise to T effector cells, which enter the systemic circulation and, upon encountering the antigen that they are programmed to recognize, release lymphokines that initiate a local inflammatory response. Immunoblasts also give rise to memory cells that enter the systemic circulation. These memory cells are capable of similar activities as the processed T lymphocytes; they recognize antigen and can be stimulated to divide. Memory cell production is essentially an expansion of the number of cells capable of recognizing a given antigen. The lymphokines released by primed effector cells that encounter their stimulating antigen directly and, indirectly, by stimulation of other white blood cells produce a local inflammatory response. Actions of lymphokines include direct tissue damage, chemotactic factors, stimulation of mitosis, increased phagocytic activity of macrophages, and factors that inhibit migration of some cell types from the area [67]. Only a small percentage of lymphocytes in an area of skin exhibiting a delayed hypersensitivity response are specifically stimulated by antigen [231]. Most cells in the lesion are recruited by lymphokines. Histologically, the response has been described as a hyperproliferative epidermis with intracellular edema, spongiosis, intraepidermal vesiculation, and mononuclear cell infiltrate by 24 h. The dermis shows perivenous accumulation of lymphocytes, monocytes, and edema. No reaction occurs if the local vascular supply is interrupted, and the appearance of epidermal changes follows the invasion of monocytes. Vascular changes (i.e., increased blood flow) occur early (2–6 h) in the response.

The histology of the response varies somewhat by species; for example, a higher proportion of polymorphonuclear cells in the cellular infiltrate have been observed in DTH reaction sites of mice than in guinea pigs or humans [151]. These differences may be due, in part, to mixed immune responses. Mice develop both antibody and DTH responses to haptens applied to the skin [5]. Exposure via the skin is believed to preferentially lead to DTH in guinea pigs and humans. Pichler has provided detailed immunologic information [355].

The biological processes necessary for producing hypersensitivity in predictive tests are often grouped into two phases: induction of the capability to respond and elicitation of a response. Induction has been referred to as the *afferent phase*, the initial exposures through clonal expansion and the release of memory cells that enter the system circulation. Elicitation, referred to as the *efferent phase*, consists of local recognition of the antigen by the memory cells, their release of lymphokines, and the activity of inflammatory mediators that are generated locally, which produce the dermatitis. All standardized predictive tests in guinea pigs and some early tests in mice [5,113,206] use the efferent phase response as an indication of immune reactivity to the chemical. The local lymph node assay (LLNA) in mice uses stimulation of lymphocytes [163–165] in local draining lymph nodes during the afferent phase as the endpoint.

Modulation of the development of DTH in experimental animals and in humans is complex. The intrinsic biological variables controlling sensitization can be influenced by the selection of animals likely to be capable of mounting an immune response to the hapten. The extrinsic variables of dose, vehicle, route of exposure, adjuvant, etc., can be manipulated to develop sensitive predictive assays. The method of skin exposure is important. Keratinocytes produce interleukins [67], important regulatory proteins for the induction of DTH. Langerhans cells express Ia antigen and may act as antigen-presenting cells [60,267,304]. Intradermal injection in animals bypasses the processes but ensures entry of the chemical into the skin.

Vehicle plays an important role in percutaneous penetration and hence presumably in sensitization. The theory, at least with regard to flux, should be simple: maximum

solubility leads to maximum thermodynamic activity and enhanced flux. The experimental literature only partially documents this presumed truth. Analysis of the relevant literature provides a partial interpretative key to this fundamental area [203]. Increasing the dose per unit area increases the sensitization rate. Upadhye and Maibach [306] reviewed in detail the influence of area application on the development of human sensitization. When early publications viewed as dogma were examined with statistical methods, the findings failed to be significant, yet dose per unit area and occlusion [198,199] appear to be highly important variables.

Application of haptens to damaged skin (i.e., irritated, tape-stripped) increases the sensitization rate, perhaps partially by increasing penetration. Although effects of vehicle, dose level, and damaged tissue have been studied more extensively in guinea pigs and humans than in mice, until conclusive studies are reported, it is prudent to consider their influence in the design of all studies. Repeated applications to the same site are more effective for inducing sensitization than applications to new sites each time [91,198]. The incidence of sensitization increases with increased number of exposures [198], and an interval between exposures of 2–6 days increases the sensitization rate [5,165,198]. This may be due to the booster effect of memory cells. Materials such as Freund's complete adjuvant (FCA) nonspecifically enhance the development of immune responses but may selectively trigger humoral immunity in some species [47]. Treatment of animals with adjuvant, either simultaneously or shortly after hapten exposure, increases sensitization rates [199,201]. The development of DTH is under genetic control; within the human and guinea pig populations, all individuals do not have the capability to respond to a given hapten [198]. The status of the immune system will determine if an immune response can be induced; for example, animals may become tolerant to a hapten, and pregnancy may suppress expression of the allergy [177].

Appropriate planning and execution of predictive sensitization assays are critical. All too often, techniques are discredited when, in fact, the performance of the tests was inferior or the study design (e.g., choice of vehicle or dose) was inappropriate. The first priority is to choose an appropriate experimental design. Often the assay to be used is chosen on a pro forma basis without realizing the inherent weaknesses and strengths of the method. A common error in choosing an animal assay is using FCA when one wishes to determine dose–response relationships. The adjuvant provides such sensitivity that dose–effect relationships are muted.

Choice of dose and vehicle appropriate to the assay and the study question is the second priority. Although dose must be sufficient to ensure penetration, it must be below the irritation threshold at challenge to avoid misinterpretation of irritant inflammation as allergic. For example, quaternary ammonium compounds, such as benzalkonium chloride, rarely sensitize, but they have been identified as allergens in some guinea pig assays. Knowing the irritation potential of compounds allows the investigator to appropriately design and execute these studies. Vehicle choice determines, in part, the absorption of the test material and can influence sensitization rate, ability to elicit response at challenge, and the irritation threshold. Inappropriate selection of vehicle and dose effectively invalidates studies.

Sensitization assays often are assigned to novices when they should be performed and read by persons experienced with the method being used. Experienced investigators will recognize marginal reactions that should be further investigated and positives that may be irritant in nature, and they will be able to assist in the estimation of risk associated with the proposed use of the material. Working with laboratories and personnel with extensive experience greatly decreases errors and increases the reliability of all of the standard assays described later [140,160,161,169,264].

Data from various sensitization assays have been broadly used to determine the likelihood of induction of clinical allergic contact dermatitis in populations to be exposed. Interpretation of these assays requires experience, judgment, and sophistication because each assay has its own strengths, weaknesses, and limitations. Central to the issue of interpretation is the definition of allergic contact dermatitis in humans and animals; for example, in the mouse, the endpoint is documented reliably only by measuring ear swelling. In humans, the patch test, albeit a highly valuable bioassay, has often been misinterpreted. Guidance for proper interpretation is found in the original references for many of the assays discussed later; for human repeat insult patch tests, guidance is found in a review article by Stotts [292]. Several authors and groups are refining criteria for allergic contact dermatitis that are operational rather than mechanistic. Fundamentally, each of the systems acknowledges the complexity of the biological process. Each parameter is qualitative or quantitative and is multifunctional; for example, in humans, it is necessary to have a pertinent history, carefully performed patch test results with appropriate virgin controls, and sufficient follow-up to define that removal of the allergen improved clinical status [156,317].

Guinea Pig Sensitization Tests

Predictive animal tests to determine the potential of substances to induce delayed hypersensitivity in humans are conducted most often in guinea pigs. Several tests have been described. Each offers its own advantages and disadvantages; most have many features in common. All use young (1–3 months old or 250–550 g), randomly bred albino guinea pigs. To reduce the possibility of seasonal variability in reactivity, animals are maintained in facilities with a temperature of approximately 20°C ± 1°C, a relative humidity of 40%–50%, a 12 h automatic light cycle, a standard vitamin C-supplemented chow, and water available at all times. Test sites are clipped free of hair with electric clippers; some assays specify chemical depilation as well. Almost all evaluate the response as the production of visible dermatitis, using descriptive scales for erythema and edema. Because of genetic influences, sensitivity to a common chemical, such as dinitrochlorobenzene (DNCB), is usually confirmed periodically for animals from each vendor. There is disagreement as to which sex, if either, is more susceptible to sensitization.

Males are more aggressive and may damage the skin of cage mates. Some assays specify use of one sex or half of each sex. The tests differ significantly in route of exposure, use of adjuvants, induction interval, and number of exposures. Table 27.4 summarizes the principal features of the most commonly used assays to predict sensitization and assays acceptable to regulatory agencies [58,87,92,152,232,264].

Draize Test

The Draize sensitization test (DT) [75,153,166] was the first predictive sensitization test and is still widely used. One flank of 20 guinea pigs is shaved and 0.05 mL of a 1% solution of test material in saline, paraffin oil, or polyethylene glycol is injected into the anterior flank on day 0. The next day, and every other day through day 20, 1.1 mL of the test solution is injected into a new site on the same flank. Challenge follows a 2-week rest period. The opposite untreated flank is shaved, and 0.05 mL of test solution is injected into each animal. Twenty previously untreated controls are injected at the same time. The test site is visually evaluated 24 and 46 h after injection. The intensity of the responses of test animals is compared with that of controls. A larger more erythematous response than that of controls is considered a positive response. Results are expressed as the percentage of animals positive or as the ratio of positive animals tested.

Open Epicutaneous Test

The open epicutaneous test (OET) [160,166,167] simulates the conditions of human use by using topical application of the test material. The procedure determines the dose required to induce sensitization and to elicit a response in sensitized animals. The irritancy profile is evaluated by testing various concentrations (typically, undiluted, 30%, 10%, 3%, and 1%) in ethanol, acetone, water, polyethylene glycol, or

TABLE 27.4
Principal Features of Guinea Pig Sensitization Assays

Feature of Test	Draize	Open Epicutaneous Test	Buehler Assay	Freund's Complete Adjuvant Test	Optimization Test	Split Adjuvant	Guinea Pig Maximization
Number in test group	20	6–8	10–20	8–10	20	10–20	20–25
Number in control group	20	6–8	10–20	8–10	20	10–20	20–25
Induction							
Exposure route	ID	Open epicutaneous	Patch	ID	ID	Patch	ID and patch
Number of exposures	10	20–21	3	3	9	4	1 ID; 1 topical
Duration of each patch	No patch	Continuous (no patch)	6 h	5%–50%	0.1%	0.1–1.2 mL	Maximum tolerated
Concentration	0.2	Nonirritating	Slightly irritating	5%–50%	0.1%	0.1–0.2 mL	Maximum tolerated
Test group(s)	TS	TS	TS	TS in FCA	TS in FCA	TS, FCA	TS, TS + FCA, FCA
Control group	None	Vehicle only	Vehicle only	FCA only	—		FCA, FCA + V, V
Site for dosing	Left flank	Right flank	Left flank	Shoulder	Back (flank first injection)	Mid-back	Shoulder
Frequency of exposure	Every second day	Daily	Every 5–7 days	Every 4 days	Every other day	Days 0, 2, 4, 7	Day 0 (ID); day 7 patch
Duration (days)	1–18	0–20	0–14	0–9	0–21	0–9	0–9
Misc.	—	—	Nine exposure version	—	—	Dry ice treatment day 0; FCA (ID) day 4	Irritant dose or SLS Pretreatment
Rest period (days)	19–34	21–34	15–27	9–21; 22–34	22–34	10–21	9–20
Challenge							
Exposure route	ID	Open	Patch	ID; patch	ID	Patch	Patch
Number of exposures	1	2	1	2	2	1	1
Duration of exposure	—	—	24 h	—	—	24 h	24 h
Exposure day(s)	35	21 and 35	28	22; 35	14–28	22	21

Note: FCA, Freund's complete adjuvant; ID, indeterminable; SLS, sodium lauryl sulfate; TS, test substance; V, vehicle.

petrolatum. In six to eight guinea pigs, 0.025 mL of the dosing solutions is applied to 2 cm^2 areas of the shaved flanks. Vehicle solubility and use conditions (e.g., direct application to skin or dilution during normal use) are considered when selecting the concentrations to be tested. Test sites are visually evaluated 24 h after the application of test solutions for the presence or absence of erythema. The dose not causing a reaction in any animal (maximum nonirritant concentration) and the dose causing a reaction in 25% of the animals (minimal irritant concentration) are determined. During induction, 0.10 mL of test solution is applied to an 8 cm^2 area of flank skin of six to eight guinea pigs for 3 weeks or five times a week for 4 weeks. As many as six groups of animals are treated with different doses; a control group is treated with vehicle only. The highest dose tested usually is the minimal irritant concentration; lower doses are based on usage concentration or a stepwise reduction (e.g., 30%, 10%, 3%, and 1%). Solutions are applied to the same site each day unless a moderate inflammatory response develops. Each animal is challenged on the previously untreated flank 24–72 h after the last induction treatment. The minimal irritant concentration, the maximum nonirritant concentration (from the irritancy screen), and five solutions of lower concentrations are applied (0.025 mL to a 2 cm^2 area). Skin reactions are read on an all-or-none basis at 24, 48, and 72 h after the applications of the solutions. The maximum nonirritating concentration in the vehicle-treated group is calculated. Animals in test groups that develop inflammatory response to lower concentrations are considered sensitized. The dose required to sensitize is determined by comparing the number of positive animals in the test groups. The minimal concentration necessary to elicit a positive response in a sensitized animal is apparent from the challenge responses.

Buehler Test

The Buehler test [45–47,106,166] also employs topical application of the test material. An absorbent patch—20 × 20 mm Webril®, backed by Blenderm™ tape and saturated with 0.4 mL of the test material—is placed on the shaved flanks of 10–20 guinea pigs. The test concentration varies from undiluted to usage levels. An optimum concentration that produces slight erythema is selected based on an irritancy screen conducted in other animals. The patch is held in place by wrapping the animal with an occlusive wrapping. The animal is then placed in a special restrainer fitted with a rubber dam to maintain even pressure over the patch for a 6 h exposure period. This procedure is repeated 7 and 14 days after the initial exposure. A control group of 10–20 animals is patched with vehicle only. Two weeks after the last induction patch, animals are challenged with patches saturated with a nonirritating concentration of test material applied to both flanks and with the vehicle (if other than water or acetone). Wrapping and restraint are as during induction. After 6 h, the patch is removed, and the area is depilated. Test sites are visually evaluated 24 and 48 h after removal. Animals developing erythematous responses are considered sensitized (if irritant control animals do not respond). The incidence of positive reactions and the average intensity of the response are calculated.

Freund's Complete Adjuvant Test

FCA test is an intradermal technique incorporating test materials in a 50/50 mixture of FCA and distilled water. The test has been significantly modified since originally described [166]. The latest published description [167] is summarized here. A 6 × 2 cm area across the shoulders of two groups of 10–20 guinea pigs is shaved and used as the injection site. Animals of one group are injected with a 5% solution of the test material in FCA/water; injection volume is 0.1 mL. Control animals are injected with FCA/water. Injections are repeated every 4 days until three injections are given. The minimal irritating and maximum nonirritating concentrations following topical application of 0.025 mL solutions to a 2 cm^2 area of skin are determined in a minimum of four naive guinea pigs (see OET procedure). Twenty-one days after the first induction injection, 0.025 mL of the minimal irritant concentration, the maximum nonirritant concentration, and two lower concentrations is applied to 2 cm^2 areas of the shaved flank. Test sites are not covered and are evaluated for the presence of erythema at 24, 46, and 72 h after application. The minimum nonirritating concentration in FCA/water-treated controls is determined. Animals injected with the test material during induction that respond to lower doses are considered sensitized. The incidence of sensitization and the threshold of concentration for elicitation of the response in these animals are calculated.

Optimization Test

The optimization test resembles the DT but incorporates the use of adjuvant for some induction injections and both intradermal and topical challenges [166,167,217]. Injections during induction are 0.1 mL of 0.1% concentration of test material in 0.9% saline or in 50/50 FCA/saline. In total, 10 injections are given. On day 1 of the first week, one injection into the shaved flank and one into a shaved area of dorsal skin are given. Two and four days later, one injection into a new dorsal site is given. The test material is administered in saline during the 1st week. During the 2nd and 3rd weeks, test material is administered in FCA/saline every other day to a shaved area over the shoulders. Twenty test animals are treated; 20 controls are injected with saline during week 1 and FCA/saline during weeks 2 and 3. The intensity of the 24 h responses during week 1 is calculated as reaction volume. Thickness of a skin fold over the injection site is measured with a caliper (mm), and the two largest diameters of the erythematous reaction are recorded (mm). The reaction volume is calculated by multiplying fold thickness times both diameters and is expressed as microliters. The mean reaction volume of each animal to the intradermal injections using saline as a vehicle (week 1) is calculated. Animals are challenged with 0.1 mL of 0.1% test material in saline 35 days after the first injection. The challenge reaction volume for each animal is calculated and compared to the mean reaction volume for that animal. Any animal developing a reaction

volume at challenge greater than the mean plus one standard deviation during induction is considered sensitized. Vehicle control animals are injected with saline at challenge. A second challenge is conducted 45 days after the first injection. A nonirritating concentration of the test material in a suitable vehicle is applied to the flank skin, away from injection sites; 0.05 mL is applied to an area of approximately 1 cm^2.

The area is covered with a 2 × 2 cm piece of filter paper backed by an occlusive dressing, which remains in place for 24 h. Reactions are visually evaluated using the four-point erythema scale of the Draize primary irritancy scale (see Table 27.9). The control animals are patched with vehicle alone. The number of positive animals in the test group is statistically compared with the number of pseudo-positive animals in the control group using the exact Fisher test. Separate comparisons of intradermal and epicutaneous challenges are made. A p-value of ≤0.01 is considered significant. To classify materials as strong, moderate, weak, or nonsensitizers, a classification scheme has been devised using results of the exact Fisher test and number of positives detected (Table 27.5)

Split Adjuvant Test

The split adjuvant test [166,200,201] uses skin damage and FCA as adjuvants; application of the test material is topical. An area of back skin just behind the scapulas of 10–20 guinea pigs is clipped, shaved to glistening, then treated with dry ice for 5–10 s. A dressing of a layer of loose mesh gauze and stretch adhesive with a 2 × 2 cm opening over the shaved area is placed around the animal and secured by adhesive tape. This dressing remains in place throughout induction. Approximately 0.2 mL of creams or solid test material or 0.1 mL of liquid is spread over the test site and covered with two layers of #2 filter paper backed by occlusive tape and attached to the dressing by adhesive tape. The concentration tested varies by irritancy potential, use conditions, etc. Two days later, the filter paper is lifted from the test site, the test material is reapplied, and the filter paper covering is replaced. On day 4, the filter paper cover is removed; two injections of 0.075 mL FCA are given into the edges of the test site, the test material is reapplied, and the site is resealed. On day 7, the test material is reapplied, and on day 9, the dressing is removed. Twenty-two days after the initial treatment, animals are challenged by topical application of 0.5 mL of test material to a 2 × 2 cm area of the shaved mid-back. The test site is covered by filter paper backed with adhesive tape, held in place by wrapping the animal with an elastic adhesive bandage secured with adhesive tape. A group of naive controls (10–20 animals) is treated by the same procedure at challenge. The dressing is removed 24 h after application, and the test site is evaluated visually at 24, 48, and 72 h, using a seven-point descriptive visual scale. Sensitization of individual animals is indicated by significantly stronger reactions than reactions of controls.

Guinea Pig Maximization Test

The guinea pig maximization test (GPMT) [166,198,199,316] combines FCA, irritancy, intradermal injection, and occlusive topical application during the induction period. The shoulder regions of two groups of 20–25 guinea pigs are shaved. Two identical sets of intradermal injections of 0.1 mL 50/50 FCA/water; test material in water, paraffin oil, or propylene glycol; and the same dose of test material in FCA/vehicle are placed in a 2 × 4 cm area. Seven days later, the test article is placed on filter paper over the injection site. The filter paper is covered with approximately 4 × 8 cm occlusive surgical tape and secured in place with an elastic bandage wrapped around the animal. If the test material is nonirritating, the test site is pretreated with 10% sodium lauryl sulfate (SLS) in petrolatum on day 6 to provoke an irritant reaction. If a vehicle other than petrolatum is used for topical application of the test material, the filter is saturated with the solution. Control animals are patched with the vehicle alone. The dressing is removed from the animals 48 h after application. Test and control animals are challenged on the shaved flank with the highest nonirritating concentration, with approximately one half of the highest nonirritating concentration, and with the vehicle. Solutions are applied to 1 × 1 cm pieces of filter paper secured in place as during induction; patches are removed 24 h later. The challenge area is shaved, if needed, 21 h after patch removal. Reactions are evaluated visually 24 and 48 h after patch removal. The intensity of responses to test material and vehicle in the test group is compared to the responses in controls. Reactions are considered positive when they are more intense than the response to vehicle and the responses to the test material in controls. Based on the incidence of positives in the test group, test materials are rated as weak to extreme sensitizers (Table 27.6).

TABLE 27.5
Classification Scheme for the Optimization Test

Interdermal Positive Animals (%)	Epidermal Positive Animals (%)	Classification
NS, 0–30	NS, 0	Nonsensitizer
S, 30–50	NS, 0–30	Weak sensitizer
S, 50–75	S, 30–50	Moderate sensitizer
S, >50	S, >75	Strong sensitizer

Note: S, statistically significant; NS, not statistically significant by exact Fisher test.

TABLE 27.6
Classification of Materials by Maximum Test

Sensitization Rate (% Responding at Challenge)	Grade	Classification
0–8	I	Weak
9–28	II	Mild
29–64	III	Moderate
65–80	IV	Strong
81–100	V	Extreme

Sensitization Tests in Mice

Although guinea pigs have been the animal of choice for predictive sensitization assays for 50 years, interest and activity in developing and validating standardized predictive assays in mice have been intensive during the last 10 years. Classical guinea pig sensitization assays are relatively costly and time consuming. All use subjective endpoints, and data interpretation is prone to difficulties. Manipulations of the animals are sufficiently stressful in some assays to alter normal physiological parameters. With the development of new techniques for studying DTH [36] and evaluating cellular response [115], it has become possible to study the response in other laboratory animals [5,64,65] in shorter time frames. Numerous less subjective techniques for evaluating the allergic response have been proposed, including changes in the water content of challenged ears [36], measurement of ear thickness with an engineer's micrometer [7,307], and responses of lymphocytes [7,222,223,253]. Although numerous approaches to developing a predictive sensitization assay in mice have been proposed, only two methods have been sufficiently developed to warrant consideration as standardized assays. The test site for each is the mouse ear, yet these methods employ distinctively different approaches. The mouse ear swelling test (MEST) uses both topical and injection exposures for induction and a topical challenge of the pinnae in which visual evaluation is replaced by measuring ear thickness with an engineer's micrometer. The LLNA consists of a topical induction followed by measurement of the mitotic activity of the draining lymph node. LLNA is unique out of all predictive assays in evaluating the response of the efferent phase of the response. A third mouse sensitization assay, the vitamin A enhancement test (VAET), has been used for the evaluation of ingredients of consumer products but has not been used by a sufficient number of laboratories to be considered standard. In the VAET, the reactivity of the immune system is heightened by maintaining animals on a diet with high doses of vitamin A for a preconditioning period and throughout induction and challenge. Challenge is topical and is assessed by measuring ear thickness. These assays are contrasted in Table 27.7.

Local Lymph Node Assay

Kimber and colleagues [162–164,238] investigated measuring lymphocyte proliferation as an alternative approach to visual evaluations or measurement of edema of the mouse ear. They found that exposure of the dorsum of the ear pinnae to sensitizers produced hyperplasia of T cells in auricular lymph nodes. A dose divided into three exposures over three

TABLE 27.7
Principal Features of Human Sensitization Assays

Feature	Complete Schwartz–Peck	Shelanski–Shelanski	Repeat Insult Patch Tests Draize	Griffith–Voss–Stotts	Modified Draize	Human Maximization
Number of subjects	200	200	200	200	100–200	25
Induction						
Exposure site	Upper arm	Upper arm, same site	Upper arm or back; naive site for each exposure	Upper arm, same site	Upper arm or back Same site	Upper arm or lower back Same site
Number of exposure	1	15	10	9	10	5
Duration of exposures	24–72 h	24 h	24 h	24 h	48–72 h	48 h
Frequency of exposure	—	3 per week	3 per week	3 per week	3 per week	24 h between patches
Evaluation schedule	At removal, 24 h, 48 h	At removal	At removal	48–72 h	30 min after removal	Before each application
Miscellaneous	4-week usage period	Fatiguing index	Pilot group	Continuous exposure	SLS/irritation adjuvant	Rest period duration
Challenge						
Exposure site	Upper arm	Upper arm	Upper arm or back	Upper arm	Upper arm or back	Lower back, upper arm
Duration of exposure	24–72 h	48 h	48 h	24 h	72 h	SLS 1 h; 48 h
Evaluation schedule	At removal, 24 h, 48 h	At removal	At removal	48 h, 96 h	At removal, 24 h	At removal, 24 h, 48 h
Miscellaneous	—	—	Naive test site	Original and naive test sites	Naive test site; may use two 48 h exposures	Sensitization index

Note: SLS, sodium lauryl sulfate 5% for induction adjuvancy and 10% at challenge.

consecutive days produced a more intense response than the same dose delivered in a single exposure [165]. In a limited trial, Balb/c mice were more sensitive than other strains [164]. Initial studies used 24 h cultures of excised lymph node cells labeled with ^{3}H-thymidine, with or without exogenous IL-2 [163,164]; however, to simplify the assay, the method was modified to expose proliferating cells to ^{3}H-thymidine in situ via intravenous injection. The method described is the final method used in intralaboratory validation studies that have been reported [13,276]. A complete list of references was made available in 1999 by an independent review evaluation of the method sponsored by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [233]. Several new reviews have recently been published [346–348].

The LLNA employs multiple topical in vivo doses of the material of interest to the mouse ear. This is followed by in vitro evaluation of mitotic activity of cells from draining lymph nodes. At least three dose levels are evaluated in separate groups of four CBA/ca mice. Experimental animals between 8 and 12 weeks of age are used. Either males or females may be used, but each assay should use only a single sex.

Vehicle selection and test concentration are based primarily on the solubility and viscosity of the solution or suspension. Investigators ascertain that the doses selected are nontoxic to the animals. Vehicles shown to be acceptable include 4:1 acetone/olive, methyl ethyl ketone, dimethylformamide, propylene glycol, dimethyl sulfoxide, and 2.5% hydroxypropyl cellulose in methanol. Investigators have proposed testing three consecutive concentrations from the series 100%, 50%, 25%, 10%, 5%, 2.5%, 1.0%, 0.5%, 0.25%, 1.0%, 0.05%, and 0.001%.

For three consecutive days, 25 μL of the appropriate test solution or the vehicle alone is applied to the dorsal surface of the pinnae. Five days after the first exposure, 250 mL phosphate-buffered saline (PBS) containing 20 μCi methyl thymidine is injected via the tail vein of each animal. Five hours after injection, animals are euthanized by carbon dioxide asphyxiation. Draining auricular lymph nodes are excised and pooled with nodes from other animals in the same group. A single-cell suspension is prepared by gently passing the nodes through stainless steel, 200-mesh gauze with the plunger of a syringe. The cell suspension is centrifuged at 190 × g for 10 min, and the pellet then washed twice with 10 mL PBS. Cells are resuspended in 3 mL of 5% trichloroacetic acid (TCA) and incubated overnight at 4°C. The precipitated macromolecules are recovered by the centrifugation, the supernate is removed, and the precipitate is resuspended in 1 mL 5% TCA. The suspension is transferred to 10 mL scintillation fluid, and disintegrations/min are counted using a β scintillation counter. Disintegrations per lymph node are calculated for each experimental group and the control group. The ratio of ^{3}H-thymidine incorporation into the test group and the control is calculated for each dose. Some investigators prefer to pool the lymph nodes from all animals in the dosage group. If the ratio is 2–3 for any dose, the material is considered a sensitizer.

Several groups have reported comparisons of the LLNA with various guinea pig assays and have suggested variations of the method [11,12,144,161,162,276]. It is clear that LLNA is not as stringent as some guinea pig assays; however, it is expected to retain utility as a rapid screening assay for materials with strong sensitization potential, as it offers advantages in the low number of animals used, lower cost, and less time required for conducting the assay. A validation meeting to review the strengths and weaknesses of the assay has been reported [233]. Certain materials, such as metals, have not been reliably identified as allergens. The validation attempt matched LLNA results with guinea pig Buehler and maximization assays. Both have false positives and false negatives. No attempt was made to determine the clinical relevance of the LLNA data, nor the benchmarks used. Key to the clinical relevance of this assay will be a more precise series of databases that permit clinical collaboration between the LLNA and human experience; that is, how does one relate the LLNA data with the frequency of allergic contact dermatitis in humans?

Mouse Ear Swelling Test

Gad and coworkers [113,114] used ear thickness measured with a caliper-type engineer's micrometer to evaluate the response of mice after challenge with potential sensitizers. They optimized a protocol in which tape-stripped skin sites that had been injected with FCA were topically exposed to the test material each day for 4 days. Seven days after the last topical exposure, animals were challenged by topical application of the test material to one ear. Early work also showed that Balb/c, CF-1, or SW mice could be used in the assay. Females were selected because their less aggressive behavior allows group housing with minimally damaged ears. Responses of animals less than 5 weeks old or more than 13 weeks old were weaker than animals 6–10 weeks of age. Administration of induction doses to the stomach region yielded a higher rate of sensitization than application to the back of the animals. The efficacy of the induction method was increased somewhat by both tape stripping and preinjection of the test site with FCA. Exposure via occlusive patches during induction did not increase the sensitivity of the assay, and in some cases, the response was diminished when the patching system was employed. Their final protocol incorporated these findings.

For the MEST, 6- to 8-week-old CF-1, Balb/c, or SW female mice are gang housed in direct bedding cages. Following a 5- to 7-day quarantine period, the fur of the abdomen is shaved by electric clippers from 10 to 15 test animals and 5 controls. Animals with damaged pinnae are excluded from testing at this time. After the area is shaved, it is tape stripped with Dermaclear®, a surgical adhesive tape, until the skin appears glossy. Then a divided dose of 0.05 mL FCA is injected intradermally with a tuberculin syringe fitted with a 30-gauge needle. The FCA is injected into two sites within the shaved area but along the borders. Following the injection of adjuvant, 100 μL of vehicle containing the test material or vehicle alone (controls) is applied to the center of the shaved area. The abdomen is allowed to dry, and the mouse is then returned to its cage. The process of

tape stripping and application of appropriate material to the abdomen is repeated daily for the next 3 days.

Vehicle is chosen based on solubility and chemical compatibility with the test substance. Acetone, methyl ethyl ketone, or 70%, 80%, and 95% ethanol in water have been shown to be acceptable vehicles. Mixed ethanol/olive oil systems are not satisfactory. The dose selection is based on dermal irritation toxicity range-finding studies prior to testing each compound. Four groups of two mice each are subjected to the induction procedure, including shaving, tape stripping, and application of the test material, and the ears are then exposed as during challenge. At least four concentrations are evaluated in the range study. Minimally irritating or nonirritating concentrations are selected for the induction application. The highest nonirritating concentration is used at challenge.

Challenge is performed 7 days after the last topical induction application by applying 20 μL of the test material in vehicle to one ear of each animal (test and control) and 10 μL of the vehicle to the opposite ear. Ear thickness is measured before application of the challenge dose and 24 and 48 h after the challenge application. Animals are lightly anesthetized with ether, and the thickness of both pinnae is measured with an engineer's micrometer. Positive respondents are defined as animals in which the ear dosed with the test material shows at least a two- to threefold greater increase in thickness than the vehicle-treated control ear. The control group should not show greater than 10% increase in ear thickness for the test to be considered valid. If the control groups show more than a 10% increase, the study should be repeated using lower doses. The percentage of respondents is calculated. In addition, the degree of ear swelling is calculated by dividing the thickness of the ear to which the test material was supplied by the thickness of the vehicle-treated control ear. Measurements from all animals in the test group are included. No additional explanations or examples of the use of the degree of ear swelling in interpreting results are available. A later paper proposed that the data generated by MEST (and classical guinea pig assays) could be used to calculate a potency index of sensitization [112].

The original paper of Gad et al. [114] included validation studies of 72 compounds. They reported a false-negative rate of only 2% and no false positives when MSET was compared to GPMT data on the same materials. The incidence of sensitization in MSET was consistently lower than that produced by GPMT. Similar findings were reported for comparisons between the Buehler assay and MEST in the same paper; however, published guinea pig data used for comparisons included data from other topical guinea pig techniques that did not employ the restraint procedure specified by Buehler. Intralaboratory validation of the method includes a comparison of test results for eight materials tested in five laboratories [114]. That report indicates that MSET did not identify weak sensitizers in two of three laboratories. Other studies have confirmed that the incidence of positive response in MEST is consistently lower than in GMPT, and weak and moderate sensitizers are not identified correctly [63,78]. The MEST has been accepted by the Environmental Protection Agency (EPA) for registration of chemicals under the Toxic Substances Control Act [59].

Although not reported in papers describing this method, the type of micrometer used may affect interpretation of the test results. Van Loveren [309] compared the use of spring-loaded, caliper-type instruments with screw and friction thimble micrometers. Spring-loaded instruments were best. Electronic instruments have been used in other types of immunological investigations. In our experience, the use of anesthetic can be eliminated by operator training prior to handling animals.

Vitamin A Enhancement Test

Miller and colleagues [206,209,221] decreased the dose of strong sensitizers required to induce sensitization by maintaining the animals on diet supplemented with high levels of vitamin A acetate. The mechanism of the increase in response was studied using radioisotopes, but because of ease in performing the assay, ear thickness measurement was selected for use in the predictive assay. Principal features of the test included a preconditioning period for the diet of 28 days, six exposures to the shaved abdomen and thorax during the 12-day induction period, and challenge 4 days later. Results from the test group were compared statistically to those of the controls, and a 50% increase over the response to controls was observed that indicated sensitization. The minimally irritating concentration was used for induction, and the highest nonirritating dose was used at challenge (determined by dose response in separate groups of mice). The vehicle was selected based on nonirritancy and solubility of the test substance. An obvious difficulty with the method was the long conditioning period required prior to the study. General comments concerning choice of micrometer for MEST also apply to VAET. The test was never widely adopted or submitted to formal validation procedures.

Human Sensitization Assays

Chemicals can be tested for their ability to induce contact hypersensitivity in panels of human volunteers from whom informed consent is obtained. Human studies should be undertaken only after the results of predictive tests in animals are available if the test material is a new compound or if it contains significantly increased levels of common ingredients. Testing higher doses in animals provides some margin of safety for potential human subjects. Generally, materials identified as sensitizers in animals are not tested on humans; however, if the potential benefit of the material warrants, a small group of human subjects may be tested with materials inducing sensitization in animals. Such situations should be reviewed by an institutional review board (IRB). Test subjects should be informed of the risks, and the number of subjects should be limited (additional subjects can be exposed if members of a small group do not respond).

Subjects should be randomly selected; however, some precautions are indicated. Some investigators believe intact draining lymph nodes adjacent to the application site are

the preferred site to induce sensitization, and patches should not be placed on areas adjacent to mastectomies. Persons with unilateral mastectomy who wish to participate may be tested by applying patches to the opposite sides of the body. Test materials should not be applied on scar tissue. Recurrence of skin conditions in remission (e.g., psoriasis and eczema) has been associated with patch testing and other minor physical traumas. Subjects at risk should be informed of this possibility and encouraged to consult their dermatologist prior to testing. Subjects should not be tested with materials to which they are known to be allergic (demonstrated by diagnostic patch test or in previous predictive assays). It is prudent to routinely question potential subjects concerning their history of dermatologic disease and allergies. Allergic contact dermatitis to materials already in commerce is sometimes detected by early induction patches. This indicates that, under patch conditions, the material may elicit a response in presensitized individuals. Although the incidence of preexisting sensitization to the material should be considered in risk assessment decisions regarding marketing, detection of preexisting sensitization may not be helpful in evaluating the ability of a material to induce sensitization. Records of previous responses of individuals participating in multiple predictive assays should be reviewed prior to testing to eliminate subjects presensitized to components of the product.

Although numerous variations have been reported, four basic predictive human sensitization tests are in current use: (1) single induction/single challenge patch test, (2) repeated insult patch test (RIPT), (3) RIPT with continuous exposure (modified Draize), and (4) maximization test. The principal features of human sensitization assays are summarized in Table 27.7. As originally described, all methods used customized patches, and patch selection was governed by available adhesive systems. Description of customized patches would be of historical interest only; as currently conducted, most human assays use similar patches. Occlusive patches, consisting of a nonwoven pad (usually Webril®) of four-ply gauze sponges backed by an occlusive surgical tape, such as Blenderm™, are available commercially or may be custom made in strips of four or five pads. Acceptable alternatives include the Hill Top Chamber® [157], which contains a Webril pad inside an occlusive plastic disk backed by porous tape; the Duhring chamber [109], a stainless steel disk that contains a Webril pad; and the large Finn Chamber®. Duhring and Finn chambers usually are secured in place by porous surgical tape. Occasionally, semiocclusive patches made of Webril backed by porous tape may be used. Semiocclusive patches are decidedly inferior to occlusive patches in the induction of sensitization.

For assays other than maximization, usually 150–200 subjects are tested. Henderson and Riley [136] showed statistically that, if no positive reactions are observed in 200 randomly selected subjects, as many as 15/1000 of the general population may react (95% confidence). As the sample size is reduced, the likelihood that the test will not correctly predict adverse reactions in the general population increases.

Results of the RIPT, modified Draize test, and human maximization tests have been accepted as valid by regulatory agencies; however, some sponsors routinely use one of the methods described and defines its use as the *standard of the industry*. This is a simplistic view of the methods and their strengths and weaknesses. As for most toxicity endpoint, the method used should provide a reasonable exaggerated exposure over the anticipated exposure from use of the product. The device group of the FDA held several meetings to review details of sensitization procedures. These deliberations led to a guidance document [55] for evaluating skin sensitization to chemicals in natural rubber products. The modified Draize procedure is recommended. Tests guidance for transdermal products evaluated by the FDA is available on the FDA website (www.fda.gov/ohrms/dockets/98fr/990236Gd.pdf).

Schwartz–Peck Test (and Modifications)

A single application induction patch followed by a single application patch test was described by Schwartz [277,278] and Schwartz and Peck [279] with a usage test of 1 month after challenge to verify patch results. The test has been modified by some to eliminate the usage test [43], to eliminate patching altogether, and to place a usage period between induction and challenge patches [299]. The term *complete Schwartz–Peck method* refers to a single induction patch, usage period, and single challenge patch test. This may also be referred to as the *Traub–Tusing–Spoon method*. Incomplete Schwartz–Peck tests do not incorporate a usage period. A patch saturated with the test material, diluted if necessary, is applied to the outer upper arm of 200 test subjects and remains in place for 24–72 h. The dose tested and duration of patch contact vary with intended use. Cosmetics may be tested without a covering (open application) or with semiocclusive patches. The test site is visually evaluated at patch removal and at 24 and 48 h after removal for erythema and edema. A 4-week normal usage period follows the induction patch in the complete Schwartz–Peck test, with a challenge patch applied to the same site on the upper arm at the conclusion of the usage period. For the incomplete Schwartz–Peck test, a second patch procedure is performed 10–14 days after the induction patch. Duration of contact and evaluation of the site are similar to those during induction. The development of dermatitis at challenge that is much stronger than during induction signifies sensitization.

Schwartz originally described the incomplete Schwartz–Peck test. A usage test was to be conducted after the challenge patch using 1000 different subjects. Although Schwartz and Peck referred to their assay as a *prophetic patch test*, experience has shown that only potent haptens will induce sensitization in this assay [169]. In fairness, it should be noted that the test was originally designed to evaluate the effect of nylon garments on the skin. It was intended to detect adverse effects, irritation, and secondary irritation (sensitization). The mechanism of skin allergy was not understood when the test was designed. Although the test was useful for its original purpose, unfortunately, its use was expanded without considering new information generated by immunologists. Clearly, the assay

is inferior to all other predictive human sensitization assays; however, a few groups continue to use the method.

Repeat Insult Patch Test

Three major variations on the RIPT are in common use: (1) the Draize human sensitization test [74,75], (2) the Shelanski–Shelanski test [279–281], and (3) the Voss–Griffith test [45,122,123,315]. Although the Shelanskis first published a description of a RIPT, they based its development on a verbal description of a method Draize was revising [281]. Voss modified the Shelanski–Shelanski test [316], and his assay was later modified by Griffith [123]. As one would expect, the three assays have much in common. However, there are significant differences in the assay as originally described. Several groups at the FDA evaluated various RIPT protocols, leading to guidances that are published on their websites (see www.FDA.gov, medical devices section).

In the Draize human sensitization test, an occlusive patch containing the test material is applied to the upper arm or upper back of approximately 200 volunteers. The patch remains in place for 24 h and is then removed. The test site is evaluated at patch removal for erythema and edema. A second patch test is applied to a new site 24 h after the first patch is removed. This process is repeated until 10 patches are applied. For convenience, the test may be run on a Monday-to-Friday schedule, with subjects removing their own patches on Saturday (72 h between Friday and Monday applications). Ten to fourteen days after the application of the last induction patches, subjects are challenged via a patch applied to new sites. Duration of contact is 24 h, and sites are visually evaluated at removal of the patch. The response at challenge is compared to the responses to patches applied early in induction, and the incidence of sensitization is reported.

Like the Draize RIPT, the Shelanski–Shelanski test employs occlusive patches that remain in contact with the skin of the upper arm for 24 h. The patching cycle is the same; however, patches are placed on the same test site each time, and a total of 15 sets of patches are applied during induction. The test site is evaluated before application of a new patch to the site. If inflammation has developed, the patch is placed on an adjacent uninflamed site. Two to three weeks after the induction period, subjects are challenged by the application of a patch that remains in place for 48 h. Test sites are evaluated at patch removal for erythema and edema, and the incidence of positive response is reported. Patch responses during induction were considered by Shelanski and Shelanski to be evidence of *skin fatigue* (cumulative irritation); the time of development (i.e., number of patches) was reported as a fatigue index. Voss [316] reduced the number of 24 h patch exposures to nine over a 3-week period. At challenge, 2 weeks after the last induction patch, duplicate patches applied to the original test site are worn for 24 h. Patch sites are evaluated 48 and 96 h after patch application. A pilot group of 10–12 subjects was tested prior to exposing the full panel of 60–70 subjects.

Griffith later published more method details [122,123]. A maximum of four dissimilar materials was tested simultaneously, and duplicate challenges were applied to the sites of induction and to the opposite arm, thus testing areas drained by different regional lymphatics. The concept of a rechallenge of subjects with reactions difficult to interpret was also introduced. The number of subjects was increased to 200 by conducting tests on multiple panels. Stotts [292] presented detailed examples of proper interpretation of human repeat insult patch tests. Sensitization is characterized by challenge reactions stronger than reactions early in the induction phase, by persistence of responses through delayed readings, by delayed appearance of response, or by weak responses in a few subjects when the material has not produced irritation in the panel. Examples of patterns of responses indicating presensitization and weak responses that warrant rechallenge are also presented in the paper.

As currently conducted, the differences in the Draize and Voss–Griffith RIPTs are minimal. Many investigators apply patches to the same site during induction and refer to the procedure as a Draize RIPT. The value of multiple grades at challenge is widely recognized. Multiple test materials are tested simultaneously in all RIPTs for reasons of efficiency and economy. Although the distinctions between the Draize and Voss–Griffith procedures have blurred with common usage, the Shelanski–Shelanski test, with five to six more induction applications, remains distinct.

Marzulli and Maibach [215,216] have suggested modifications to the RIPT that provide advantages in compliance. Instead of having the volunteers remove the patch, laboratory staff performs this function. Typical application remains at thrice weekly, but the patch remains in place for 48, 48, and 72 h cycles. Hence, chemical/skin exposure is continuous for 3 weeks rather than three 24 h exposures per week. An additional modification involves dosing several groups at ascending doses to allow the determination of the minimal induction concentration.

Human Maximization Test

Kligman [170] reviewed the common human predictive sensitization test methods in use in 1966 and found them to be unsatisfactory in inducing sensitization to nine clinical allergens. In panels of 200 subjects, the Shelanski–Shelanski method induced sensitization to four materials, the original Draize test and the complete Schwartz–Peck test induced sensitization to two allergens each, and the incomplete Schwartz–Peck test failed to induce sensitization to any allergen. Kligman concluded [171] the following:

- Emphasis must shift from prophecy to the more practical objective of identifying potential allergens.
- Once the allergenic potential is known with reasonable certainty, a judgment of risk might be ventured after examining all the pertinent variables.

This represented a profound change in the intent of predictive sensitization assays. Based on his studies of factors affecting rates of sensitization in predictive assays [168,175], Kligman designed the human maximization test [175].

He later modified the procedures somewhat to reduce the difficulties in performing the test and in interpreting the test [175]. The maximization test uses irritancy as an adjuvant. During induction, irritating compounds are tested at a concentration that produces moderate erythema within 48 h. If materials are nonirritating, the test site is pretested with a 24 h patch of 5% SLS. Additional pretreatment SLS patches may be applied before each patch application until a brisk erythema is produced. Induction concentrations are at least five times higher than use levels; petrolatum is a preferred vehicle. Often, custom-made Webril/Blenderm™ patches or plastic or metal chambers are used. Patches are applied to the outer aspect of the arm or lower back, and up to four dissimilar materials may be tested at one time. Wrapping with extra tape is often necessary to ensure occlusion. Bandage sprays may be used to ensure sealing of the test site. Five sets of patches are worn on the same site for 48 h each, with a 24 h rest period between removal and reapplication.

Following a 2-week rest period, an SLS provocative patch is applied to prepare the skin for challenge. A patch saturated with a 2.5%–5.0% solution of SLS is applied to previously untreated sites on the lower back. SLS concentration is based on the season and on individual subject response. The SLS patch is removed after 1 h, and a patch containing the test material is applied. A control site is patched with SLS (1 h) and petrolatum (48 h) to aid in interpretation of the results. The patch is removed 48 h after application, and the test sites are evaluated. Test sites are reexamined 24 and 48 h after patch removal. The number of subjects developing a positive response is reported, and a sensitization index based on percentages of subjects responding is assigned to the test material. In common practice, the human maximization procedure is performed on either the outer upper arm or the back. Although it is clear that the maximization test is a sensitive tool for the detection of allergenicity, the skin damage produced is dramatic and unacceptable to many subjects. This assay is little used today for this reason and because the 25 subject panel has been shown incapable of identifying some allergens.

Modified Draize Human Sensitization Test

The RIPT procedure was modified to provide for continuous patch exposure. Materials are applied to the outer upper arm each Monday, Wednesday, and Friday until 10 patches have been applied during a 3-week period [213,214]. Patches remain in place until approximately 30 min before the application of a fresh patch. This brief rest period allows some clearing of responses to tape and facilitates grading. Fresh patches are applied to the same site unless moderate inflammation has developed; the patches are placed on adjacent, noninflamed skin, should inflammation become pronounced. This produces a continuous exposure of 504–552 h (some investigators apply only nine patches) compared to a total exposure period of 216–240 h for RIPT of comparable induction applications. In addition, induction concentration was increased to levels above usage exposure. Two weeks after induction, subjects are challenged by exposure of a new site to a patch of 72 h duration at a nonirritating concentration. Test sites are evaluated at patch removal and 24 h after removal. Jordan and King [155] proposed modifying the challenge procedure to two consecutive 48 h patch periods. The modified Draize test has been selected as the test of choice for chemicals in natural rubber products [55]. However, numerous causes of false positive predictive allergenic contact dermatitis exist and are beyond the province of this chapter. Lachapelly and Maibach [358] provide extensive information that helps separate false and true positives.

In Vitro Assays for Allergic Contact Dermatitis

As our understanding of cell biology of DCH has increased, in vitro assays to replace diagnostic patch testing and as early screening predictive assays have been proposed. Much of the work toward diagnostic procedures has centered on measuring the effect of cytokines released by sensitized lymphocytes on target cells as a marker for allergy. Proposals for in vitro predictive assays have focused on the afferent phase of antigen binding and stimulation of target cells. To date, neither approach has proved satisfactory, but some limited experimental successes have been reported.

Migration inhibition of peritoneal exudate cells from capillary tubes has been reported to be inhibited by the antigen to which donor guinea pigs were sensitized [116]. Inhibition of macrophage migration is medicated by soluble factors produced by sensitized lymphocytes in the presence of sensitizing antigen [76]. This factor, identified as migration inhibition factor (MIF), has been shown to aggregate macrophages, increase macrophage adherence to glass, and decrease macrophage mobility [70]. Rocklin et al. [269] reported that MIF was produced by highly purified populations of proliferating T cells. Moorehead et al. [230] later demonstrated that MIF production is dependent on Ia/T cell subsets. Mitogenic factors, such as phytohemagglutinin, initiate mitotic activity and transform lymphocytes in their blast forms. Pearmain et al. [255] demonstrated that tuberculin produced the same effect in peripheral blood leukocyte cultures from sensitive patients but not from unsensitized patients. Similar work experiments were conducted in nickel-sensitive subjects [6]. Lymphocytes from unsensitized controls also underwent blast transformation when exposed to nickel. The nonspecificity of nickel and mercury transformation was confirmed by numerous investigators [248,253,258]. Using ^{14}C-thymidine uptake to measure blast transformation, MacLeod et al. [193] demonstrated transformation in over half of the nickel-sensitive subjects and no unsensitized controls.

Experimental studies of blast transformation in which animal or human subjects were intentionally sensitized have been somewhat more successful. Investigators [115,222] have demonstrated that lymphocytes from guinea pigs sensitized with DNCB and incubated with dinitroflurobenzene (DNFB) were transformed to a greater degree than cells from unsensitized controls (note that a dinitrophenyl group would attach to protein using either material). Tritiated thymidine was used to measure blast formation when exposed to DNFB conjugated to epidermal protein. Similar responses were demonstrated

using human volunteers and epidermal extracts for conjugation [223]. Miller and Levis [222] produced the same effect using leukocyte and erythrocyte membranes for conjugation.

Cytotoxicity consistent with that produced by lymphotoxin has been demonstrated in experimentally induced allergic contact dermatitis [72]. Peripheral lymphocytes from DNFB-sensitized guinea pigs were incubated with DNFB-coated radiolabeled chick erythrocytes. Increased radioscope leakage was produced by lymphocytes from sensitized animals than produced by controls. Similar effects were demonstrated using epidermal cells [295].

The use of exclusively in vitro assays for predicting sensitization potential of various schemes has been proposed. In one hypothetical system [36], binding to Langerhans cells would be measured. If no binding occurred, the activation state of the Langerhans cell would be evaluated. If no activation was detected, the material would not be considered to induce sensitization. If Langerhans cells were activated, an autologous lymphocyte blastogenesis assay would be preformed. Current commercial activity in producing skin recombinants may make this type of approach feasible. Blomberg et al. [31] reviewed the use of in vitro assays to study mechanisms of contact dermatitis. There is great enthusiasm for developing such assays to serve as substitutes for testing on animals and humans [349,350]. Taken together, the extensive experimental literature on in vitro assays provides hope that one of several of these methods will not only be *validated* but also sufficiently developed to provide not only hazard information but information to support risk assessment of such materials as well.

Skin Irritation and Corrosion

Historically, skin irritation has been described by exclusion as localized inflammation not medicated by sensitized lymphocytes or antibodies (e.g., that which develops by a process not involving the immune system). The application of some chemicals destroys tissues directly, producing skin damage (including necrosis) at the site of application. Chemicals producing necrosis that result in the formation of scar tissue are described as *corrosives*. Chemicals producing inflammation after a single exposure are termed *acute irritants*. Some chemicals do not produce acute irritation from a single exposure but may produce inflammation following repeated application to the same area of skin. The cumulative irritation from repeated exposures was originally called *skin fatigue*, but the term *cumulative irritation* prevails [279]. Because of the possibility of skin contact during transport and the wide use of many chemicals, regulatory agencies have mandated that chemicals be screened for their ability to produce skin corrosion and acute irritation. These studies have been conducted in animals using standardized protocols; however, recent efforts to replace animal studies with in vitro or human assays have had some success. It is not appropriate to conduct screening studies for several acute irritation or corrosion in humans. Acute irritation can be evaluated in humans after animal studies have shown that the risk of systemic toxicity is low and if the material is known to be noncorrosive. Tests for cumulative irritation in both animals and humans have been reported. In general, cumulative irritation is evaluated in humans unless the toxicity of the material necessitates testing in animals (pesticides, industrial chemicals).

The processes that result in any form of skin irritation are not well understood. In addition to destroying tissue directly, chemicals can disrupt cell functions or trigger the release, formation, or activation of autocoids. Autacoid that are generated following exposure of tissue to some chemicals produce low increases in blood flow, increase vascular permeability, attract white blood cells in the area, and damage cells indirectly. The additive effects of the autocoid mediators would result in local skin inflammation. No agent has yet met all the criteria to establish it as a mediator of skin irritation [261]; however, histamine, 5-hydrotryptamine, prostaglandins, leukotrienes, kinins, complement reactive oxygen species, and products of white blood cells [246] have been strongly implicated as mediators of some irritant reactions.

We have studied the process by which chemicals produce acute skin irritation following open topical applications to the ear of the mouse [228,250,252]. The time course of the inflammatory responses varied from compound to compound and was independent of vehicle and applied dose. Because the differences in time course could not be attributed to differing rates of penetration, this suggested that the materials tested triggered different inflammatory processes. Differences in the irritation process triggered by three chemicals were confirmed by histology, albumin leakage, and changes in the rate of blood flow. Using a series of pharmacological antagonists of putative mediators of irritation, enzyme inhibitors to prevent the formation of suspected mediators, and agents that deplete the body of serum mediators, we confirmed that different pathways of mediator involvement existed for skin irritation. The implications for this finding are clear. A battery of in vitro assays would be required to screen materials for skin irritation; no single assay would be effective.

Many factors may modulate the development of skin irritation (Table 27.8). As with DCH, these factors have been classified as extrinsic or intrinsic [186,205,216,333]. Some extrinsic factors have been shown experimentally to be important considerations in designing predictive tests for skin irritation. A few investigators have also considered intrinsic variables when designing studies.

Like other toxic responses, skin irritation is related to dose. If the duration of contact and the dosing procedure are held constant, the intensity of the response increases as the concentration of the solution increases. Under patch test conditions, the rate of increase in intensity decreases as the concentration increases [335]. The type of appliance, chamber, and tape used to secure patches in place influence the intensity of irritant responses [58,169,179]. More intense inflammatory responses are produced as the degree of occlusion is increased. The search for good techniques of occlusion ultimately led to the development of the Finn, Duhring, and Hill Top chambers now routinely used in patch testing.

TABLE 27.8
Factors Influencing Sensitivity of Skin to Development of Irritation

Variables	References
Extrinsic	
Degree of occlusion	Magnusson and Hersle [196,197]
Choice of vehicle	Patrick et al. [254]
Frequency of dosing	Kligman and Wooding [177], Frosch and Kligman [109]
Duration of exposure	Wooding and Opdyke [337]
Dose (concentration)	Patrick et al. [254], Kligman and Wooding [177]
Temperature	Rothenborg et al. [271]
Environmental conditions	Hannuksela et al. [132], Carter and Griffith [54]
Altered barrier function (including abrasion)	Frosch and Kligman [106]
Chemical damage	Finkelstein et al. [97,98], Patrick and Maibach [251]
Tape stripping	Kligman [172]
Intrinsic	
Anatomical site	Magnusson and Hersle [196,197]
Concomitant disease	Skog [285], Bjornberg [25]
Species differences	Davies et al. [71]
Age	Rockl et al. [268]
Gender (effect disputed)	Wagner and Purschel [317], Bjornberg [26], Frosch and Kligman [107]
Race	Weigand et al. [325], Weigand and Gaylor [324]

Increases in occlusion may be accompanied by local increases in surface temperature, and increased temperature is believed to predispose subjects to irritation. The temperature of solutions used in immersion assays is usually around 105°F [124,155,242]. Although systematic studies demonstrating that those temperatures were necessary were not presented, increased temperature has been shown to be necessary to reproduce irritant dermatitis in some instances [269].

The influence of vehicle in diagnostic patch testing for allergy is well recognized. Similar effects are seen when irritation is studied. These effects are demonstrated convincingly in open systems; for example, a dose of croton oil that produces no measurable edema in the mouse ear when applied in olive oil produces the maximum response when applied in acetone [252]. Patch occlusivity and interactions between vehicles and adhesives used in patch systems also influence the intensity of response and make it more difficult to demonstrate vehicle effects under patch conditions. In most predictive irritation tests, the choice of solvent is related to conditions, and water is the solvent often used.

Dosing schedules have been developed that maximize the development of the responses of interest. In general, the longer the duration of contact to the same dose of a given chemical, the greater the intensity of the response. Multiple exposures at frequent intervals are the basis for most cumulative irritation assays, although there is some disagreement on the optimal time between exposures. In developing the soap chamber test (discussed later), Frosch and Kligman [110] varied both the frequency and the duration of exposure in order to produce a more sensitive test.

The seasonal variability in human response to normal exposure to irritants is well documented [131]. Conducting usage studies in late fall and winter increases the discriminating ability of the test [54]. Some investigators have demonstrated similar effects using patch test procedures [168] and small-scale exaggerated exposure tests [155]. Although the basis for this variability is not well understood, it is believed to be due in part to changes in the barrier properties of the skin. Investigators have experimentally altered the barrier properties of skin to develop assays that are more sensitive. Alterations vary from the abrasion of Draize-type tests [76] and the chamber scarification test [106] to tape stripping to remove the outer surface of the epidermis [172]. Pretreating the test sites with damaging agents increases the reactivity of the skin to other chemicals [96,97,251]. Although these extrinsic factors modify the barrier function, intrinsic factors governing barrier properties are also important [211]. Barrier function would be expected to contribute to responses observed in screening tests used to identify sensitive subjects [107,111]. The demonstration that persons with some skin disease develop more intense response to irritants [25,283] and have diminished barrier function [309] was not unexpected. Susceptibility to the development of irritant responses is presumed to be under genetic control. The prevalence of irritant responses in atopic individuals supports this theory. The response to identical patches applied to different sites is convincing evidence of regional variation in susceptibility to irritants. The reactivity of the various sites appears to correlate to the ability of chemicals to penetrate the skin in that area. Regional differences in skin response are not limited to humans [311].

Susceptibility to irritation is believed to vary by age, gender, and race. Children may develop inflammatory responses to lower levels of a variety of chemicals than adults [266]. The inflammatory response to some materials is decreased in the elderly [173,294]. The skin of women may be more sensitive to irritants than men [315]; however, sex differences in reactivity were not confirmed by other investigators [26]. Higher doses of irritants are required to produce inflammation of black skin [322,323]. This difference in reactivity disappeared when black skin was tape stripped, leading some investigators to hypothesize that black skin forms a more efficient barrier. Berardesca and Maibach [18] questioned whether these differences are more real than apparent.

The principles of toxicology should be remembered when designing and conducting any animal (and human) assay for skin irritation. One should consider dose–response relationships. Draize scores require careful interpretation; they are best interpreted by comparison to related compounds of formulations with a history of human exposure. Knowledge of intended human use (and foreseeable misuse) permits a more rational interpretation. With occlusive application techniques,

remember that occlusion increases the permeability of some but not all moieties. Although there is a consistent, reasonably good correlation between responses in rabbits and humans, occasional inconsistencies have occurred [117]. Different species exhibit widely varying reactivity under identical test conditions, especially in substances with only minor irritant potential; thus, the accuracy of the Draize test and other animal testing as it relates to humans has been questioned [117,135,159,252]. In addition, results from the animal methods differ due to the subjective visual test scoring. These differences occur most frequently in the assessment of the toxicity of mild irritants and colored material [241]. Wise investigators conduct carefully planned and executed tests in humans when rabbit tests indicate that materials are possible irritants. This is particularly true when one wishes to compare the irritancy potential of mild irritants. One should follow the guidelines of the National Academy of Science (NAS) committee [232] when this course of action is taken. The clinical and basic knowledge of what is now called the *irritant dermatitis syndrome* continues to advance, and interest in irritant dermatitis has led to an international symposium being held approximately every 3 years. Several textbooks summarize current advances [85,308,351].

Irritation Tests in Animals: Draize-Type Tests

Primary irritation and corrosion are most often evaluated by modifications described by Draize and colleagues in 1944 [76]. The Federal Hazardous Substances Act (FHSA) adopted one modification as a standard procedure [57]. The backs of six albino rabbits are clipped free of hair.

Each material is tested on two 1 in^2. sites on the same animal; one site is intact and one is abraded in such a way that the stratum corneum is opened but no bleeding produced. Abrasion can be performed using the tip of a hypodermic needle drawn across the skin repeatedly or commercial instruments such as the Berkeley scarifier [130].

Materials are tested undiluted, and 0.5 mL liquid or 0.5 g solid or semisolid material is applied. In some cases, the skin may be moistened to help solids adhere to the site, or an equal volume of solvent may be used to moisten the material.

Each test site is covered with two layers of 1-inch square surgical gauze secured in place with tape. The entire trunk of the animal is then wrapped with rubberized cloth or other occlusive impervious material to retard evaporation of the substances and to hold the patches in one position. The wrappings are removed 24 h after application, and the test sites evaluated for erythema and edema using a prescribed scale (Table 27.9). Evaluations of abraded and intact sites are recorded separately. Test sites are evaluated again 48 h later (72 h after application) using the same scale.

The reproducibility of the FHSA procedure [311,324] and the relevance of test results to human experience have been questioned [71,140,216,260,288]. Numerous modifications to the Draize procedure have been proposed to improve its prediction of human experience. Proposed modifications include changing the species tested [230], reduction of the exposure period, the use of fewer animals, and testing on intact skin only [80,126,226]. A few investigators have supplemented visual evaluation with other techniques [219,220], but these additions have not been considered essential for the standard method. Several governmental bodies have used their own modifications of the Drake procedure for regulatory decisions. The FHSA, DOT, Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), and OECD guidelines are contrasted to the original Draize methods in Table 27.10. Cruzan et al. [66] proposed a composite test that meets requirements of major agencies.

Summaries and evaluations of the scores vary. Draize reported values for individual animals at each time point, combined the erythema and edema values at each time point, and then averaged the 24 and 72 h evaluations for intact and abraded sites separately. He also calculated a primary irritation index (PII) of averaged intact and abraded sites. Agents producing a PII of 2 were considered only mildly irritating, 2–5 moderately irritating, and more than 5 severely irritating. The primary irritation calculated for the FHSA is essentially the PII of Draize. A minimum PII of 5 defines an irritant by CPSC standards. The method of the National Institute of Occupational Safety and Health does not combine responses of abraded sites and includes probable effects on normal and damaged skin in their evaluation.

Although vesiculation, ulceration, and severe eschar formations are not included in the Draize scoring scales, all Draize-type tests are used to evaluate corrosion as well as irritation. When severe reactions, which may not be reversible, are noted, test sites are observed for a longer period. Delayed evaluations usually are made on days 7 and 14; however, evaluations have been made as late as 35 days after

TABLE 27.9
Draize Scoring System in Albino Rabbits[a]

Description	Score Assigned[b]
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely visible)	1
Well-defined erythema	2
Moderate-to-severe erythema	3
Severe erythema (beet redness) to slight formation (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

[a] The scale as defined by Draize and adopted by various regulatory agencies.
[b] The primary irritation index (PII) is calculated by averaging the erythema values and averaging the edema values from all animals and then combining the averages (maximum PII = 8).

TABLE 27.10
Comparison of Skin Irritation Tests Based on the Draize Method

Feature	Draize	FHSA	DOT	FIFRA	OECD[a]
Number of animals	3[b]	6	6	6	3
Abrasion	Abraded and intact	Abraded and intact	Intact	2 abraded and 2 intact	Intact
Dose liquids	0.5 mL undiluted	0.5 mL undiluted	0.5 mL	0.5 mL undiluted	0.5 mL
Dose solids	0.5 g	0.5 g in solvent	0.5 g	0.5 g moistened	0.5 g moistened
Wrapping materials	Gauze and rubberized cloth	Impervious material	—	—	Semiocclusive
Length of exposure	24 h	24 h	4 h	4 h	4 h
Evaluated at[c]	24 h, 72 h	24 h, 72 h	4 h, 48 h	0.5 h, 1 h, 24 h, 48 h, 72 h	0.5 h, 1 h, 24h, 48 h, 72 h
Treatment at removal	Not specified	Not specified	Skin washed	Skin wiped, not washed	Skin washed
Excluded from testing	—	—	—	Materials with pH < 2 or >11.5	Materials with pH < 2 or >11.5

Note: DOT, Department of Transportation; FHSA, Federal Hazardous Substances Act; FIFRA, Federal Insecticide, Fungicide, and Rodenticide Act; OECD, Organization for Economic Cooperation and Development.

[a] Although other species are acceptable, the albino rabbit is the preferred species.

[b] Draize tested four materials on six rabbits. Three abraded and three intact sites were tested with each material.

[c] Times listed are after removal for FIFRA and OECD. Times listed for Draize, FHSA, and DOT are after the application of the test material.

application. The EPA bases interpretations on 7-day observations. The basic exposure procedures of the OECD guidelines for skin irritation or corrosion have been further modified to test for corrosion during shorter periods [243]. Under a directive of the EEC, a shorter 3 min exposure was added (with no wrapping procedure). The United Nations' recommendations for the transport of dangerous goods are based on exposure times of 4 h, 1 h, and 3 min, with the recommendation that the 1 h exposure be conducted first. Evaluations are made 1, 24, 48, and 72 h and 7 days after dosing.

The Draize method has generally erred on the side of safety in that it overpredicts the severity of skin damage produced by chemicals, thus providing a safety factor for those exposed. One criticism often repeated is that the test is not sensitive enough to separate mild from moderate irritants. The purpose when designing the Draize test was to identify chemicals that posed a severe hazard to the public, not to compare products. Criticisms of the Draize test have been embraced by groups supporting the elimination of animal testing as they serve to demonstrate that use of the method is unwarranted. These criticisms overlook the tremendous value the test has provided in warning consumers, workers, and manufacturers of potential dangers associated with specific chemicals so appropriate precautions can be taken. Although Draize-type tests may be replaced by in vitro assays at some time in the future, we have no widely accepted in vitro substitutes at present [261]. Although a few assays have been validated as reproducible, chemical relevant data are often minimal.

Non-Draize Animal Studies

Animal assays to evaluate the ability of chemicals to produce cumulative irritation have been developed [255]; however, they are not required by any regulatory agency. The impetus for their usage is largely the development of products that are better tolerated by consumers and industrial workers. Although many such tests have been described, few are used extensively enough to summarize here. Even those used more often are not as well standardized as Draize-type tests, and many variables have been introduced by multiple investigators.

Repeat application patch tests using several species where diluted materials are applied to the same site each day for 15–21 days have been reported [255]; the guinea pig or rabbit is most commonly used. Patches used vary considerably, with gauze-type dressings and metal chambers being the extremes. Some authors recommended testing the materials with no covering, presumably with a restraining collar to prevent grooming of the area and ingestion of the material. A material of similar use or that produces a known effect in humans is included in almost all of the repeat application procedures as a control. The degrees of inflammation produced by the materials in a single assay are compared. Test sites are evaluated for erythema and edema using the scales of the Draize-type tests or more descriptive scales developed by the investigator. Although interpretation ratings such as *slight*, *moderate*, or *severe* irritant are not usually made, the data from cumulative irritancy assays in rabbits have been used to predict reactions in humans. Other investigators have used multiple applications with shorter time periods to evaluate materials [155].

A 5-day dermal irritation test in rabbits was used to compare consumer products [193]. After the animals' backs were shaved, 0.5 mL of test materials was spread over a 5 × 4.5 cm area of skin. The test sites were protected from grooming by placing the animals in a leather harness or Elizabethan collar. After 4 h, sites were cleaned and graded using the Draize scoring system. This procedure was repeated daily for 5 days. The authors showed good agreement between this assay and 21-day human patch tests of liquid detergents, after-bath colognes and hair preparations; the technique was less satisfactory for other types of materials.

The guinea pig immersion assay has been used to evaluate the irritancy of aqueous detergent solutions [41,242,326]. Ten guinea pigs are placed in restraining devices that are immersed in a 40°C test solution for 4 h. The apparatus is designed to maintain the head of the guinea pig above the solution. Immersion is repeated daily for three treatments. The flank is shaved 24 h after the final immersion, and the skin is evaluated for erythema, edema, and fissures. A photographic grading scale for this assay was presented in MacMillan et al. [194]. Only materials of limited toxic potential are suitable for this assay because systematic absorption of a lethal dose is possible. Concentrations of test materials vary somewhat but are usually below 10% to limit systematic toxicity of the agents. A second group of animals is usually tested with a reference material as a control for the material of interest.

An open application procedure in guinea pigs uses microscopic examination of skin biopsies of sites treated with weak irritants to rank materials [4]. Biopsies are taken after three daily applications of 10% of solvent or 5% aqueous test solutions to 1 cm areas of the shaved flank. Sites are evaluated visually for erythema and edema, and 3 μm histological sections are stained with May–Grünwald–Giemsa under oil immersion to evaluate microscopically epidermal thickness and dermal infiltration. A composite score reflecting the macroscopic evaluation, number of applications before the development of visible response, the epidermal thickness, and the cellular response is used to rank chemicals. Although this method provides information on pathogenesis of the response to each chemical, the extensive processing may limit its application to special studies.

Uttley and Van Abbe [308] developed a mouse ear test in which undiluted shampoos were applied to one ear daily for 4 days. The degree of inflammation was quantified visually as vessel dilation, erythema, and edema. The degree of inflammation produced by materials of interest was compared to that produced by a reference material tested on another group of mice. One confounding factor with this assay may be the use of anesthetics to facilitate performance of the procedure, which may alter the development of inflammation.

To distinguish between mild and moderate irritants in an acute exposure test, Finkelstein and colleagues [97,98] used pretreatment of test sites with an irritant and enhanced visualization of the response by injection of Trypan blue to increase test sensitivity. The technique was performed in anesthetized rabbits, rats, or guinea pigs. A circular area of the shaved abdomen was painted with a 20% solution of formaldehyde and then was allowed to dry for 5 min. This was repeated three times, and then 1 in. cotton flannel pads saturated with test material were applied to each site. A control substance of known irritancy was tested in each study. Pads were secured in place, and the entire trunk was then wrapped in polyethylene. A solution of Trypan blue was injected into subcutaneous tissue away from the dosage sites. The dye was absorbed and served as a marker for plasma leakage because it spontaneously binds to albumin. After 16 h, patches were removed and the degree of bluing at each site was evaluated on a 0%–100% scale. In light of work comparing the reactivity of dorsal and abdominal animal skin [311], one wonders if the enhanced sensitivity was due in part to choice of test site. Another study reported quantifying the amount of Evans blue dye recovered from rat skin after exposing the skin to inflammatory agents [142].

A few tests in which material is not applied topically have been developed that claim to evaluate the intrinsic irritancy of test materials. The persistence of edema in the skin of depilated juvenile white mice following intracutaneous injection of solutions has been used to assess local irritation [44,319]. The number of wrinkles observed when piercing (is there a better word?) the skin with thin pincers is counted before and at selected time points through 6 h after injection of 0.01 mL test solution. Although test animal number has varied between 8 and 25, the developers considered 20–25 optimal. An obvious limitation of this method is that materials must be administered as isosmotic solutions, which requires substantial pretest formulation. Although the developers claim this procedure has good predictive power for eye, skin, and mucosal irritation, it has not been adopted extensively, and no validation studies comparing this method to the standard assays have been published.

Justice et al. [156] described a repeat animal patch test for comparing the irritation potential of surfactants. Solutions were applied to the clipped backs of immobilized albino mice with saturated cotton-tipped applicators. Test sites were covered with rubber dams to prevent evaporation. This process was repeated seven times at intervals of 10 min. The skin was then evaluated microscopically for epidermal erosion.

Brown [42] used both open and closed exposures to rank surfactants for skin irritation potential. Tests ranged from 6 h patch exposures each day for 3 consecutive days in rabbits to daily open application to the skin of rabbits, guinea pigs, or hairless mice for up to 4.5 weeks. Good agreement among the test methods was not obtained, and none of the methods gained wide acceptance, although they are similar to techniques developed by others later.

We have used an assay in which dilute solutions of surfactants and other chemicals are applied to one ear of five or six mice each day for 4 days [251]. Ear thickness measurements at various time points after each treatment were used to quantify the degree of inflammation. Multiple groups (at least four) were tested with different doses of the material. The dose producing a 50% maximum response following a single treatment, and the slopes of the dose–response lines for the chemicals were compared. Pretreating the ear with croton oil or TPA 72 h before application of the material of interest increased the sensitivity of the assay. Although the procedure was useful for most surfactant-based products, it is not suitable for oily and highly perfumed materials because animals attempt to remove the materials by grooming. Moloney and Teal [227] also used ear thickness to quantify inflammatory changes produced by *n*-alkanes applied to the ears of mice. They dosed animals twice per day for 4 days to produce inflammation. Dithranol-induced skin irritation and the modulating effects of different pharmacological agents, such as the corticosteroid and the lipoxygenase

and cyclooxygenase inhibitor studies, were studied using the mouse ear model [310].

Human Irritation Tests

Because only a small area of skin must be tested, it is possible to conduct predictive irritation assays in humans, provided that systematic toxicity (from absorption) is low and informed consent is obtained. Although regulatory agencies do not routinely require testing in humans, human tests are preferred to animal tests in some cases because of the uncertainties of interspecies extrapolation. New materials, those of unknown or unfamiliar composition, should be tested on animal skin first to determine if application to humans is warranted [233]. Patch test responses generally heal rapidly, within a week or so. More severe reactions should be evaluated periodically over a longer period to determine how the inflammatory response is resolved. Some subjects may develop changes in pigmentation level at the test site following severe responses. It is prudent to arrange for medical consultation whenever human clinical tests are undertaken. Lamel et al. [356] summarize the minimal quantitation for hyperpigmentation [354].

Single-Application Irritation Patch Tests

Many forms of single-application patch tests have been published. The duration of patch exposure has varied between 1 and 72 h. Custom-made apparatus to hold the test material has been designed [195,280,282], and a variety of adhesives that are no longer commercially available have been used [197]. Although the individual assays provided important information to the investigators of the period, they were never standardized or gained widespread acceptance.

The single-application patch procedure outlined by the NAS [232] incorporates important aspects of assays used by many investigators. The procedure is similar to FHSA tests in rabbits. Commercial patches, chambers, gauze squares, or cotton bandage material, such as Webril, applied to either the intrascapular region of the back or to the dorsal surface of the upper arms are expected to produce equivalent reactions [174]. Patches are secured in place with surgical tape without wrapping the trunk or the arm. For new volatile materials, a relatively nonocclusive tape, such as Micropore®, Dermicel™, or Scanpore®, should be used. Increasing the degree of occlusion with occlusive tapes such as Blenderm™ or chamber devices such as the Duhring and Hill Top chambers generally increases the severity of responses. A 4 h exposure period was suggested by the NAS panel; however, it is desirable to test new materials and volatiles for shorter periods (30 min to 1 h), and many investigators apply materials intended for skin contact for 24–48 h periods. Subjects should routinely be instructed to remove patches immediately if any unusual discomfort develops. After the exposure period, the patches should be removed, the area cleaned with water to remove any residue, and the test site marked by study personnel.

Responses are evaluated 30 min to 1 h after each patch removal (to allow hydration and pressure effects to subside) and again 24 h after the patch is removed. Persistent reactions may be evaluated for 3–4 days. The Draize scale for erythema and edema (Table 27.9) has been used for grading human skin responses; however, the scale has no provision for scoring papular, vesicular, or bullous responses. Integrated scales ranging from 4 to 16 points have been published (see Table 27.11) and are generally preferred to the Draize scale. Up to 10 materials can be tested simultaneously on each subject. Skin reactivity differs by body region, and some patch sites may receive more pressure (e.g., from chairs or clothing); therefore, the location where the materials are placed on the skin (e.g., upper right back and lower left back) should be systematically varied within each study. Each study should include at least one reference material. Scores from all subjects are averaged for each material, and comparisons made between standards and other test materials. Some investigators have accepted an average difference of one unit on the grading scale as meaningful. Other investigators analyze the data by standard nonparametric statistical tests. It is also possible to test multiple doses and calculate the ID_{50} [176].

Wooding and Opdyke [337] investigated the effects of modifying some test parameters on the intensity of the response,

TABLE 27.11
Human Patch Test Grading Scales

A. Simple Patch Test Grading Scale	
0	Negative, normal skin
±	Questionable erythema not covering entire area
1	Definite erythema
2	Erythema and induration
3	Vesiculation
4	Bullous reaction
B. Detailed Human Patch Test Grading Scale	
0	No apparent cutaneous involvement
½	Faint, barely perceptible erythema or slight dryness (glazed appearance)
1	Faint but definite erythema, no eruptions or broken skin *or* No erythema but definite dryness; may have epidermal fissuring
1-1/2	Well-defined erythema or faint erythema with definite dryness; may have epidural fissuring
2	Moderate erythema, may have a *few* papules or deep fissures, moderate-to-severe erythema in the cracks
2-1/2	Moderate erythema with barely perceptible edema *or* Severe erythema not involving a significant portion of the patch (halo effect around the edges), may have a few papules *or* Moderate-to-severe erythema
3	Severe erythema (beet red). May have generalized papules *or* Moderate-to-severe erythema with slight edema (edges well defined by raising)
3-1/2	Moderate-to-severe erythema with moderate edema (confined to patch area) *or* Moderate-to-severe erythema with isolated eschar formations or vesicles
4	Generalized vesicles *or* Eschar formation *or* Moderate-to-severe erythema and/or edema extending beyond patch area

and the intensity of inflammation has been shown to increase after removal of patches in some cases [67,263]. Kooyman and Snyder [180] used 6 h exposures to 8% solutions of bar soaps and evaluated test sites 24 h after patch application. Griffith et al. [125] reported using single-application patch tests with exposures of less than 24 h to evaluate laundry detergents containing enzymes. Justice et al. [156] varied exposure time between 18 and 24 h to test bar soaps, liquid detergents, and laundry detergents. Others have suggested that 48 h patch exposures are more suitable for some products [269].

A standardized procedure for evaluating the irritation potential of new chemicals in humans as a replacement for the Draize rabbit test has been proposed [14,15,79,125,337,338]. The method has been tested in several laboratories, and results appear reasonably reproducible. The classification of irritancy is based on a comparison of the length of exposure to the chemical being tested vs. the length of exposure producing irritation following the application of a 20% solution of sodium laurel sulfate. Chemicals producing irritation after shorter exposures than used for SLS are considered irritants and would be labeled as such. Each test subject is exposed to the undiluted test material in occlusive chambers (e.g., Hill Top Chamber®) and to a 20% solution of SLS. Length of exposure begins with a 15 min exposure with evaluation at removal and at 24 and 48 h. If no response is observed, then another set of patches is applied and worn for 30 min. This process of patching, evaluation, and patching for a longer exposure interval is repeated until the subject responds to the SLS exposure or until a 4 h exposure has been completed. The test was developed for chemicals but may have used for evaluating consumer products as well. For most household chemicals and cosmetics, this cycle can probably be shortened considerably. Nixon et al. [236] used a 4 h FHSA-type procedure (including abrasion) to evaluate a range of household products.

Repeat-Application Irritation Patch Tests

The term *skin fatigue* was used to explain the development of inflammation late in the induction phase of sensitization tests without positive responses at challenge [279]. The phenomenon was also referred to as *secondary irritation* and later as *cumulative irritation*. The human RIPT for skin allergy was modified to evaluate skin irritation. As with single-application patch test, many investigators developed their own version of the repeat application patch test. Most were patterned after human sensitization studies with 24 h exposures with or without a rest period between patches. Kligman and Wooding [177] applied the Litchfield and Wilcoxon probit analysis to cumulative irritation testing with the calculation of IT_{50} and ID_{50} values, and statistical comparison of those values for different materials. Their early work forms the basis for the 21-day cumulative irritation assay, which is currently widely used.

The cumulative irritation assay, as described by Lanman et al. [191], was developed to compare antiperspirants, deodorants, and bath oils to provide guidance for product development. A 1 in. square of Webril® was saturated with liquid or up to 0.5 g of viscous substances and applied to the skin. The patch was applied to the upper back and sealed into place with occlusive tape. After 24 h, the patch was removed, the area evaluated, and a fresh patch applied. The procedure was repeated for up to 21 days. The sensitivity of the assay was increased by increasing the number of test subjects from 10 to 24. The IT_{50}, as described by Kligman and Wooding [177], was used to evaluate and compare test materials.

Modifications of the cumulative irritation assay have been reported. Intensity of response has been evaluated using other evaluation schemes, the interval between application of fresh patches has been varied, and other methods of data evaluation have been proposed [19,53,262]. The newer chamber devices have replaced Webril with occlusive tape in some laboratories. Some investigators currently use cumulative scores to compare test materials and do not calculate an IT_{50}. The necessity of 20 applications has been questioned [19]. Although the procedure came to be known as the 21-day cumulative irritation assay, the number of applications used was varied by Lanman, depending on the types of materials to be tested; 21 days was the maximum period of testing. Kligman and Wooding performed their studies on surfactants in 10 days. Lanman found that 21 applications were necessary to discriminate between baby lotions. The number of applications used to rank materials should be chosen based on the class of material being studied.

Numerous other human repeated application schedules have been used for comparing commercial products. Finkelstein et al. [97,98] described tests using either a 5–6 h or a 17–18h exposure each day for 14 days. Test sites were evaluated 1 h after patch removal. Modifications of this procedure have also been used to evaluate shaving creams and toilet soaps [285].

Repeated application patch tests on intact skin fail to predict some adverse reactions due to repeated application to *damaged* skin (e.g., acne, shaved underarms, or sensitive areas such as the face) [16]. The chamber scarification test [106,108,110] was developed to evaluate materials that would normally be applied to damaged skin. Light-skinned Caucasians who develop severe erythema with edema and vesicles following a 24 h exposure to 5% SLS in Duhring chambers applied to the inner forearm are preselected subjects. Six to eight 10 mm^2 areas on the midvolar forearm are scarified with eight criss-cross scratches made with a 30-gauge needle. Four scratches are parallel, with another four at right angles. In scarifying the tissue, the bevel of the needle is to the side and is drawn across the tissue at a 45% angle with enough pressure to scratch the epidermis without drawing blood. Duhring chambers containing the test material (0.1 g for ointments, creams, or powders or Webril® saturated with 0.1 mL for liquids) are placed over the scarified areas and are secured in place with nonocclusive tape wrapped around the forearm. Fresh chambers containing the same materials are applied daily for 3 days. The test sites are evaluated on a 0–4 scale (Table 27.12) 30 min after the removal of the last set of chambers. The responses are averaged, and materials are classified as low (0–0.41), slight

TABLE 27.12
Grading Scale for the Chamber Scarification Tests

0	Scratch marks barely perceptible
1	Erythema confined to scratches
2	Broader bands of erythema with or without rows of vesicles, pustules, or erosions
3	Severe erythema with partial confluence with or without other lesions
4	Confluent, severe erythema sometimes with edema, necrosis, or bulla

(0.5–1.4), moderate (1.5–2.4), or severe irritants. A scarification index (SI) may be calculated by dividing the score on intact skin by the score from the scarified site. The SI is used to estimate the relative risk for damaged and normal tissue; it is not used to rank test materials.

Although bar soaps produce erythema when tested by conventional patch test techniques, the typical clinical response is dryness and flaking with occasional erythema and fissuring. Frosch and Kligman [110] developed the soap test chamber test to compare the *chapping* potential of bar soaps. Sensitive subjects are preselected as described for the chamber scarification test or by ammonium hydroxide blistering time [89]. Duhring chambers fitted with Webril® pads are used to apply 0.1 mL of an 8% solution of soap to the forearm. Chambers are secured in place by encircling the arm with porous tape. Patch contact time is 24 h on day 1 (Monday) and 6 h each day for the next 4 days (Tuesday through Friday). Test sites are monitored each day before the application of fresh solutions. If severe erythema is noted, dosing is discontinued. Unless treatment was discontinued before the fifth exposure, skin reactions are evaluated on day 8 (Monday). This test has shown good agreement with skin-washing procedures but has overpredicted irritant responses to some materials [105].

Exaggerated Exposure Irritant Tests

Although patch tests have been useful in detecting differences in the irritation potential of some materials, in some cases, predicted differences were not apparent when materials were used by consumers. Exaggerated exposure tests have been developed to bridge the gap between responses occurring during product use and patch testing. Perhaps the oldest nonpatch irritancy test still in use is the arm immersion technique [179], in which the relative irritancy of two soap or detergent products is compared. As originally described, soap solutions of up to 8% were prepared in troughs. Temperature was maintained at 105°F while subjects immersed one hand and arm to just above the elbow in one test solution and the other arm in a solution containing a second product. The period of exposure varied between 10 and 15 min, three times a day, for 5 days or until observable irritation was produced on both arms.

In most volunteers, the first sign of irritation was erythema of the antecubital surface of the arm [155,179]. Later, the hands developed dryness and cracking. These observations led to the development of separate assays on the antecubital area and the hands. Numerous versions of the antecubital washing test (also known as the flex washing test and elbow crease washing test) have been used. Published methods compare two products; however, dosing regimes differ somewhat. Investigators have used two [105] or three [124,292] washing procedures per day, and some specify that lather is allowed to remain on the skin for a brief period. Erythema and edema are evaluated as endpoints for all studies. Frosch [106] used a similar procedure on the cheeks to evaluate toilet soaps. A simple 1–4 (i.e., slight, moderate, severe, very severe) grading scale is used to evaluate the severity of the response. Products can be compared in terms of the average grades or the number of washes producing an effect. Some investigators have tested up to four samples per forearm by washing in glass cylinders and then rinsing the area [144].

At least two types of hand immersion procedures have been used. In small studies (i.e., 10 subjects), relatively concentrated solutions (up to 2%) of two materials are tested. Up to four hand-dishwashing products have been compared at near-use concentrations in studies on 64 subjects using a Latin-square dosing pattern (Bannan, E. A. (1975): Personal communication.). Exposure conditions have varied from two to three 10–15 min immersions each day [124] to a single 30 min exposure each day (Bannan, E. A. (1975): Personal communication.). Grading scales for this type of assay focus on scaling and cracking as well as erythema.

Evaluation of skin conditions before and after use in the home has also been used to compare the irritation potential of various products. These tests represent skin tolerance studies, as either irritation or allergy could be detected. The clinical method published by Johnson et al. [154] has been varied to include tests of bar soaps, laundry soaps and detergents, and dishwashing detergents. Essentially, the method is a double-blind crossover study with usage periods of 2 weeks [54]. Skin condition is evaluated by a dermatologist before the study and after the use of each product. Magnification of the area is used to facilitate grading using a 0–10 scale. Tests are conducted using large panels (more than 300 subjects per product), and up to eight materials can be evaluated simultaneously using a Latin-square design. The principles used in conducting this type of large-scale usage study have been applied to laundry powders for diapers and bar soaps used in infants [84,124] and to fabric softeners in adult populations [321]. Special emphasis should be placed on the statistical design of clinical trials of this type to ensure validity of the study [1].

Use of Bioengineering Devices in Irritancy Evaluations

Measurements of biophysical parameters of skin function have been proposed as adjuncts to visual elevations of the inflammatory response [210,212,297,298,334]. In many instances, investigators constructed their own instruments to perform these measurements. As the availability of commercial instruments increases, assessment of the biophysical changes in skin has become widely used to supplement visual evaluations. The benefits of the methods vary and include more objective measurements of erythema (as change in blood flow), more precise determination of the color change, and measurement of parameters of damage that cannot be evaluated visually. When combined with various techniques

of exposure, it is possible to vastly decrease the number of subjects and probable clinical relevance of the studies.

Laser Doppler velocimetry has been used to quantify the increased blood flow to inflamed tissue. This device is an optical technique for the estimation of microcirculation, based on the Doppler principle. When the laser beam, a 632 nm helium–neon (He–Ne) laser source, is directed toward the tissue, reflection, transmission, absorption, and scattering occur. Laser light backscattered from moving particles, such as red cells, is shifted in frequency according to the Doppler principle, whereas radiation that is backscattered from nonmoving structures remains at the same frequency. Detailed guidelines for using this device are provided by the Standardization Group of the European Society of Contact Dermatitis [23].

Skin reflectance spectrophotometry has been used to measure the color change in skin associated with inflammation. Polychromatic light is directed into the skin. The reflected light is collected in an integrating sphere and guided to a monochromator, where the light is split into five bands in the spectral range of 355–700 nm. Melanin content and oxygenized and deoxygenized hemoglobin are analyzed at different spectra, and the relative changes are expressed as a percentage of chromophore content in control skin [3,24].

Changes in transepidermal water loss (TEWL) and electrical resistance have been detected before inflammatory changes are apparent. Early investigations clearly established that chemicals that provoked inflammation increased TEWL. TEWL reflects the integrity of the water-barrier function of stratum corneum as determined by the use of an evaporimeter (open chamber method, where the skin capsule is open to the atmosphere). TEWL is calculated from the slope provided by two hygrosensors precisely oriented in the chamber. Air movement and humidity are the drawbacks of this method. A report reviewing interindividual, environmental, and instrument-related variables with respect to the Evaporimeter EPI (ServoMed), its use, and good practice guidelines can be obtained from Pinnagoda et al. [258]. Malten and Thiele [211] showed that increase in TEWL occurred before visible inflammation when ionic, polar, water-soluble substances (e.g., sodium hydroxide, soaps, detergents) were used as irritants. Unionized polar irritants, such as dimethyl sulfoxide, and unionized, nonpolar, water-soluble irritants, such as hexanediol diacrylate and butanediol diacrylate, did not induce increased water loss until visible inflammation occurred. This method thus seems to be possibly valuable for detecting irritancy with no perceivable irritancy for ionizable, polar, water-soluble substances. These techniques have been used to compare the irritancy potential of soaps tested at near-use levels [133].

Water content of the skin can be estimated by electrical measurements of skin resistance and capacitance. Subtle degrees of skin damage can be measured using skin resistance before skin inflammation occurs [297]. The corneometer is used to register the electrical capacitance of the skin surface. The principle of this instrument is to decipher distinctly different dielectric constants of water and other constitutional materials (fewer than 7) with a probe applied to the skin at constant pressure. Another instrument working on similar principle is the skin hygrometer, which measures conductance of the skin using high-frequency electric current of 3.5 MHz with the help of a sensitive probe [295]. Several other devices using different frequencies and technologies have been developed to measure water content, including instruments that measure impedance, resistance, phase angle, microwave transmission, and photo-acoustic methods. Noninvasive bioengineering techniques, such as colorimetry, remittance spectroscopy, measurement of surface pH, and skin surface topography, have been reviewed by Berardesca and Maibach [17,18]. One unique new way of evaluating irritancy uses in vivo measurements of the water-binding capacity of the stratum corneum after occlusion [17]. Bioinstrumentation techniques have now also been used in in vitro studies. These techniques permit more clinically realistic bioassays that more closely mimic use experience. The five volumes on skin bioimaging (reference needed) and the volume by Serup et al. detailed experimental documentation [357].

In Vitro Assays of Skin Irritation and Corrosion

Since 1980, much effort has been expended toward developing in vitro alternatives to Draize-type tests for both eye and skin irritation. Approaches to development of an in vitro model include cell toxicity [32,34], measurement of inflammatory mediators, effect on cell recovery and survival [81,178,229], effect on cellular physiology [249], cell morphology [32,34], biochemical endpoints [225], and effect on membranes [27] and artificial membranes [118,120] constructed to release dye indicator. Some investigators have included metabolic activators in their systems [35]. Nonmammalian cells [48] have also been used.

In vitro assays for skin irritation are being explored as tools in toxicologic research and as aids in formulating mild products, as well as for replacement of the Draize-type tests. This mirrors the overall use of in vitro assays during the last 25 years [15]. Numerous proposals for the validation of the methods have been put forth; however, tests in intact animals or humans are currently the only means of assessing the potential irritation hazard from skin exposure [287]. In vitro assays for skin corrosion have been validated and accepted.

Two commercial systems, Skin2® and EpiDerm™, have been used extensively for skin irritation testing. Skin2 [99,302,303] is a three-dimensional coculture of human fibroblasts and keratinocytes. In Skin2, human neonatal fibroblasts are seeded on a nylon mesh to which they attach and lay down collagen. When the proper degree of confluence is reached, human keratinocytes are seeded onto the fibroblast culture. The epidermis is exposed to air, and a partially differentiated stratum corneum develops. Several cytotoxicity assays and assays for the release of inflammatory mediators are available for use with these systems. EpiDerm [51] consists of a multilayered cornified epithelium with no dermal element. It appears to have a well-differentiated stratum corneum. Perkins et al. [256] used these systems to evaluate skin corrosion in vitro using the dimethyltriazoldiphenyl tetrazolium-formazan (MTT) [52] cell viability assay with some success.

SKINTEX™ and Corrositex® [101–104] are described as membrane barrier/protein matrices. They are two-component systems consisting of a barrier matrix that contains an indicator dye. Dye release is expected to correlate with protein disruption and denaturation. The second compartment is a reagent system that increases in turbidity when exposed to irritants. Corrositex has been accepted by the DOT as an alternative to the Draize test for skin corrosion [145] and has been validated for limited purposes by ICCVAM [343].

Several European investigators have reported success in using excised rat or human skin to assess skin corrosivity [14,239,240,318,330] based on changes in transcutaneous electrical resistance (TER). A rubber O-ring is used to secure full-thickness skin (including dermis), epidermal surface uppermost, onto the top of small tubes. These tubes are then suspended in a larger tube containing an electrolyte solution in distilled water. Applied to the surfaces of at least three disks is 150 μg of the test material. After 24 h, the skin is rinsed, the surface is treated with ethanol, and electrolyte solution is added to the skin surface. The TER is then measured using a commercial instrument. Values above 11–12.5 kΩ/disk are indicative of corrosivity (varies by investigator and source of skin). With the judicial use of reference materials to set the threshold values for classifying materials as corrosive, this method appears to be reproducible.

Contact Urticaria and Urticaria-Like Syndromes

Circumscribed, erythematous, evanescent areas of edema involving the epidermis and superficial portions of the dermis are referred to as *urticaria* [314]. Classically, the reaction has been described as a wheal-and-flare response that develops within 30–60 min after exposure of the skin to certain agents. Symptoms of immediate contact reactions can be classified according to their morphology and severity; itching, tingling, and burning with erythema is the weakest type of immediate contact reaction. Local superficial wheal-and-flare with tingling and itching represents the prototype reaction of contact urticaria. Generalized urticaria after local contact is rare but can occur from strong urticants. Signs in other organs appear with the skin symptoms in cases of immunologic contact urticaria syndrome. Urticaria and angioedema (edematous processes in the deep dermis, subcutaneous, and submucosal tissue) may persist for up to 72 h [62]. The strength of the reactions may vary greatly, and often the entire range of local signs—from slight erythema to strong edema and erythema—can be seen from the same substance if different concentrations are used in skin tests [182]. Not only the concentration but also the site of the skin contact can affect the reaction. A certain concentration of contact urticant may produce strong edema and erythema reactions on the skin of the upper back and face, but only erythema on the volar surfaces of the lower arms or legs. In some cases, contact urticaria can be demonstrated only on slightly or previously eczematous skin, and it can be part of the mechanism responsible for the maintenance of chronic eczemas [2,202,237]. Some agents, such as formaldehyde, produce urticaria on healthy skin following repeated but not single applications to the skin. Diagnosis of immediate-contact urticaria is based on a thorough history and skin testing with suspected substances [62]. Skin tests for human diagnostic testing have been summarized [314]. Because of the risk of systemic reactions, such as anaphylaxis, human diagnostic tests should be performed only by experienced personnel with facilities for resuscitation on hand.

Contact urticaria has been divided into two main types: immunologic and nonimmunologic [183]. Several reviews list agents suspected to cause each type of urticarial response [184,185,314]. Some common urticants are listed in Table 27.13. Nonimmunologic contact urticaria occurs without previous exposure in most individuals and is the most common type. The reaction remains localized and does not cause systemic symptoms or spread to become generalized urticaria. Typically, the strength of this type of contact urticaria reaction varies from erythema to a generalized urticarial response, depending on the concentration, skin site, and substance. The mechanism of nonimmunologic contact urticaria has not been delineated, but a direct influence upon dermal vessel walls and nonimmunologic release of mast cell mediators are possible mechanisms [278]. Nonimmunologic urticaria produced by different agents may involve different combinations of mediators [183].

The most potent and best studied substances producing nonimmunologic contact urticaria are benzoic acid, cinnamic aldehyde, and nicotinic esters. Under optimal conditions, more than half of a random sample of individuals

TABLE 27.13
Agents Reported to Cause Contact Urticaria in Humans

Immunologic mechanism
Grains
Nuts
Bacitracin
Parabens
Seafood (protein extracts)
Penicillin
Butylated hydroxy toluene
Nonimmunologic mechanism
Aspirin
Balsam of Peru
Benzoic acid
Cayenne pepper
Cinnamic aldehyde
Codeine
Dimethyl sulfoxide
Unknown mechanisms
Lettuce/endive (probably immunologic)
Cassia oil
Formaldehyde

Note: More comprehensive lists and the original references can be found in Amin, S. et al., Contact urticaria syndrome, in *Dermatotoxicology Methods: The Laboratory Worker's Vade Mecum*, Marzulli, F.N. and Maibach, H.I., eds., Taylor & Francis, New York, pp. 161–176, 1998.

shows local edema and erythema reactions within 45 min of application of these substances if the concentration is high enough. Benzoic acid and sodium benzoate are used as preservatives for cosmetics and other topical preparations at concentrations from 0.1% to 0.2% and are capable of producing immediate contact reactions at the same concentrations. Cinnamic aldehyde at a concentration of 0.01% may elicit an erythematous response associated with a burning or stinging feeling in the skin [215]. Mouthwashes and chewing gums contain cinnamic aldehyde at concentrations high enough to produce a pleasant tingling or lively sensation in the mouth that enhances the sale of the product. Higher concentrations produce lip swelling of typical contact urticaria in normal skin. Eugenol in the mixture inhibits contact sensitization to cinnamic aldehyde and inhibits nonimmunologic contact urticaria from this same substance. The mechanism of the quenching effect is not certain, but competitive inhibition at the receptor level may be the explanation [109].

Immunologic contact urticaria is an immediate type 1 allergic reaction in people previously sensitized to the causative agent [314]. The molecules of a contact urticant react with specific IgE molecules attached to mast cell membranes. The cutaneous signs are elicited by vasoactive substances, including histamine, released from mast cells. The role of histamine is conspicuous, but other mediators of inflammation (i.e., prostaglandins, leukotrienes, and kinins) may influence the degree of response. Immunologic contact urticaria reactions can extend beyond the contact site, and generalized urticaria may be accompanied by other symptoms, such as rhinitis, conjunctivitis, asthma, and even anaphylactic shock. The term *contact urticaria syndrome* was therefore suggested by Maibach and Johnson [206]. The name has been accepted for a symptom complex where local urticaria occurs at the contact site with symptoms in other parts of the skin or in target organs, such as the nose and throat, lung, and gastrointestinal and cardiovascular systems. Fortunately, the appearance of systemic signs is rare, but it may be seen in cases of strong hypersensitivity or in a widespread exposure and abundant percutaneous absorption of an allergen.

Foodstuffs are the most common causes of immunologic contact urticaria. The otolaryngeal area is a site where immediate contact reactions frequently are provoked by food allergens, most often in atopic individuals. The actual antigens are often proteins or protein complexes. As a proof of immediate hypersensitivity, specific IgE antibodies against the causative agent typically can be found in the patient's serum using the RAST technique and skin test for immediate allergy. The passive transfer test (Prausnitz–Kustner test) also often gives a positive result.

Predictive assays for evaluating the ability of materials to produce nonimmunologic contact urticaria have been developed. No predictive assays for immunologic contact urticaria have been published. Lahti and Maibach developed an assay in guinea pigs using materials known to produce urticaria in humans. One-tenth of a milliliter of the material is applied to one ear of the animal; it is also applied to the opposite ear as a control. Ear thickness is measured prior to application and then every 15 min for 1 or 2 h after application. The swelling response is dependent on the concentration of the eliciting substance. The maximum response is about a 100% increase in ear thickness, and it appears within 50 min after the application of a contact urticant. In histologic sections, marked dermal edema and intra- and perivascular infiltrate of heterophilic granulocytes appear 40 min after the application of test substances. This assay is the predictive test of choice for nonimmunologic contact urticaria if animals are to be tested. Guinea pig body skin reacts with quick-appearing erythema to cinnamic aldehyde, methyl nicotinate, and dimethyl sulfoxide but not benzoic acid, sorbic acid, or cinnamic acid. Analogous reactions can be elicited in the earlobes of another animal species. Cinnamic aldehyde and dimethyl sulfoxide produce a swelling reaction in the guinea pig, rat, and mouse. Benzoic acid, sorbic acid, cinnamic acid, diethyl fumarate, and methyl nicotinate produce no response in the rat or mouse, but the guinea pig ear reacts to all of them [183]. This suggests that either there are several mechanisms of nonimmunologic contact urticaria or that differences are due to the relative sensitivity of the species to the mediators.

Materials can also be screened for nonimmunologic contact urticaria in humans. A small amount of the test material is applied to a marked site on the forehead, and the vehicle is applied to a parallel site. The areas are evaluated at approximately 20–30 min after the application for erythema or edema [314]. Differentiating between nonspecific irritant reactions and contact urticaria may be difficult. Strong irritants, such as hydrochloric acid, lactic acid, cobalt chloride, formaldehyde, and phenol, can cause clear-cut immediate healing if the concentration is high enough, but the reactions usually do not fade away within a few hours. Instead, they are followed by signs of irritation; erythema and scaling or crusting are seen 24 h later. Some substances have only urticant properties (e.g., benzoic acid and nicotinic acid esters), some are pure irritants (e.g., SLS), and some have both of these features (formaldehyde and dimethyl sulfoxide). Contact urticaria reactions are much less frequently encountered than either skin irritation or skin allergy [185]; however, increasing awareness of contact urticaria has expanded the list of etiologic agents and perhaps will lead to the development of adequate predictive assays for detecting causative agents of other forms of urticaria.

Subjective Irritation and Paresthesia

Cutaneous application of some chemicals elicits sensory discomfort, tingling, and burning without visible inflammation. This noninflammatory painful response has been termed *subjective irritation* [107,111]. Materials reported to produce subjective irritation include dimethyl sulfoxide, some benzoyl peroxide preparations, and the chemicals salicylic acid, propylene glycol, amyl-dimethylparamino benzoic acid, and 2-ethoxyethyl-*p*-methoxy cinnamate, which are ingredients of cosmetics and OTC drugs. Pyrethroids, a group of broad-spectrum insecticides, produce a similar condition that may lead to temporary

numbness, or paresthesia [9,50,177]. As in subjective irritation, the nasolabial folds, cheeks, and periorbital areas are frequently involved. The ear is particularly sensitive to the pyrethroids. Herbst et al. investigated an assay to quantify subjective irritation using a pyrethroid chemical model [353].

Only a portion of the human population seems to develop nonpyrethroid subjective irritation. Frosh and Kligman [107] found that they needed to prescreen subjects to identify *stingers* for conducting predictive assays. Only 20% of subjects exposed to 5% aqueous lactic acid in a hot humid environment developed stinging responses [107]. All stingers in their series reported a history of adverse reactions to facial cosmetics, soaps, etc. A similar screening procedure by Lammintausta et al. [188] identified 18% of their subjects as stingers. Prior skin damage, such as sunburn, pretreatment with surfactants, and tape stripping, increases the intensity of responses in stingers. Persons not normally experiencing a response report pain upon exposure to lactic acid or other agents that produce subjective irritation [107]. Attempts to identify reactive subjects by association with other skin descriptions (e.g., atopy, skin type, degree of skin dryness) have not yet been fruitful; however, data show that stingers develop stronger reactions to materials, causing nonimmunologic contact urticaria and some increase in TEWL and blood flow following the application of irritants via patches than those of nonstingers [187].

The mechanisms by which materials produce subjective irritation have not been investigated extensively. Pyrethroids act directly on the axon by interfering with the channel-gating mechanism and impulse firing [312]. It has been suggested that agents causing subjective irritation act via a similar mechanism because no visible inflammation is present. An animal model was developed to rate paresthesia to pyrethroids and may be useful for other agents [50,98]. The test site is the flank of 300 to 450 g guinea pigs. Both flanks are shaved, and animals are housed individually in observation cages. A volume of 100 µL of the test material is spread over approximately 30 mm^2 on one flank. The animal's behavior is monitored by an unmanned video camera for 5 min and at 0.5, 1, 2, 4, and 6 h after the application of the materials. Subsequently, the film is analyzed for the number of full turns of the head made to the control and pyrethroid-treated flanks. Head turns were usually accompanied by attempted licking and biting of the application sites. It was possible to rank pyrethroids for their ability to produce paresthesia using this technique. The ranking corresponded to the ranking available from human exposure.

As originally published, the human subjective irritation assay required the use of a 110°F environmental chamber with 80% relative humidity [107]. Volunteers were seated in the chamber until a profuse facial sweating was observed. Sweat was removed from the nasolabial fold and cheek. A 5% aqueous solution of lactic acid was then rubbed briskly over the area. Those who reported stinging for 3–5 min within the first 15 min were designated as stingers and used for subsequent tests. Subjects were asked to evaluate the degree of stinging as 0 = no stinging, 1 = slight stinging, 2 = moderate stinging, and 3 = severe stinging. Stinging was evaluated 10 s and 2.5, 5, and 8 min after the application of the test material. Other investigators [187] used a 15 min treatment with a commercial facial sauna to produce facial sweating. The subjects turn away from the sauna for application of the test materials, then turn back to face the sauna for the observation period. The facial sauna technique is less stressful to both subjects and investigators and produces similar results.

Advances in understanding the somatosensory processes in humans are leading to the development of more objective methods for evaluating skin sensory effects [336,339]. Although these approaches are not yet sensitive enough to warrant use in predictive assays, they have been used to study pain and itch responses to histamine [341] and to some solvents [340]. In time, these techniques will undoubtedly be applied to predictive testing.

QUESTIONS

27.1 A chemically exposed occupational worker developed generalized urticaria and shortness of breath at the work site. Differential diagnosis includes
- a. Photoirritation
- b. Allergic contact dermatitis
- c. Contact urticaria syndrome (answer)

27.2 On introduction of a revised topical formulation, complaints of acute burn, sting, and itch increase beyond the expected level. Likely diagnostic possibilities include
- a. Photoallergic contact dermatitis
- b. Sensory irritation (answer)
- c. Photoirritation

27.3 Topical formulations may be contraindicated during pregnancy because of concern regarding
- a. Irritant dermatitis
- b. Allergic contact dermatitis
- c. Percutaneous penetration (answer)

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28 Inhalation Toxicology

Joseph D. Brain, Wolfgang G. Kreyling, and John J. Godleski

CONTENTS

INTRODUCTION

The respiratory tract has a unique proximity to the environment. The same thinness and delicacy that qualify the air–blood barrier for the rapid exchange of oxygen and carbon dioxide reduce its effectiveness as a barrier to inhaled microorganisms, allergens, carcinogens, and a wide variety of toxic particles and noxious gases. The surface area of the more than 300 million alveoli is typically 100–150 m^2. The thickness of the air–blood barrier—the distance between inspired air and circulating blood—is less than 1 μm. This is small compared to the barrier between blood and the external environment in the gut or in the skin. Clearly, any discussion of toxicology must include the respiratory tract as an important site for interactions between the internal and external environments.

Not only does anatomy make us vulnerable to airborne threats, so does physiology. The weight of the air breathed each day greatly exceeds the weight of ingested food and water. Adults, depending on their size and physical activity, breathe from 10,000 to 20,000 L of air each day. A wide variety of toxic particles and gases enter the body with this air and are potentially hazardous. In this chapter, we describe particle and gas exposures, discuss how particles deposit in the respiratory tract, and describe diverse physiologic mechanisms that prevent the accumulation and deleterious action of inhaled particles and gases reaching the airways and parenchyma.

It is important to remember that exposure to aerosols or gases do not define the dose produced, nor does it say anything about the anatomic distribution of that dose. Regardless of the aerosol exposure problem being studied, it is essential to quantify the dose and to know as much as possible in regard to the location of particle retention or gas uptake. Our understanding of the biologic effects of inhaled agents stems in part from our understanding of factors important to their uptake and fate. An essential tool for experimental inhalation toxicology is knowledge of how to generate and to deliver aerosols. Ultimately, at the policy level, we must better control the exposure of the public and workers to toxic aerosols and gases to prevent respiratory disease and other pathologic consequences in other organ systems.

This chapter will give the reader an overview of the principles and methods used by the inhalation toxicologist. It will emphasize the methods used in assessing the effects of aerosols and gases on the respiratory system following inhalation. A bibliography is provided for the reader who is interested in a more in-depth treatment of the topics presented here.

ANATOMY AND FUNCTION OF THE RESPIRATORY TRACT

An overview of the anatomy, function, and physiology of the mammalian respiratory system is presented to clarify issues that face the inhalation toxicologist. The respiratory system can be divided into three major components: (1) nasopharyngeal, (2) tracheobronchial, and (3) pulmonary. These components represent distinctly different functions, clearance processes, and susceptibilities to inhaled substances.

Nasopharyngeal Region

The nasopharyngeal region includes the nose, epiglottis, glottis, pharynx, and larynx. As the entry port for inspired air, the nostril openings (nares) and nasal cavity serve to remove the larger inhaled particles (through impaction in the turbinates and filtration by nasal hairs) and to condition the incoming air (by moderating the temperature and raising the humidity). Given its location, the nose is exposed to the highest concentrations of inhaled substances within the respiratory tract; accordingly, the nasopharyngeal region is the subject of considerable investigation.

Nose

The nasal airway is divided into two passages by the nasal septum. Each passage extends from the nostrils to the nasopharynx. The nasopharynx is the airway posterior to the termination of the nasal septum and proximal to the soft palate. Air moves through the nares into the nasal cavity that contains turbinates (bony structures lined by well-vascularized and innervated respiratory or olfactory mucosal tissue) covered by a continuous layer of mucus. The nasal mucus layer is moved distally by underlying cilia to the oropharynx, where it is swallowed into the esophagus. The turbinates project into the airway lumen from the lateral walls into the main chamber of the nose. These increase the inner surface area of the nose, which in humans is about 150–200 cm^2 or about four times that of the trachea (Guilmette et al. 1989).

The nose functions as both an air conduit to the lungs as well as the organ of the sense of smell (olfaction). Though there are some similarities in the nasal passages of most mammals, there are definite interspecies differences in nasal architecture (Figure 28.1). Humans have relatively simple noses with breathing as the primary function, while other mammals have more complex noses with olfaction as the primary function. Although humans (and some nonhuman primates) use both nasal and oral breathing, most laboratory rodents used in inhalation toxicology studies (e.g., rats, mice, hamsters, and guinea pigs) are obligate nose breathers due to the close apposition of the epiglottis to the soft palate. Differences in nasal airflow patterns are due to variation in the shape and complexity of the turbinates. The human nose has three turbinates: superior, middle, and inferior. These structures are relatively simple compared to turbinates in laboratory animal species, such as the dog, rat, and mouse, which have complex folding and branching patterns (Figures 28.1 and 28.2) specialized for olfactory function (Harkema et al. 2006).

The mucus lining at the air–mucosal cell interface of the nasal structures is produced by mucous (goblet) cells in the surface epithelium and subepithelial glands in the lamina propria. The surface epithelial cell populations lining the nasal passages differ among species. These differences include the distribution of nasal epithelial populations and the types of

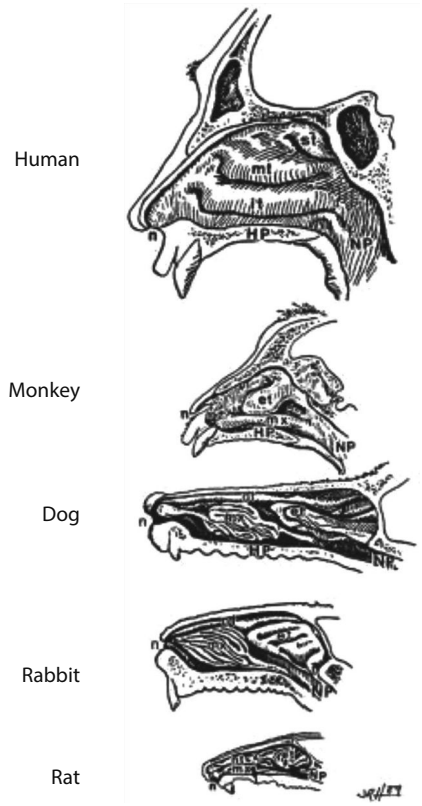

FIGURE 28.1 Diagrammatic representation of the exposed mucosal surface of the lateral wall and turbinates in the nasal airway of the human, monkey, dog, rabbit, and rat. HP, hard palate; n, naris; NP, nasopharynx; et, ethmoturbinates; nt, nasoturbinate; mx, maxilloturbinate; mt, middle turbinate; it, inferior turbinate; st, superior turbinate. (From Harkema, J.R. et al., *Toxicol. Pathol*., 34(3), 252, 2006. With permission.)

nasal cells within these populations. There are four distinct nasal epithelial populations in most animal species. These include the squamous epithelium of the nasal vestibule; ciliated pseudostratified cuboidal/columnar epithelium in the main chamber and nasopharynx; nonciliated cuboidal/columnar epithelium (transitional) lying between squamous epithelium and the respiratory epithelium in the proximal or anterior aspect of the main chamber; and olfactory epithelium, located in the dorsal or dorsoposterior aspect of the nasal cavity (Harkema et al. 2006).

The olfactory epithelium has specialized neural cells for its primary role of odor detection. Olfactory cells process odors to help identify predators, prey, and hazardous substances/conditions. The olfactory epithelium covers a much greater percentage of the nasal cavity in rodents compared to humans. About 50% of the nasal cavity surface area in F344 rats is lined by this sensory neuroepithelium (Gross et al. 1982). Olfactory epithelium in humans is limited to an area of about 500 mm^2, which is only 3% of the total surface area of the nasal cavity (Sorokin 1988). There are three cell types of olfactory epithelium: the olfactory sensory neurons, the supporting (sustentacular) cells, and the basal cells. The neurons are bipolar cells interposed between the sustentacular cells (Vollrath et al. 1985, Farbman 1994). The dendritic portions of these neurons extend above the epithelial surface and terminate into the olfactory knob from which protrude immotile cilia (Menco 1983). These cilia provide an extensive surface area for reception of odorants. The ciliary membranes contain odorant receptors responsible for chemical interaction with odors. Odorant receptors are G protein–coupled, seven transmembrane domain proteins (Buck and Axel 1991, Mombaerts 1999, 2001). The nasal cavity of a mouse has approximately two million olfactory sensory neurons (Harkema et al. 2006). The axial portion of the neurons form nerve bundles that form the olfactory nerves and travel to the olfactory bulb of the brain. Tissue spaces or lymphatic spaces along these nerves are pathways by which toxicants may directly translocate to the brain (Oberdorster et al. 2004).

The supranuclear cytoplasm of sustentacular cells has abundant smooth endoplasmic reticulum and xenobiotic-metabolizing enzymes including hydrolytic enzymes (especially carboxylesterases) necessary to detoxify inhaled substances (such as aromatic esters). Sustentacular cells also contribute to the regulation of the ionic composition of the overlying mucous layer, which may affect the chemical interactions between odors and their odorant receptors. Basal cells are the progenitor cells for olfactory epithelium. Exposure to inhaled substances may injure the epithelium leading to either temporary or permanent loss of smell (anosmia). Importantly, the olfactory cells differ from other nerve tissue cells because they have the capability to regenerate following injury, provided the olfactory epithelium and their progenitor cells are not completely destroyed (Keenan et al. 1990, Bergman et al. 2002).

In addition to the cells described previously, there are several other types of more rare scattered cells called microvillus cells, distinctive from neuronal and sustentacular cells (Menco and Morrison 2003). Bowman's glands, located in the underlying lamina propria, are simple tubular-type glands composed of small compact acini. Ducts from these glands pass through the basal lamina to the luminal surface. Bowman's glands contain neutral and acidic mucosubstances that contribute to the mucous layer covering the luminal surface of the olfactory epithelium (Harkema et al. 2006).

Transitional epithelial cells of rodents located anteriorly in the nose have no secretory granules but do have abundant smooth endoplasmic reticulum in their apices (Harkema et al. 1987). These cells also have xenobiotic-metabolizing enzymes, including cytochromes P-450. The majority of the nonolfactory nasal epithelium of laboratory animals and humans is ciliated respiratory epithelium. About 46% of the nasal cavity in F344 rats is lined by respiratory epithelium (Gross et al. 1982). These cells are responsible for the movement of mucus and the materials trapped within from the nasal surface. Nonciliated cells among the ciliated cells are rich in metabolizing enzymes. There is yet another specialized epithelium, the lymphoepithelium, in animal nasal airways that covers discrete focal aggregates of nasal-associated lymphoid tissue in the underlying lamina propria. The correlate of rodent nasal-associated lymphoid tissue in

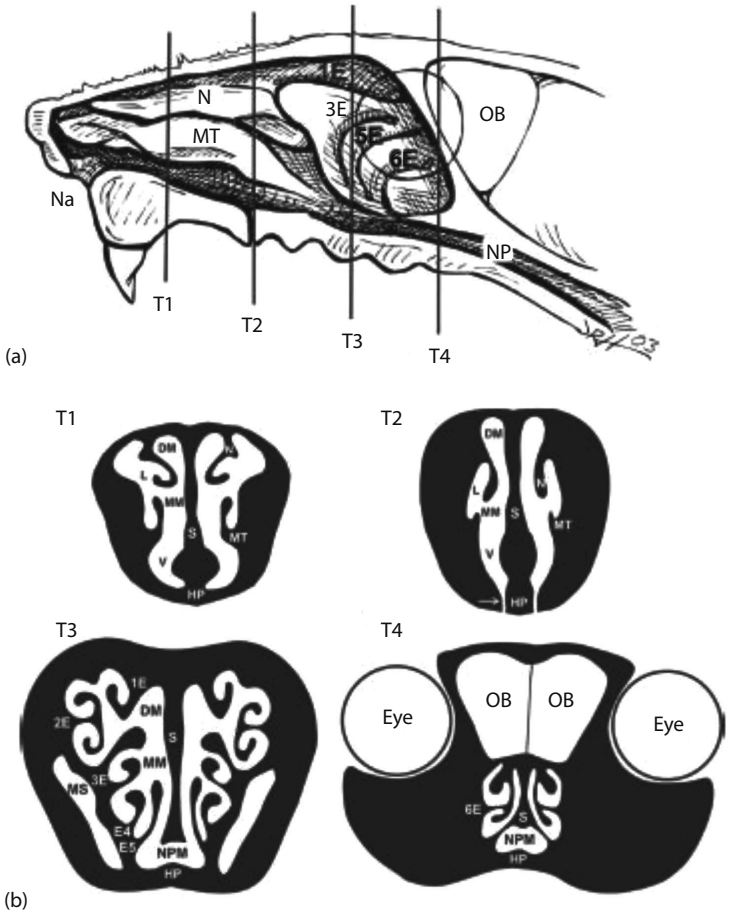

FIGURE 28.2 (a) Illustration of the lateral wall and turbinates in the nasal passage of a mouse. Vertical lines indicate the location of the anterior faces of four cross-sectional nasal tissue blocks routinely sampled for light microscopic examination (T1–T4). (b) Anterior faces of selected tissue blocks from the proximal (T1) to the distal (T4) nasal airway. Note the complexity of the olfactory structure in T3. In T4, the air conduit is smaller with the eye, the olfactory bulb of the brain, and facial muscles/sinuses (not labeled but part of the inferior black areas of the illustration taking much more space). N, nasoturbinate; MT, maxilloturbinate; 1E–6E, 6 ethmoturbinates; Na, naris; NP, nasopharynx; HP, hard palate; OB, olfactory bulb of the brain; S, septum; V, ventral meatus; MM, middle meatus; L, lateral meatus; DM, dorsomedial meatus; arrow in T2, nasopalatine duct; MS, maxillary sinus; NPM, nasopharyngeal meatus. (From Harkema, J.R. et al., *Toxicol. Pathol*., 34(3), 252, 2006. With permission.)

humans is Waldeyer's ring, the oropharyngeal lymphoid tissues composed of the adenoid, bilateral tubule, palatine, and lingual tonsils (Brandtzaeg 1984). These lymphoid structures in both species have roles in immune responses.

Larynx

The larynx is found at the upper part of the trachea in most vertebrate animals, and contains the vocal cords. The walls of the larynx are made of cartilage. Sound is produced by air passing through the larynx, causing the walls of the larynx to vibrate. The pitch of the sound produced can be altered by the pull of muscles, which changes the tension of the vocal cords. The surface of the larynx is covered with squamous epithelium. There are interspecies differences among laboratory rodents in the anatomy of sensitive areas of the laryngeal mucosa. In Sprague–Dawley rats, the mucosa covering the epiglottis differs from that of the Syrian golden hamsters in that it is thinner and composed of a mixture of cell types. In contrast, the cartilage of the hamster is much more prominent and forms a distinct protrusion into the lumen at the base of the epiglottis. These anatomic differences can play a role in the use of this information in extrapolating these findings to man (Renne et al. 1993).

Tracheobronchial Airways

The tracheobronchial airways conduct inspired air through a series of branching ducts ending at the terminal bronchioles. Many differing epithelial cell types line this airway and are distinct on the basis of ultrastructural morphology, functionality, and sensitivity to inhaled materials. Considerable variation exists in the abundance of each of these cell types at defined airway levels among species. However, the tracheobronchial epithelia contains predominantly basal, ciliated mucous, Clara, and neuroendocrine cells (Jeffery 1983). Complex layers of airway mucus lines these airways. It consists of an epiphase and hypophase with scattered amounts of surface active material (Yoneda 1976) (Figure 28.3).

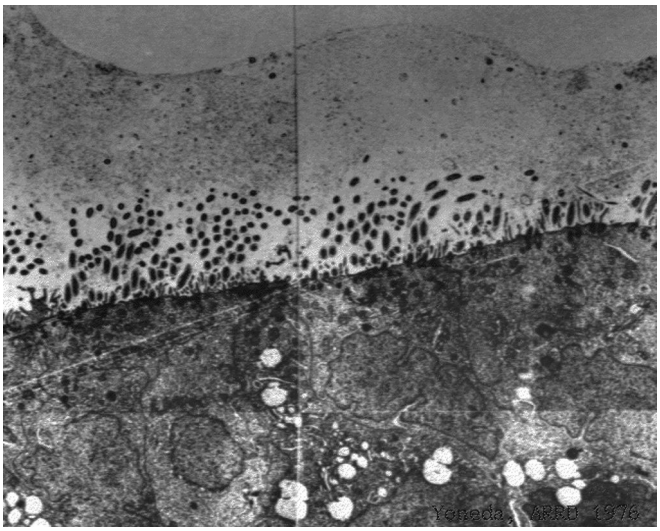

FIGURE 28.3 Large airway of a rat fixed with osmium vapor to preserve the mucous layer. The upper layer of mucous is the epiphase; it is denser and often has fragments of surface active materials. Surrounding the cilia is the watery hypophase. Cilia are seen in cross section and longitudinal sections. Airway epithelial cells make up the bottom half of the figure. (Reprinted from Yoneda, K., *Am. Rev. Respir. Dis*., 114(5), 837, 1976. With permission.)

Mucociliary clearance is a protective mechanism by which contaminants of inhaled air are trapped or dissolved in the mucous layer and then removed by ciliary transport. The two critical components of the system are the of mucus blanket and ciliated cells. Methods to define the composition of mucous include carbohydrate histochemistry and cytochemistry, autoradiography, immunohistochemistry, and biochemistry (Lamb and Reid 1969, Rose et al. 1979, Spicer et al. 1983, St. George et al. 1985). Assessment of mucociliary escalator transport function has been useful in measuring the biological response to inhaled gases and cigarette smoke in particular.

Marked species differences exist in the geometric structure of the tracheobronchial region. Typically, the branching patterns of the tracheobronchial tree are either monopodal or regular dichotomous. The monopodal pattern is characterized by long tapering airways with asymmetric, smaller lateral branches that leave the main tube at a shallow (<60°) angle as seen in cats, dogs, hamsters, horses, mice, monkeys, pigs, rabbits, and rats. In contrast, regular dichotomous branching, typical of humans, involves division of a tube into nearly identical equal-diameter smaller tubes with approximately equal branching angles. Variations in symmetry of branching, airway diameters and lengths, and number of airway generations can affect deposition of particles in the respiratory tract and can account for observed differences. Mathematical airway models suggest that the uptake kinetics of inhaled gases (oxygen, nitrogen dioxide, and sulfur dioxide) vary substantially among rats, dogs, and humans. Such variations are at least partially the result of anatomical and physiological differences in their airways (Tsujino et al. 2005).

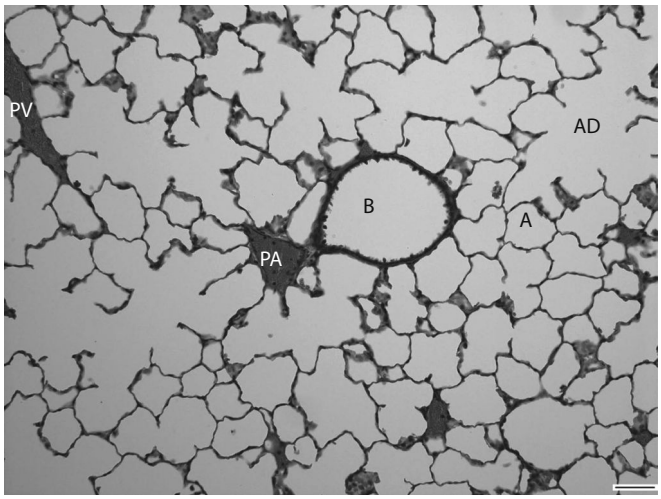

FIGURE 28.4 Histology of the pulmonary region of the rat lung illustrating the thinness of alveolar septae. B, bronchiole; PA, pulmonary artery; AD, alveolar duct; A, alveolus; PV, pulmonary vein. Bar = 50 μm.

Pulmonary Region

The pulmonary region is composed of respiratory bronchioles, alveolar ducts, and alveoli (Figure 28.4). The alveolus is the functional gas exchange unit of the lung between the air and blood compartments. The alveolus itself has walls formed by epithelial cells. Major cell types of alveolar epithelium are known as type I and type II cells. The type I epithelial cell is very thin, has a smooth surface, and covers nearly the entire alveolar surface. The type II cell contains numerous microvilli, is metabolically active, and manufactures and secretes surfactant(s), which, by reducing surface tension at the air–liquid interface (ALI) of the alveolus, reduces the tendency for alveoli to collapse.

Another important cell type in the lung is the alveolar macrophage (Brain 1985, 1988). This is a large, mononuclear cell that functions to engulf foreign particulate material deposited in this region. These cells are scavengers of inhaled particulate material, and their location on the alveolar surfaces preserves the sterility of the deep lung by keeping inhaled bacteria and particles from accumulating. Similar to the nose, larynx, trachea, and bronchi, the bronchioles are lined by ciliated mucosa with columnar goblet cells that manufacture and secrete mucus. The cilia and mucus work together in a coordinated fashion to propel particulate material entrained in the mucus away from the lungs.

Measurement of breathing pattern, lung flows and volumes, lung capacities, forced expirograms, and blood gas and pH are important in determining not only the functional performance of the lung but also are used to approximate the dose of a substance received under experimental conditions. Reliable values for these parameters can be obtained during exposures using whole-body plethysmographs (Diaz et al. 2011). A comparison of typical breathing characteristics of a number of commonly studied species is shown in Table 28.1.

TABLE 28.1
General Breathing Characteristics of Common Species

Species	Mass (g)	Frequency (Breaths/min)	Tidal Volume (mL)	Minute Ventilation (mL)
Human	70,000			
Rest		12	750	9000
Light exercise		17	1700	28,900
Dog	10,000	20	200	3600
Monkey	3000	40	21	840
Guinea pig	500	90	2.0	180
Rat	350	160	1.4	240
Mouse	30	180	0.25	45

Allometric scaling factors ($Y = kM^x$; M in kg)	k	x	k	x	k	x
	53.5	–0.26	7.69	1.04	411	0.78

Source: Adapted from Boggs, D.F., in *Treatise on Pulmonary Toxicology*, Vol. 1, *Comparative Biology of the Normal Lung*, Parent, R., ed., CRC Press Inc., Boca Raton, FL, 1992.

GENERATING AND CHARACTERIZING EXPOSURES TO INHALED PARTICLES AND GASES

National and international regulatory agencies have implemented testing guidelines regarding the conduct of inhalation toxicity studies. These guidelines attempt to standardize testing procedures so that toxicity data may be obtained and compared using a defined protocol. Test data generated in this fashion may be used in a variety of ways: (1) to investigate the relationship between exposure concentrations and adverse effects, (2) to provide information on the mechanism of toxicity and permit a reasoned extrapolation of the experimental animal data to potential human health risks, (3) to form the basis for dose-level selection for repeated-exposure studies, or (4) to categorize the inhalation toxicity of a test material relative to other substances. Such data are the foundation for establishing science-based exposure limits for the protection of human health and appropriate toxicity classification and labeling of substances.

Regulatory agencies have adopted standardized protocols for both short-term and long-term inhalation testing and are harmonizing protocols internationally. Guidance on general study design, exposure conditions, measurement frequencies, and major end points of concern have been published for acute, subchronic, and chronic inhalation toxicity testing (OECD 2006b, EPA 2006c).

Exposure Systems

Inhalation exposure systems involve the harmonious integration of several subsystems whose design, construction, and operation are critical to a safe, functional system allowing generation of meaningful results. The various subsystems are shown schematically in Figure 28.5 and include a conditioned air supply system, a suitable vapor or aerosol generator for the test substance, an atmosphere dilution and delivery system, one or more exposure chambers, an atmosphere sampling and analytical system, and an exhaust/scrubbing system. There are several inhalation modes of exposure to evaluate substances appropriate spontaneous breathing animals and ventilated animals (i.e., nose only or whole body for the former and controlled ventilation of the intubated animal for the latter). While the design, operational parameters, testing objectives, and technical requirements for these approaches are similar, there are several issues to consider when choosing one mode versus another (Table 28.2). Specific

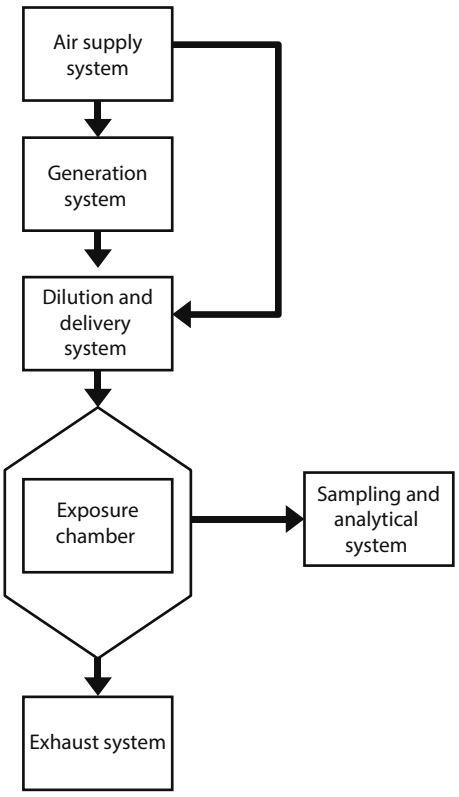

FIGURE 28.5 Components of an inhalation exposure system.

TABLE 28.2
Testing Guidelines for Acute Inhalation Toxicity Studies

Criteria	U.S. EPA OPPTS 870.130[a]	Europe OECD 436[b]	Japan MAFF[c]
Study design			
Species[d]	Rat	Rat	Rat
Sex and number per group	≥5 of each sex[e]	3 male, 3 female	5 male, 5 female
Age	Young adult, 8–12 weeks	8–12 weeks	Young adult
Weight range	<±20% of mean weight per sex	<±20% of mean weight per sex	Not specified
Exposure time	4 h after equilibration of chamber concentration	4 h after equilibration of chamber concentration	4 h
Observation period (days)	14	14	14
Limit test (mg/L)[f]	2	5	5
Exposure conditions			
Chamber temperature	22°C ± 2°C	22°C ± 2°C	22°C ± 2°C
Relative humidity (%)	30–70	30–70	40–60
Airflow	≥10 air changes/h	Not specified	Not specified
Chamber oxygen concentration	At least 19%	At least 19%	At least 19%
Particle size distribution	1–4 μm MMAD	1–4 μm MMAD	Not specified
Chamber loading	Total animal volume <5% chamber volume	Total animal volume <5% chamber volume	Not specified
Frequency of measures			
Airflow	Monitor continuously, record >3 times/exposure	Prefer continuously	Not specified
Particle size	2–4 times/exposure	Atleast twice during each 4 h exposure	As needed to determine consistency
Exposure concentration	2–4 times/exposure	Either continuously or atleast twice during each 4 h exposure	Not specified
Temperature and humidity	Monitor continuously, record ≥3 times/exposure	Prefer continuously, or atleast 3 times during each 4 h exposure	Not specified
Nominal concentration[g]	Required but frequency not specified	Prefer continuously	Not specified
End points			
Morbidity and mortality	Yes; daily	Yes; daily	Yes
Clinical observations	Yes; daily	Yes; daily	Yes
Body weights	Yes; weekly	Yes; daily	Yes
Gross pathology	Yes; at termination	Recommended	Recommended
Histopathology	Optional; in animals with gross changes at necropsy	Discretionary	Discretionary

[a] This harmonized guideline was developed in 1998 by the Office of Prevention, Pesticides and Toxic Substances to blend the testing requirements for the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Toxic Substances Control Act (TSCA) with the Organisation for Economic Co-operation and Development (OECD).

[b] OECD Guideline for the Testing of Chemicals, Acute Inhalation Toxicity, Section 436, 1-4, 2009. At the time of publication, a revised, harmonized OECD guideline had not been issued.

[c] Requirements for Safety Evaluation of Agricultural Chemicals. Testing Guidelines for Toxicology Studies, Ministry of Agriculture, Forestry and Fisheries (Japan), Acute Inhalation Toxicity Study, 1985.

[d] Mammals are specified in the test guidelines, but rats are generally preferred.

[e] At least five experimentally naive animals are used of each concentration, and they should be of one sex. After completion of the study in one sex, at least one group of five animals of the other sex is exposed to establish that animals of this sex are not markedly more sensitive to the test substance.

[f] If exposure at the limit concentration (LC) (or where this is not possible due to the physical characteristics of the test substance) produces no observable toxic effects, a full study may not be necessary.

[g] Amount of test material delivered to the generation system divided by the air volume used during the exposure.

components typically used for test substance generation and analysis and animal exposure are shown in Figure 28.6.

Conditioned Air Supply

A sufficient amount of clean, conditioned air for chamber operation must be supplied. The availability of high-quality, contaminant-free (e.g., particles or organics) air for chamber supply is essential, especially for long-term inhalation studies. Ordinarily, ambient air is dried, filtered, and purged of organic vapors prior to adjusting its temperature and humidity to the desired levels. Where this is not practical for very small, low airflow chambers with limited numbers of animals, a commercial source of compressed purified air may be utilized. The size of the air-conditioning equipment should

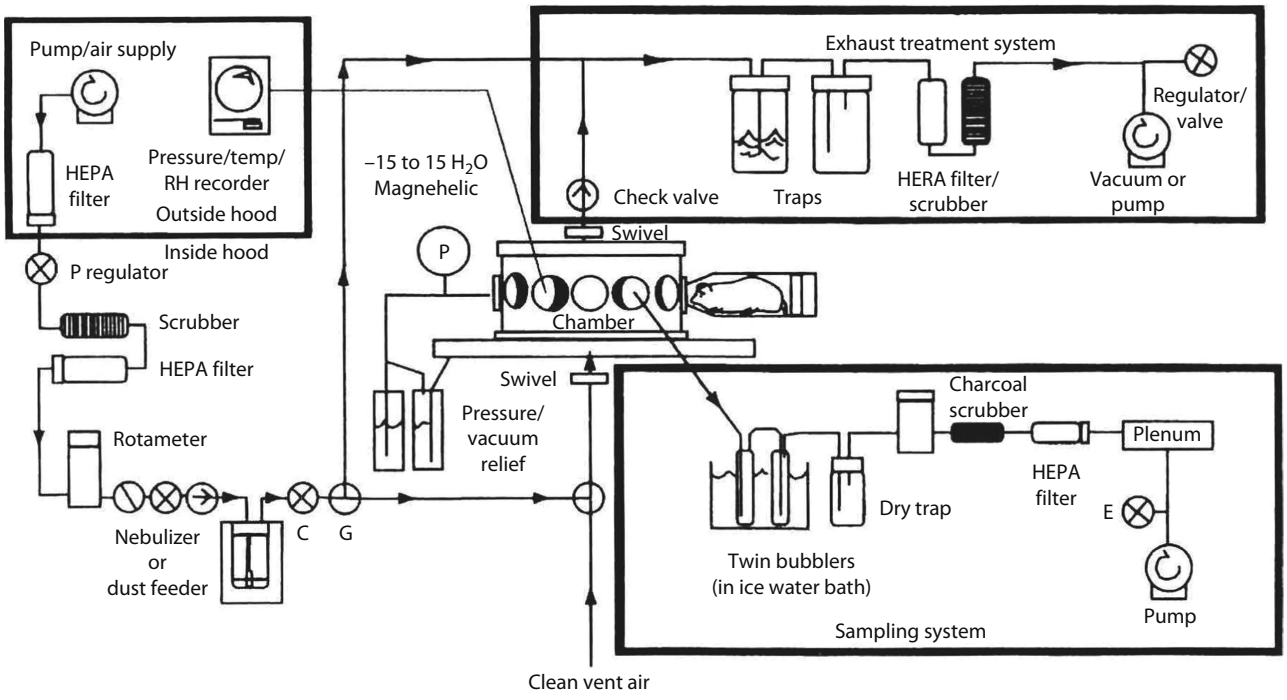

FIGURE 28.6 Details of components used for air supply and test atmosphere generation, dilution and sampling, and exhaust in a nose-only inhalation exposure system.

be scaled to allow some excess flow capacity for the required number of chambers operating at the desired flow rates.

Airflow rate, temperature, and humidity need to be maintained within fairly tight ranges to ensure environmental conditions are acceptable to the well-being of the test species. Excursions beyond the normal range could result in varying overall exposures and could lead to altered test species responses to any given concentration. Typically, environmental conditions are targeted to a temperature from 20°C to 24°C, humidity from 30% to 70%, and airflow greater than 10 air changes/h. Modern inhalation systems have been automated for chamber monitoring and exposure control, which eliminate the need for manual adjustments and data logging. It is important that these conditions remain relatively consistent for the duration of the exposure (and from exposure to exposure).

Generation of Test Atmospheres

Generation of Gases and Vapors

Gases are the simplest atmospheres to generate. They can be metered by flow meters, syringe drives, diffusion tubes, or other techniques into a dilution airstream, allowed to mix, and then introduced into the exposure chamber. A number of flow dilution devices are available. Vapors of either liquid or solid compounds can be formed by heating within a temperature-controlled heating device (care must be taken to prevent chemical decomposition or reaction with air or water at elevated temperatures used to generate vapor). Another technique, depending on physical properties such as boiling point, viscosity, or chemical purity, is to use an infusion pump to meter the liquid test substance onto a heated surface. Other liquid materials may be vaporized in fritted glass bubblers. Frequently, nitrogen is used to vaporize the test substance to minimize chemical reaction or oxidation with air at elevated temperatures. The saturated stream can then be diluted with filtered air or oxygen to raise the oxygen concentration to 19%–21% and to adjust the test substance level to the desired concentration. The application of an automatic proportional-integral-derivative (PID) control algorithm to an inhalation exposure system operating with a concentration monitoring system that samples on a 30 min cycle was shown to produce exposures that accurately achieved and maintained the targeted concentration (Wong 2003).

Special care must be taken when generating vapors from mixtures of test substances with low boiling points. If test atmospheres are generated from the liquid mixture by flash evaporation, the composition of the resulting vapor will be representative of the composition of the liquid mixture. In contrast, if the headspace of the liquid mixture is tapped, the resulting vapor composition in the mixture will reflect the partial pressures of the components with a disproportionate amount of the component with the highest vapor pressure relative to the other components. This is a problem in longer-term inhalation studies, because the composition of the test atmosphere will vary from day to day as the higher vapor pressure components of the mixture are depleted as the mixture is consumed.

Generation of Aerosols

The generation of particulate materials in a uniform manner is considerably more difficult than vapor generation. While two decades ago the focus of inhaled particle dosimetry and toxicology was on micron-sized particles with a characteristic

diameter d of $15 > d > 0.5$ μm, this has changed substantially since currently there is great interest in the potentially hazardous effects associated with the inhalation of ambient ultrafine particles (UFPs) or engineered nanoparticles (ENPs). Several definitions for nanomaterials have been formulated by various national and international bodies including the U.S. FDA (FDA 2007), SCENIHR (2007), ISO (ISO/TS 2008), OECD (2008), Health Canada (Canada 2010), EC Recommendation (EC 2011). Both definitions span the same size range from about 1 to 100 nm with some uncertainties about how to deal with particles or clusters <1 nm and also slightly particles >100 nm. While UFPs—particularly those generated incidentally by human activities such as combustion—are usually a complex mixture of many chemical compounds mostly originating from burning and further modified by chemical reactions according to the thermodynamic condition of the ambient atmosphere, ENPs are specifically designed to offer specific physicochemical properties used for specific functional particle behavior and responses with neighboring materials.

Human ENP toxicology is particularly interested in possible reactions of biological systems, such as organs and tissues as well as body and cellular fluids. Ecotoxicology focuses on responses of ecosystems within aquatic and terrestrial environments. The prominent interest in UFP and ENP relates to changes of the physicochemical properties during shrinkage from micron-sized to nanometer-scaled particles such as quantum physics phenomena (e.g., quantum dots), catalytic and photocatalytic properties (e.g., gold ENP <3 nm or titanium dioxide ENP), ultraviolet (UV)-light absorption (e.g., titanium dioxide, zinc oxide), and the increasing ratio of surface atoms or molecules to internal atoms or molecules within the particle lattice when particle size decreases. This may lead to defects in the outer lattice or may result in unsaturated and hence highly reactive molecules on the high curvature surface of ENP and UFP thereby forming of oxygen and nitrogen radicals, and other highly reactive biomolecules altering homeostasis when compared with submicrometer particles and micron-sized particles. The altered physicochemical properties, together with the increased reactivity of ENP, inspired great hopes for new functional particles and triggered new products and their applications in almost all technological, scientific, and medicinal fields (EC 2005, NCI 2013, EC 2013). At the same time, concerns have been raised that the altered physicochemical properties, together with the increased reactivity of ENP, may also lead to unknown and unexpected biological responses either in human and mammalian bodies or in ecological systems, which may lead to adverse health effects and disturbances of the delicate balance of environmental ecosystems.

Particulate materials may be generated from dry powders or from liquids or by evaporation and subsequent condensation. The generation of aerosols using dry dispersion techniques presents problems that are unique to each dust being studied. In toxicology testing, particle concentration and size distribution must remain constant over long periods of time. The powder being tested must be dispersed into unitary particles of respirable size rather than agglomerates. This requires a means of continuously metering a powder into the aerosol generator at a constant rate, a means of disrupting particle clumps/agglomerates, and a way of suspending the resulting discrete particles into the airstream. Note that disaggregation of particle aggregates is usually not occurring due to chemical bonds within the aggregates. Most materials contain particles of irregular size and shape, which means that monodisperse aerosols rarely are produced and that, in the generating system, the airborne particle size distribution will differ from that in the original powder.

Simple dust metering systems use a gravity feed of loose powder into an airstream, usually assisted by screw-driven conveyors, agitators, or vibrators. Volumetric feeders are useful to deliver specific amounts of powder from a reservoir or hopper into an aerosol generator. The classical Wright dust feed, for example, uses a scraping mechanism to remove a finely ground powder from the surface of a cylinder packed with a test substance. Other metering systems use metal screws or brushes to transport powders at user-selectable rates to the aerosol generator.

Venturi Dispersion of Powders

Aerosol generation involves the dispersion of a powder by supplying sufficient energy, such as a high-velocity airstream, to a relatively small volume of the bulk powder to separate the particles by overcoming their own attraction forces. Hydrophobic materials, such as talc, are more easily dispersed than hydrophilic materials, such as limestone or quartz, because particle agglomeration or clumping is minimal. Thus, dry powders are considerably easier to disperse than humidified ones. The metered powder may be dispersed and agglomerates broken up directly by a turbulent air jet, or the dust-laden airstream can be passed through an impactor or fluidized bed, for example, according to the simple design and easy-to-operate Venturi principle: the flow in the Venturi tube creates suction near the constricted area to feed powders into the device, and the shear flow downstream of the Venturi tube disperses particles into aerosol form. Powders are fed into the Venturi disperser by a screw feeder.

Elutriators or settling chambers have been used to selectively remove large agglomerates and are also useful in dispersion. Clean, dry air can be used to generate aerosols, but it should be noted that extremely dry air (relative humidity less than 50%) can induce strong electrostatic charges on particles favoring agglomeration and reducing their dispersability. As has been discussed, steps must be taken to ensure that the particles generated are respirable for the test species.

Willeke et al. (1974) describe fluidized bed aerosol generators that are capable of a very stable output of particles from 0.5 to 3 μm. This generator uses a fluidized bed of glass or metallic beads (100–200 μm) into which the powdered test substance is added. The mechanism of dispersion involves the agitation of the particles by the beads and their subsequent entrainment into the airstream from both particle impaction, as the beads collide with one another, as well as aerodynamic turbulence. The entire system also acts as

an elutriator, preventing large particles and agglomerates from leaving the system. This means of generation can only be used for dry, nonadhering powders and produces electrically charged aerosols, which need to be neutralized upon leaving the fluidized bed. Compared to some other dust generators, fluidized bed generators do not generally have high particle outputs, limiting their use in acute toxicity testing.

Lee et al. (1983) used a high-pressure air impingement device (microjet) to separate the finer fibrils from a para-aramid fiber matrix. Once separated from the bulk fiber matrix, the smaller fibers pass through a cyclone into the exposure chamber. This system was used to generate fiber mass concentrations as high as 18 mg/m^3 or fiber count concentrations as low as 2.5 fibers/cm^3 for periods ranging from 2 weeks to 2 years. The microjet is a versatile aerosol generator, finding practical applications for both fibrous and nonfibrous materials. By increasing airflow to the secondary high-pressure air jets, the microjet can be adjusted either to disrupt particle agglomerates or to affect some reduction in particle size through trituration in the oval inner chamber. Other examples of the use of this technology for particle dispersal are described by Cheng et al. (1985) and Bernstein et al. (1984). Bernstein et al. (1984) described a brush-feed micronizing jet mill that produces a relatively wide concentration range of respirable particles. Test concentrations from 0.22 to 7.48 mg/L were maintained with particles less than 3 μm. Only at higher aerosol concentrations were these small particle sizes not attainable.

Aerosolization of Carbon Nanotubes

Carbon nanotubes (CNTs) either consisting of only a single layer of carbon atoms in an annular wall (single-walled carbon nanotube [SWCNT]) of the tube or with double, triple, and multiple layers of carbon atoms in concentric annular walls (multi-walled carbon nanotube [MWCNT]) are being produced worldwide in increasing quantities because of great interest in applications resulting from their unique physicochemical properties. Hence, potential respiratory exposures during production and handling lead to an urgent call for inhalation studies to determine their potential toxicity. Baron et al. (2008) described a design for a generation system producing respirable aerosols at several mg/m³ for a 1-week mouse exposure. The starting material used in their experiments was as-produced powder from the high-pressure carbon monoxide method that was sieved to number 6 mesh (<2.3 mm). An acoustic feeder system was developed that handled the SWCNT powder without causing compaction of the material. The feed rate was adjustable, allowing output concentrations as high as 25 mg/m^3. The powder particles were reduced in size using a mill that produced high shear forces, tearing the agglomerates apart. The resulting aerosol was size-separated using a settling chamber and two cyclones to produce a respirable aerosol. The mass output efficiency of the entire system for producing a respirable aerosol from bulk material was estimated to be about 10%. This exposure system is now being used at the National Institute for Occupational Safety and Health (NIOSH) lab; a first comparison versus oropharyngeal aspiration showed increased inflammatory response in the mouse model as a result of the more equally dispersed deposition of inhalable SWCNT agglomerates versus large agglomerates in the aspirated SWCNT suspension (Shvedova et al. 2008).

There is an increasing need to reach the peripheral lung with dry powder medications as well as with collected ambient aerosols in environmental research projects. In a novel aerosol generator to create short-duration, high-concentration exposures, a fixed volume of compressed air was used to create a short burst of a highly concentrated aerosol in a 300 mL holding chamber. Collected diesel soot was deagglomerated to a fine aerosol with a mass median aerodynamic diameter (MMAD) of 0.55 μm. A fine powder such as 3 μm silica particles was completely deagglomerated to an aerosol with an MMAD of 3.5 μm. The aerosol system is particularly useful for peripheral lung delivery of collected ambient aerosols or dry powder pharmaceuticals following a minimal effort in the formulation of the powder (Gerde et al. 2004).

Given the interest in the relationships between ambient aerosols and morbidity/mortality, researchers are attempting to study collected ambient particle samples in animals. The Harvard Ambient Particle Concentrator (HAPC) is designed to deliver concentrated ambient fine particles (<2.5 μm aerodynamic diameter) for conducting animal and human exposure studies. The HAPC consists of the following components: (a) a size-selective inlet (a high-volume conventional inertial impactor with a 2.5 μm cutpoint) and (b) a series of three virtual impactors (stages I, II, and III) with 0.15 μm cutpoints. Like conventional inertial impactors, virtual impactors separate particles from the surrounding air by particulate inertia. Particles larger than the cutpoint of the impactor possess enough momentum to cross the air streamlines into the collection nozzle; as a result, a small fraction of the total flow passes into the collection nozzle, which contains the concentrated particles (larger than the cutpoint) suspended in air. The three successive virtual impactors produce an enrichment of the aerosol mass concentration of fine ambient particles by a factor of about 30 (Sioutas et al. 1995).

Another such high-volume, ambient air particle concentrator focusing on UFPs (<100 nm thermodynamic diameter) has been described by Gupta et al. (2004). The concentrator is similar to the previously mentioned HAPC but induces particle growth due to water vapor condensation prior to the second set of virtual impactors (for concentrating) and consists of several units, including a size-selective inlet, a condensational growth unit, two virtual impactors (concentrators), a thermal size restoration device, an air cooler, and a size-selective outlet. This system was evaluated using single-component artificial aerosols with a variety of physiocochemical properties as well as ambient air. All UFPs grew and were concentrated by about the same enrichment factor of 10–20, regardless of their composition and surface properties.

Aerosol Generation by Nebulization

Atomization of solutions can be achieved by mechanical methods (i.e., nebulization) or by applying an electrostatic potential between the solution and a counter electrode (i.e., electrohydrodynamic atomization [EHDA]). One way of generating aerosol particles is by atomizing liquid solutions of a specified chemical composition. Atomizers can be categorized depending on the forces applied to break up the liquid into small airborne droplets (Hinds 1999). The most appropriate methods are pneumatic, ultrasonic, and electrospray atomization because they produce relatively small primary droplets (1–10 μm in diameter).

In pneumatic atomizers, pressurized air is introduced via an orifice in such a way that the expanding airstream moves perpendicularly to the end of a tube connected to the liquid reservoir. Due to the Bernoulli effect, the low pressure created at the tube end draws the liquid from the reservoir into the airstream. The high forces occurring at the air–liquid interface (ALI) cause the solution to break up into small liquid droplets that become airborne and are carried away by the airflow. The spray stream is then directed onto an impactor plate where the larger droplets are deposited and are either directed back to the liquid reservoir or break up into smaller droplets and exit the atomizer in the outlet stream.

Ultrasonic atomization is another way of breaking up a solution to micron-sized droplets by ultrasonically vibrating its ALI (Wood and Loomis 1927). The mechanical energy applied from a piezoelectric crystal to the system agitates the surface of the solution, thereby creating capillary waves that break up into micron-sized droplets (Faraday 1831, Kelvin 1871, Rayleigh 1883). A particle-free airstream is passed over the solution to take away the generated airborne particles. The diameter of the primary droplets produced is a function of the frequency of vibration and the physical properties of the solution, that is, surface tension and density (Biskos et al. 2008).

EHDA, or electrospraying, is a method for producing very fine monodisperse droplets from a liquid under the influence of electrical forces. By controlling the liquid flow rate and the electrostatic potential between the liquid and the counter electrode, droplets within a narrow size range, with mean diameters from nanometers up to several micrometers, can be generated. Besides generating monodisperse droplets, electrosprays are also distinguished by their self-dispersing nature due to coulombic repulsion, the possibility of trajectory control of the produced charged droplets, and the reduced risk of nozzle clogging due to the large size of the orifice compared to the size of the droplets (Zeleny 1914, Grace and Marijnissen 1994, Biskos et al. 2008).

Spray Pyrolysis for the Generation of Submicron and Nanosized Aerosols

As recently reviewed by Biskos et al. (2008), in spray pyrolysis, the atomized particles are heated in a furnace (e.g., Messing et al. [1993]), a flame (e.g., Madler et al. [2002]), or a laser beam (e.g., Cauchetier et al. [1994]). The high temperatures cause rapid evaporation of the solvent of the droplets and the initiation of interparticle reactions that lead to the final product. The physical (size, morphology, and density) and chemical properties of the particles generated by spray pyrolysis depend on the composition of the liquid precursors, the atomization method, and the operating conditions of the apparatus.

Flame spray pyrolysis can be performed using aqueous solvents for the delivery of metal and/or metalloid oxide precursors while obtaining desirably high flame temperatures for the synthesis of uniform submicron inorganic metal oxide particles. A multiple liquid channel nozzle can be used to deliver liquid for the formation of the aerosol that is combusted in the flame. One or both channels can deliver liquid with metal/metalloid precursors and/or organic fuels. Recently, a novel system for generation of engineered nanomaterials (ENMs) suitable for in situ toxicological characterization within biological matrices was developed. This Versatile Engineered Nanomaterial Generation System (VENGES) is based on industry-relevant, flame spray pyrolysis aerosol reactors that can scaleably produce ENMs with controlled primary and aggregate particle size, crystallinity, and morphology. ENMs are produced continuously in the gas phase, allowing their continuous transfer to inhalation chambers, without altering their state of agglomeration. The ability of the VENGES system to generate families of ENMs of pure and selected mixtures of iron oxide, silica, and nanosilver with controlled physicochemical properties was demonstrated recently (Sotiriou et al. 2012).

Gas-to-Particle Synthesis

Gas-to-particle synthesis can be achieved by using furnace, flame, plasma, and laser reactors, glowing wires, and spark discharges, as reviewed by Biskos et al. (2008). Material evaporation followed by subsequent nucleation and condensation in an inert gas is a widely used approach for generating aerosol particles of uniform chemical composition. The great advantage of particle synthesis from the gas phase is the high purity and the high production rates (Pratsinis and Mastrangelo 1989). In addition, when the cooling of the gas–vapor system is well controlled, the morphology and size of the particles can be precisely adjusted.

Various energy sources can be used to evaporate materials for particle synthesis in the gas phase. Furnace reactors, glowing wires, and spark discharges use electrical energy to heat and vaporize the desired materials. Flame reactors utilize the heat from combustion to oxidize the vapors, which then form clusters and nanoparticles in the cooler regions of the reactor. Plasma and laser reactors utilize the high energy of an ionized gas (plasma), whereas laser reactors employ the high energy and precision of a laser beam. Details of the various methodologies are discussed by Biskos et al. (2008). Particle synthesis from the gas phase is most appropriate for generating single-component particles. One of the great advantages of gas-phase routes for particle synthesis is that small high-purity particles within a very narrow size range can be generated in great quantities.

The process of spark discharge is initiated by gas breakdown and formation of a conducting plasma channel. The rapid discharge consists of a current associated with a high temperature (typically 2000 K), as discussed by Biskos et al. (2008). Briefly, the electrode material is evaporated in the vicinity of the spark. This is followed by rapid cooling, initially governed by adiabatic expansion and radiation. Below the evaporation temperature, cooling is dominated by thermal conduction. The cooling period at temperatures below the boiling point is relatively small, thereby leading to the formation of high concentrations of very small particles. Spark generators typically consist of a chamber of 10–100 cm^3 in volume, in which two opposing cylindrical electrode rods are mounted at an adjustable distance. The electrodes are connected to a high-voltage power supply and in parallel to a capacitor. The power supply delivers a constant current, periodically recharging the capacitor after discharge has occurred at the breakdown voltage. The spark frequency can typically be adjusted up to 1 kHz. The mean particle size can be controlled via the energy per spark, which is in turn determined by the capacitance and the distance between the electrodes. The particle mass produced per unit time is proportional to the spark frequency. Separated, unagglomerated particles a few nanometers in size can be obtained if the inert gas flow through the generator is high enough with respect to the spark repetition frequency. The nanoparticulate mass produced is typically a few g/kWh. Different electrode materials lead to different mean primary particle sizes and mass production rates. Thermal conductivity, evaporation enthalpy, and the boiling point also influence these parameters.

Recently, the methodology of spark discharge aerosol production was extended to the generation of radiolabeled iridium or titanium dioxide (TiO_2) or gold nanoparticle aerosols using previously neutron- or proton-irradiated electrodes of either iridium, titanium, or gold. A schematic drawing is presented in Figure 28.9. It shows a ventilation apparatus holding four anesthetized rats individually in four plethysmograph chambers. The rats are intubated to connect to the aerosol freshly produced by the spark discharge aerosol generator; in the latter, two iridium electrodes are mounted, which had been previously neutron irradiated in a nuclear reactor to form the ^{192}Ir radioisotope. The freshly generated nanoparticle aerosol with a median diameter of 20 nm (geometric standard deviation [GSD], 1.6) was led into an inhalation apparatus for immediate inhalation of rodents in order to study lung retention and nanoparticle clearance and translocation into the circulation leading to retention in secondary organs and tissues (Kreyling et al. 2002, 2009, Gibson et al. 2011, Möller et al. 2013).

Dilution and Delivery Systems

Once a suitable atmosphere has been generated, it must be delivered to the exposure chambers at an appropriate concentration. Dilution is effected by mixing the test atmosphere with conditioned, filtered air before its introduction to the exposure chamber. It is important that the delivery system be fabricated with materials that minimize wall losses, either through absorptive or reactive processes, and designed to minimize physical losses through aerosol deposition within ductwork. The former situation may be avoided by using nonreactive materials and the latter by minimizing the number of bends and maintaining laminar flow in the ductwork. In general, the delivery system should be nonreactive with tubing as wide, short, and straight as practical to minimize test material losses and pressure drop and in order to minimize the residence time of the aerosol. It is especially important to use electrically conductive materials in the choice of tubing materials. Substantial aerosol deposition can occur in areas of high electrostatic charge, leading to excessive and variable aerosol losses along transfer lines. A broad selection of mechanical and electrical valves and flow measurement devices is available to measure and control the flow of test material and dilution air.

Exposure Chambers

There are a number of options when using inhalation exposure chambers either for human subjects or experimental animals such as rodents or large animals: whole-body exposure chambers, nose-only or head-only exposure chambers, or whole-body plethysmographs for controlled breathing. The selection of a suitable exposure chamber depends on the exposure mode (e.g., whole-body or nose-only exposure or whole-body plethysmographs for controlled breathing), reactivity of the test material, available resources (supply of conditioned air and test material), number of animals to be exposed, and efficiency in delivering test material to the animals. Apart from the obvious difference in size, the design objectives for nose-only or whole-body exposure chambers are similar. Chambers should be constructed of nonreactive materials such as stainless steel, glass, or plastic. Although stainless steel is durable and nonreactive toward many materials, it is comparatively expensive. In contrast, glass or plastic chambers are more readily fabricated, are less expensive, and permit ready observation of animals. However, glass or plastic exposure chambers are electrically nonconductive. This may allow charge differences to accumulate within the chamber and cause test aerosol losses through electrostatic attraction. This also can contribute to unacceptable spatial and temporal variations in chamber aerosol concentrations. Normally, chambers are operated under dynamic exposure conditions (i.e., a continuous supply of air is flowing through the chamber) with a slight negative pressure within the chamber to prevent leakage of test material into the exposure room.

Whole-Body Exposure

In a whole-body exposure chamber, the subject is immersed in the atmosphere contained in the chamber, simulating environmental or workplace exposures. Whole-body exposure chambers come in a wide variety of sizes, from very small chambers that hold one animal to very large, room-sized chambers for humans. Large whole-body chambers are most commonly used for long-duration exposure studies and for large numbers of test subjects. The large chambers

are designed to house the test subjects during exposure and nonexposure periods. These large chambers are operated on a dynamic flow basis where there is a continuous flow of air through the chamber. In order to achieve a homogeneous distribution of the aerosol, great care is required to maintain a homogeneous and uniform displacement flow either horizontally or vertically from the inlet to the outlet of the chamber. A design of such a chamber for human exposure was described by Eduard et al. (2008). For dogs, a whole-body chamber for long-term exposure was described by Karg et al. (1992). Many of the rodent exposure chambers go back to the fundamental design of the Rochester chambers by Leach et al. (1959). Inside a whole-body chamber, animals may be housed individually or in groups. Recently, the design and operation criteria were critically reviewed by Wong (2007). Group-housed animals may huddle leading to the potential that an individual may inhale less of the test compound due to the filtration or reaction with surrounding animals' fur. Also, an individual might inhale air that has been exhaled and cleaned of the test compound by surrounding animals. Both of these possibilities could result in varying uptake of the compound among animals. Also, during preening, animals ingest material that has deposited on the fur. Despite those potential limitations, whole-body exposure chambers for inhalation of test aerosols and gases represent the gold standard of inhalation exposure since their application resembles physiological breathing and the behavior of the animals is not changed by restraint.

Nose Only and Head Only

The test subject is confined so that only the head or nose is exposed to the test atmosphere. A continuous flow of air is supplied to the system (Figure 28.7). Particularly in earlier designs, the test subjects inhale from the plenum containing the test atmosphere and exhale back into the same plenum (Smith et al. 1981). In this type of system, the downstream test subjects may inhale air that was exhaled from the upstream test subjects. If the airflow is too low, downstream test subjects may get a lower dose, or the dose to each test subject may be more variable (Cannon et al. 1983). This potential problem has been addressed by a system design in which the test atmosphere flows into an inlet manifold that directs the flow toward the nose or head of each test subject. The exhaled air is then carried along with the excess airflow into an exhaust system (Figure 28.8). Thus, all the test subjects are exposed to the same atmosphere (Prasad et al. 1988, Pauluhn 1994).

Smaller animals such as rodents are held in tubes (Phalen 1997). The tube is attached to the chamber so that a hole or extension from the inlet manifold directs the atmosphere flow toward the animal's nose. An adjustable back restraint is used to prevent the animal from backing out. A restraint that is open to the atmosphere can allow heat and humidity to escape. However, the test atmosphere can leak around the animal as discussed by Wong (2007). Leaks may be prevented by using a restraint system that seals the tube, though heat and moisture buildup in the tube is a concern (Phalen 1997). A sealed restraint system is desirable if the test compound is particularly toxic or hazardous. Animals inside the restraint tubes do not have access to food or water. While loading the animals in the tubes, care must be taken to position them correctly. Animals, particularly the younger or smaller ones, may attempt to turn around inside the tubes with the risk of suffocation. The airflow through the nose-only chamber may be reduced to minimize the amount of test compound used. However, if the flow through each port approaches the minute ventilation rate of the animal, the flow may be insufficient to clear the exhaled atmosphere away from the animal.

Inhalation/Ventilation of Intubated Animals

Aerosol inhalation by a ventilated and intubated animal is an invasive exposure methodology compared to whole-body or nose-only exposure. The anesthetized animal is

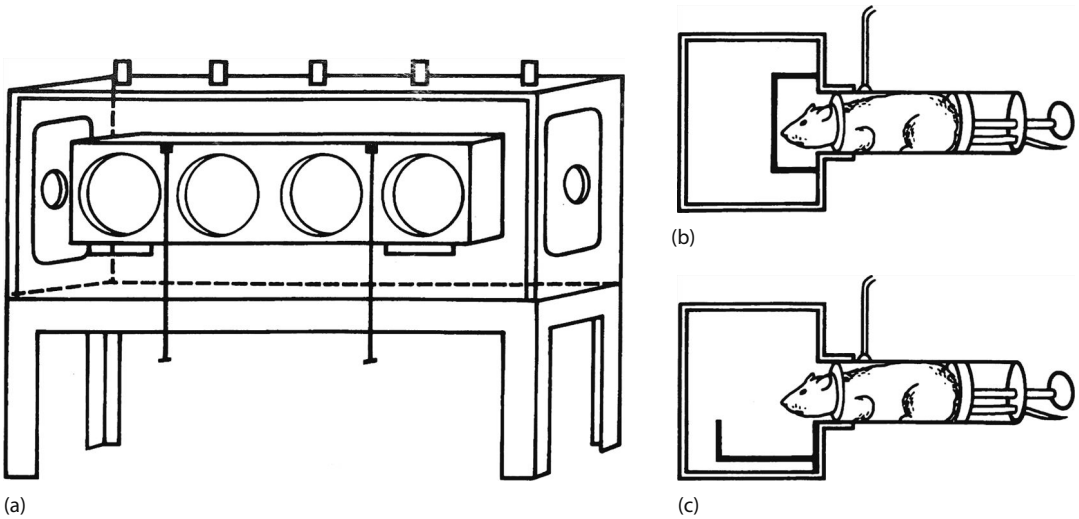

FIGURE 28.7 Airlock exposure chamber for instantaneous exposures. (a) Front view. (b) End view, atmosphere preequilibrated while rats breathe fresh air. (c) Airlock dropped for instantaneous exposure.

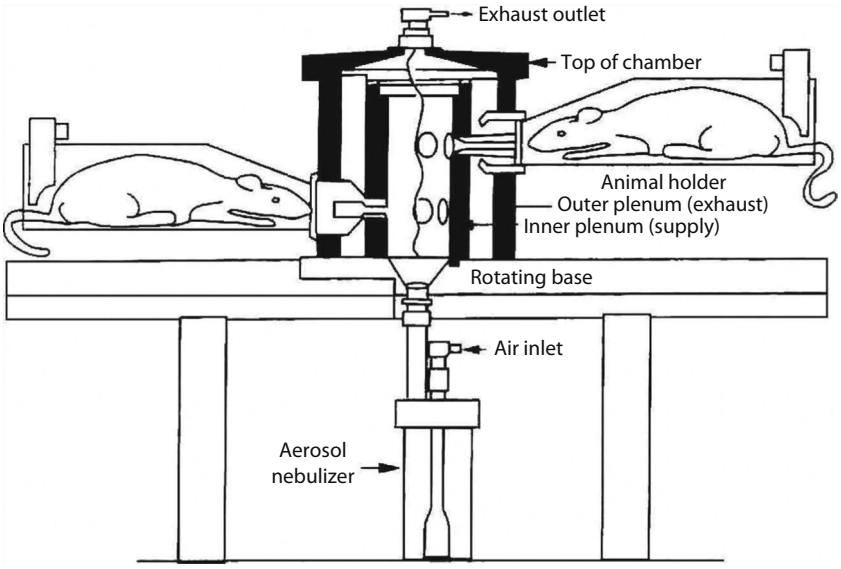

FIGURE 28.8 Exposure chamber and rodent holder designed for nose-only exposures to aerosols and vapors.

intubated and connected to a computer-controlled ventilator unit. Ventilation of the intubated animal, however, allows for higher-precision measurement of the inhaled aerosol volume during controlled breathing and of the aerosol fraction being deposited. In addition, this system allows for bypassing the extrathoracic airways and the upper part of the trachea due to the endotracheal tube being placed and usually sealed against the outside such that the aerosol delivery to the lungs can be enhanced leading to higher particle deposition probabilities. Furthermore, the particle deposition can be further optimized by certain selection of the ventilation parameters (e.g., deep and slowly breathing). Another advantage is the avoidance of any fur contamination during exposure representing a major shortcoming of whole-body exposure. Note that rodents are obligatory nose breathers that filter inhaled particles of sizes larger than 3 μm (2.5 μm) aerodynamic diameter in the case of rats (mice, respectively). Hence, inhalation exposures of aerosol particles larger than these sizes will not reach the thoracic airways and alveoli of rodents under normal breathing; however, aerosol inhalation via an endotracheal tube is an option for such exposures.

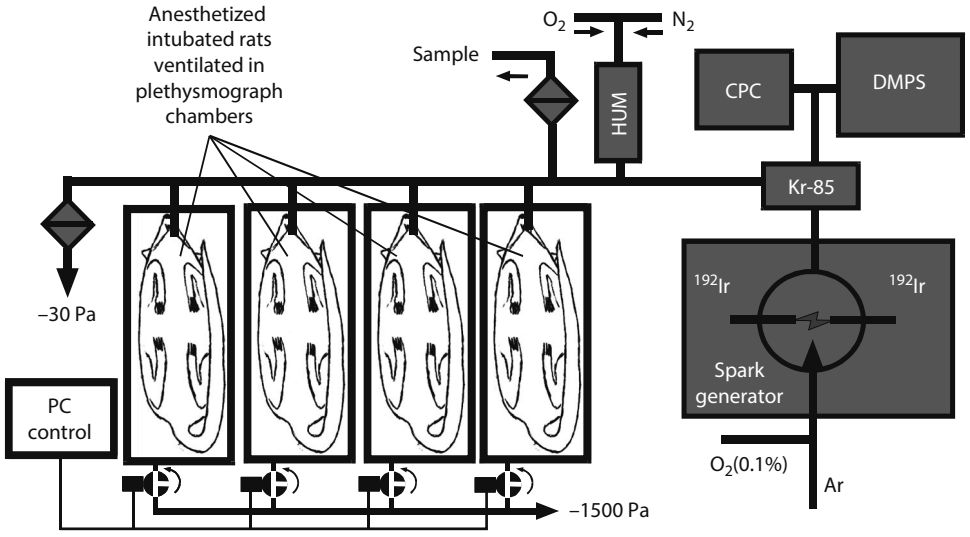

FIGURE 28.9 Schematic drawing of the ventilation apparatus and the spark discharge nanoparticle aerosol generator: Four anesthetized rats individually placed in plethysmograph chambers are intubated to connect to the aerosol. Rats are ventilated by alternately applying and releasing low pressure. The aerosol is freshly produced by the spark discharge aerosol generator in an argon atmosphere. For breathing, this aerosol is air-conditioned and supplied with oxygen and nitrogen. In the spark discharge generator, two iridium electrodes are mounted, which had been previously neutron irradiated in a nuclear reactor to form the ^{192}Ir radioisotope at a selected radioactivity. Size distribution and concentration of the aerosol are continuously monitored.

Two ventilation methods can be used: aerosol exposure via positive-pressure ventilation or via negative-pressure ventilation. In the first case, the anesthetized animal is connected via the sealed endotracheal tube to a piston-type servo ventilator controlled by a computer. When the alveolar CO_2 is low enough, the anesthetized animal does not breathe spontaneously but follows the external breathing pattern triggered by the piston-type ventilator. This system allows ventilation using physiological breathing patterns, which can individually be adjusted to the size and the behavior of the animal. This was demonstrated for dogs (Meyer and Slama 1983, Schulz et al. 1992). A similar system was scaled down and developed by the latter group for rodents to perform lung function tests, achieving such high sensitivity that distinction between the lung functions of two strains of mice was possible (Schulz et al. 2002).

A similar system called intratracheal inhalation was described earlier (Oberdorster et al. 1997, Osier et al. 1997). It consists of a closed system, allowing for the filtration and removal of potentially hazardous or radioactive particles prior to exhaust, thus making decontamination procedures significantly simpler and faster than those for standard inhalation systems. The main advantage is the rather precise estimate of the inhaled aerosol volume when the breathing flow is monitored, which is possible because the number of breaths during the study is set by the ventilation parameters. Knowing the aerosol concentration and the deposition probability or measuring particle deposition additionally, for example, by labeling the aerosol particles, the deposited fraction can be determined precisely.

In the second case, ventilation by negative-pressure ventilation, the intubated and anesthetized animal is located in an airtight plethysmograph chamber, and its lungs are connected via the endotracheal tube tightly sealed in the trachea through the chamber wall to the aerosol line with the bypassing aerosol flow. By applying a negative differential pressure of 1.5 kPa (a reasonable range is 1–2.5 kPa) to the plethysmograph chamber, the thorax expands and the animal inhales; releasing the low pressure to the ambient level, the animal exhales to its functional residual capacity (FRC). When the alveolar CO_2 is low enough the anesthetized animal does not breathe spontaneously but follows the external breathing pattern triggered by the pressure in the plethysmograph chamber (Kreyling et al. 2002, Semmler-Behnke et al. 2012). Since the entire aerosol system was maintained at a slightly lower pressure of 20–30 Pa compared to the lab pressure, the use of radioactively labeled, freshly generated nanoparticles became possible, warranting radiation protection of the operating personal. Various aerosolized materials (iridium, elemental carbon, titanium dioxide, gold, and silver) with a median diameter of 20 nm were applied for biokinetics studies (Kreyling et al. 2009, 2011, Möller et al. 2013).

A transoral/intratracheal inhalation method has been reported previously (Drew et al. 1987). In this system, anesthetized rats were intubated intratracheally and exposed to an aerosol of fibers. Unlike the previously described system, the animals were allowed to breathe spontaneously. Anesthetized rats tend to breathe more shallowly, and ventilation is less which would be expected to decrease deep lung deposition. In trained dogs, permanent tracheostomy can be used to deliver common aerosols for inhalation studies in alert, awake animals (Drazen et al. 1982, Godleski et al. 2000).

Inhalation Exposure of Aerosol Material of Limited Availability

In most animal inhalation systems, the aerosol is freshly generated and flows by such that the inhaled fraction of the aerosol is usually below 0.1% of the generated aerosol. When the material to be aerosolized is limited in its mass, such systems can no longer be used. This may be the case for hazardous materials such as radioactively labeled particles or other chemically toxic materials or for extremely expensive materials such as drugs. In this case, a bag-in-box exposure system may enhance the ratio of inhaled over generated aerosol particles up to 1%–5%. Basically, a flexible bag inside a box is filled with the freshly generated aerosol; thereby, it replaces the air between the bag and the box. Subsequently, the animals start breathing the aerosol from the bag, while air enters the space between the box and the bag replacing the inhaled aerosol volume inside the bag. This procedure allows inhalation of an aerosol of rather constant concentration until the bag is emptied. The procedure can be repeated by filling the bag with freshly generated aerosol (Bailey et al. 1985, Kreyling et al. 1999). Care is required to determine aerosol particle losses in the bag due to settling and diffusion; the inside surface needs be of electrically conducting surface in order to avoid deposition of charged particles.

Atmosphere Analysis: Measuring Size and Concentration

In many cases, analysis of the test atmospheres is the most challenging part of conducting inhalation studies. The investigator must not only sample the chamber for proper environmental conditions (temperature, relative humidity, oxygen content) but must also select, validate, and conduct analyses for the test substance(s) in terms of particle size (if a solid/liquid aerosol), concentration, and solvent concentration (if present in a mixture); the problem magnifies when testing materials are not stable in air or in the presence of moisture and multiple component substances are tested. All of these issues must be addressed and resolved to be considered a well-conducted study. This section attempts to identify some of the available analytical approaches and methods that may be useful.

Analysis of test atmospheres begins with the sampling system. An appropriately designed sampling system is central to accurate analysis of test substance concentrations in the exposure chamber. First of all, the sampler probe should be located in the immediate proximity of the breathing zone of the animals, since regional distribution differences within the chamber skew the actual concentrations inhaled by the animals. The sampling system should be designed to transmit a representative air sample from the animal's breathing zone

to the sensor or collection medium without significant losses. It is not necessary that the collection efficiency be 100%, but if sample losses cannot be avoided, at least the collection efficiency of the sampling system should be measured with the test substance and experimental conditions to be used. In addition, care is required when the aerosol is likely to dynamically change due to, for example, coagulation, growth, or deposition in the test line. Above all, the investigator should strive for a constant collection efficiency. For aerosols, the sampling system should be as straight and short as possible and designed to minimize impaction sites (i.e., those locations with bends, reducers, or turns) as well as diffusional losses of UFPs and ENPs. Rarely are aerosols monodisperse and, since most particle entrapment mechanisms are size dependent, the collection characteristics of a given sampler will change with aerodynamic or thermodynamic particle size and upon sampler airflow. Aerosol collection efficiency will also change in response to sampler particle loading. A filter will be more efficient and trap finer particles as dust collects on the surface, but efficiency will gradually decrease as the pressure drop across the filter increases, reducing airflow. In contrast, the efficiency of an electrostatic precipitator will drop as a resistive layer of particles accumulates on the collection electrode.

Sampling errors generally reflect the contribution of many small errors in the system rather than any single large source of error. The items that need to be carefully considered and evaluated in any such system include sampling train leakage and losses, variations in flow rate and sample volumes, sampler aerosol or vapor collection efficiency, sample stability in the collection media, extraction efficiency from the sampling substrate, and analytical background or interferences introduced by the sampling substrate. Additionally, the air sampling rate should be known with a high degree of precision, and this can be performed with previously calibrated rotameters, gas meters, critical orifices, displacement monitors (dry or wet test meters), or electronic mass flow sensors. Much of this technology is described in classical texts on industrial hygiene.

For gases or vapors, sampling of the test atmosphere may be either instantaneous (i.e., grab sampling) or continuous in nature. If grab sampling is conducted, samples should be taken with sufficient frequency to identify fluctuations in concentration. Samples may be collected in evacuated glass or metal containers, inflatable flexible polymer bags, or by gastight syringes. Alternatively, gas or vapor samples may be taken from remote locations by directing the sample through nonreactive sample lines by using a vacuum pump and introducing aliquots of the sample stream directly into the analytical instrument. Continuous samples can be taken in this manner by pumping (or drawing) a constant stream of gas directly through a dedicated detector or by having the analytical device continuously cycle through sample streams from multiple exposure chambers.

Gases and vapors offer the least difficulty in sampling as they follow the laws of diffusion, mix freely with the general atmosphere, and equilibrate rapidly. Sampling typically involves removal of the substance from air by scrubbing through a solvent or reagent, adsorption to a collection surface, or collection in some type of container (e.g., an impermeable, nonreactive gas bag).

An extractive sampling system for gases, vapors, and aerosols includes sample lines from the breathing zone, a scrubbing device, an airflow or volume meter to determine sample volume, and a vacuum pump. Commonly, solvent-filled impingers are used for extractive sampling. Impingers consist of a sampling tube extending through a reservoir of collecting media; the end of the tube at the bottom of the reservoir may be either open, narrowed, or fritted; the latter two modifications provide comparatively higher collection efficiency. The increased collection efficiency is due to the smaller bubbles produced by the narrow or fritted impinger tips, which allows more complete diffusion of vapor from the bubbles into the surrounding solvent. The solvent used in impingers can quickly evaporate, and, therefore, sampling cannot be conducted indefinitely.

Solid adsorbent sampling devices are used for vapors and some gases. Solid adsorbent samplers consist of a small tube (about 100 mm long × 6 mm diameter) containing a sorbent, typically charcoal, which serves as the collection medium, and a vacuum source to draw a known rate of air over the sorbent. Charcoal is an effective sorbent for many compounds since its surface structure is microporous, thereby providing a high surface area for chemical adsorption. Importantly, charcoal possesses not only a large number of pores, but the pores exist in a range of dimensions to allow molecules of different sizes to enter the pores. Thus, charcoal affords nonspecificity and can trap a wide variety of organic compounds. Other trapping agents include polymers, which can be engineered to trap certain chemicals, silica gel for inorganic substances, or molecular sieves. Once trapped, however, the sorbent must also allow the vapor/gas to be desorbed with high efficiency into a solvent for subsequent quantitation. Carbon disulfide is broadly used as a desorption solvent for charcoal tubes since it has good solvent properties and a low background with the flame ionization detector generally used in analysis. Sampling is done at relatively low airflows to allow time for chemical adsorption yet must be of brief duration to not exceed the adsorbent capacity of the sorbent. While relatively simple and cheap, use of gas sampling tubes in most toxicology studies is uncommon due to the labor involved in sample collection and preparation compared to continuous, direct reading analyzers.

Gas sampling using containment systems is also relatively uncommon in toxicology studies. They serve as temporary storage vessels to allow transport of gas or vapor samples from the exposure system to the analytical device. Examples include evacuated glass or stainless steel containers or Tedlar® or Mylar® plastic bags. While some substances are stable in such containers, there are numerous logistical and practical limitations for their routine use in inhalation toxicology studies. Gas sampling bags are physically cumbersome (their bulk depends upon the sample volume needed), require a logistical commitment to have a sufficient quantity of sample vessels available for the duration of testing and vessel transport to the analytical laboratory, require

demonstration of chemical inertness and storage stability of the substance between the interval of sample collection and analysis, need to be decontaminated if vessels are reused for other substances, and may confound results from undetected leaks or sample losses during shipment.

Instruments for gas or vapor component identification and quantitation have brief response times (seconds or minutes) and are used after air sampling. Sensors in these instruments typically utilize either absorbance of infrared or UV radiation, flame ionization or photoionization, electron capture, or chemiluminescence detection.

As with gas and vapor sampling, instantaneous (grab) or continuous (integrated) sampling techniques may also be used to collect particulate matter. Instantaneous sampling involves removing a small volume of the atmosphere and separating the particle phase by either filtration or impacting the aerosol against a solid substrate on which the deposited particles are later characterized (counted and sized and/or subjected to chemical analysis). For continuous sampling, a constant stream of the test atmosphere is directed to the analytical instrument. Analytical techniques that rely on physical forces such as gravity, impaction, electrophoresis, thermophoresis, electrical mobility, inertia, or diffusion techniques are used for particle collection; collected particles are typically measured using mass or optical properties. Yet due to the increasing interest in UFPs and ENPs, other metrics such as number concentration and the corresponding size distribution and/or particle surface metrics are required in addition to mass concentrations and according size distribution determinations. An overview of the common instrumentation useful for determining particle mass and size for use in inhalation studies is listed in Table 28.3.

Grab sampling of aerosols usually involves either impactors or filters. Fluid-filled impingers (as used for gases and vapors) have been used for aerosols, but as collection efficiency is variable, they are not commonly used. Instead, grab sampling for aerosols generally involves the use of filters for determining aerosol mass concentration in the chamber. For nonhydroscopic aerosols, the accuracy of chamber aerosol concentrations is limited by the sensitivity of the volume measuring device as the weight of collected aerosol can be determined with great precision using modern analytical scales. Hydroscopic aerosols pick up water vapor from the air and consequently can change weight during the brief interval needed for filter weighing. To more accurately measure the weight of hydroscopic aerosols, standardized weighing techniques are needed such as desiccating (especially glass or cellulose acetate fiber filters) or by equilibrating filters in a constant humidity chamber prior to weighing.

The choice of filter media available is broad and includes glass fiber and various polymer membranes (such as cellulose

TABLE 28.3
Features of Some Aerosol Monitoring Instruments

Instrument	Aerosol Size (μm)	Response Time	Measured Parameter	Factors Affecting Response	Advantages	Disadvantages
Photometer	0.3–15	Continuous	Total light scattering	Density, size distribution, refractive index	Continuous readout	Response changes with dust type
β-Attenuation monitor	1–15	1–30 min	Absorption of β-radiation	Atomic number	Direct mass measurement	Low sensitivity
Optical particle counter	0.3–15	0.1–10 min	Light scattering, size, and count	Density, refractive index	Indication of size, high sensitivity	Low resolution and accuracy
Piezobalance	0.02–10	0.5–2 min	Mass	Particle size	Direct mass measurement	Sensor cleaning
Piezobalance cascade impactor	0.05–25	1–60 min	Mass	Particle size	Direct mass measurement, size, distribution	Sensor cleaning, internal losses
Condensation nucleus counter	0.01–1	0.5–30 s	Particle count	Particle count	Small particle sensitivity	Alcohol emission
Diffusional mobility particle size	0.003–1	<1–5 min	Size based on electrical mobility	Electrical charge	Real-time display of UFP size	Measures only fine particles
Fibrous aerosol monitor	Optically visible fibers	1–1000 min	Light scattering size of fibers	Fiber length, size	Specific for fibers	Nonfibrous interferences
Aerodynamic particle sizer	0.8–15	2–10 min	Aerodynamic size	Density	Direct measure of aerodynamic diameter	Coincidence and density effects
Tapered element oscillating microbalance	0.002–15	0.01–30 min	Mass	Absorbed water	Direct mass measurement	Frequent filter replacement

Source: Adapted from Baron, P.A., *Appl. Indus. Hyg*., 3, 97, 1988.

acetate, polycarbonate, polyvinyl chloride, polyvinylidene fluoride, or Teflon®). Membrane filters are perforated with holes of defined size. Unlike fiber filters that trap particles within the filter matrix, membrane filters collect particles on the surface of the membrane. The choice of filter media is dictated by the need for high collection efficiency for particles of the size of interest, chemical inertness (to allow good recovery of the test substance for subsequent chemical analysis), and minimal pressure drop across the filter (to allow high air sampling rates). Pressure drop is especially important for membrane filters since smaller holes create a higher pressure drop, limiting airflow through the filter. To some degree, smaller-size holes in the filter membrane ensure a higher sample collection efficiency, but even larger-pore membrane filters collect many particles smaller than the pore size due to deposition by interception and/or diffusion. Membrane filters are widely used when analyzing the morphology of mineral dusts by either optical or electron microscopy.

In addition to determining aerosol chamber concentrations via measurement of collected mass on filters, the filters may be extracted and analyzed to determine test substance content. This is particularly valuable for mixed aerosol atmospheres where the content of each component must be evaluated, but this will require a specific analytical method for each component of interest. Clearly, in cases where test substances are to be extracted from the filter medium, the filter itself must be chemically unreactive with the test substance and should allow rapid and quantitative desorption of test substances for chemical analysis. Filters made from superfine glass fibers with diameters below 1 μm are available for collecting virtually all suspended particles, have high particle loading levels, and have low airflow resistance. While glass fiber filters interfere negligibly with most subsequent chemical analyses, it should be noted that glass surfaces have imbedded hydroxyl groups that may bind metals or react with some substances. Regardless of composition, filter spiking and recovery studies should be evaluated before use to determine whether the particular filter substrate will interfere with analysis.

Complementary to aerosol mass monitoring by filtration, real-time aerosol monitors can be used to provide analytical data for concentration adjustments. Light-based particle monitoring systems rely on light scattering through either refraction, reflection, or diffraction, all of which will vary with the particle's optical size. The theory and explanation behind light-scattering devices is well described by others (Van de Hulst 1957, Kerker 1969, Bohren and Huffman 1998). While light-scattering techniques for measuring aerosol concentrations provide instantaneous output and have found general acceptance for chamber monitoring, it should be noted that instrument response is a function of aerosol size, shape, color, concentration, and chemical composition. Thus, light-scattering aerosol monitors need to be calibrated against other analytical methods with the specific aerosol of interest to allow quantitative use; even then, they are subject to coincidence errors and are useful only at relatively low aerosol concentrations. These particle monitors do, however, provide immediate display of changes in concentrations and are useful to detect deviations in aerosol generator output or chamber airflow dynamics.

For particle sizing, cascade impactors are frequently used. Impactors are flow-through devices that withdraw aerosol from the test atmosphere. The impactor contains a series of impingement stages, each stage with progressively smaller nozzle dimensions. Since airflow through the impactor is constant, each stage induces progressively increasing airstream velocities coupled with sequentially reduced impaction distances to effect a progressive separation of smaller and smaller particles as aerosol progresses through the unit. Particles deposited on each stage can be weighed and/or examined microscopically. The aerodynamic median size of an aerosol may be determined with the cascade impactor by calculating the percentage of aerosol by weight on each stage using weight, radioactivity, or chemical analysis to determine the amount of material deposited on each stage. This process is shown in Figure 28.10. The same caveats with respect to selection and the use of filter media and filter weighing techniques apply to cascade impactors. A major liability when operating cascade impactors is the potential for overloading impactor stages with excessive particle mass. This may lead to the erroneous conclusion that aerodynamic sizes are smaller than in reality due to particles bouncing off of the target stage and subsequent capture on lower stages, indicating finer particle size.

Advances in electronics permit bypassing manual weighing of individual impactor stages and allows direct, nearly instantaneous readout of aerosol size. An example of a single-particle aerodynamic particle sizer is the TSI aerodynamic particle sizer. This device displays aerodynamic diameters by measuring the particle velocity in a laser light-scattering sensing zone. Particles of different sizes are accelerated to different speeds when directed through a nozzle. The airstream is directed through dual-focus laser beams, one downstream of the other. The system's circuitry measures the speed of the particle in an accelerating flow field, represented by the time taken to traverse the distance between the two lasers, and converts each time-of-flight measurement into an aerodynamic diameter. This device provides high-resolution, real-time aerodynamic measurements of particles between 0.5 and 20 μm, but can only process particles at a concentration of about a few thousand particles/cm^3; otherwise, coincidence errors occur; higher concentrations require dilution before measurement. Unlike the TSI unit, the piezobalance cascade impactor, known as the quartz crystal microbalance (QCM) by California Systems, measures mass concentrations (and aerodynamic diameters) directly. The QCM is a self-contained unit with a 6–10-stage cascade impactor that uses a quartz sensing crystal in lieu of a filter on each stage. The crystal has an inherent oscillating frequency that changes in proportion to the mass of aerosol deposited on the crystal. This allows very small amounts of mass to be detected and thus is ideal for very low (such as ambient aerosol) concentrations; size analysis of high concentrations can also be measured by very short sampling durations to avoid

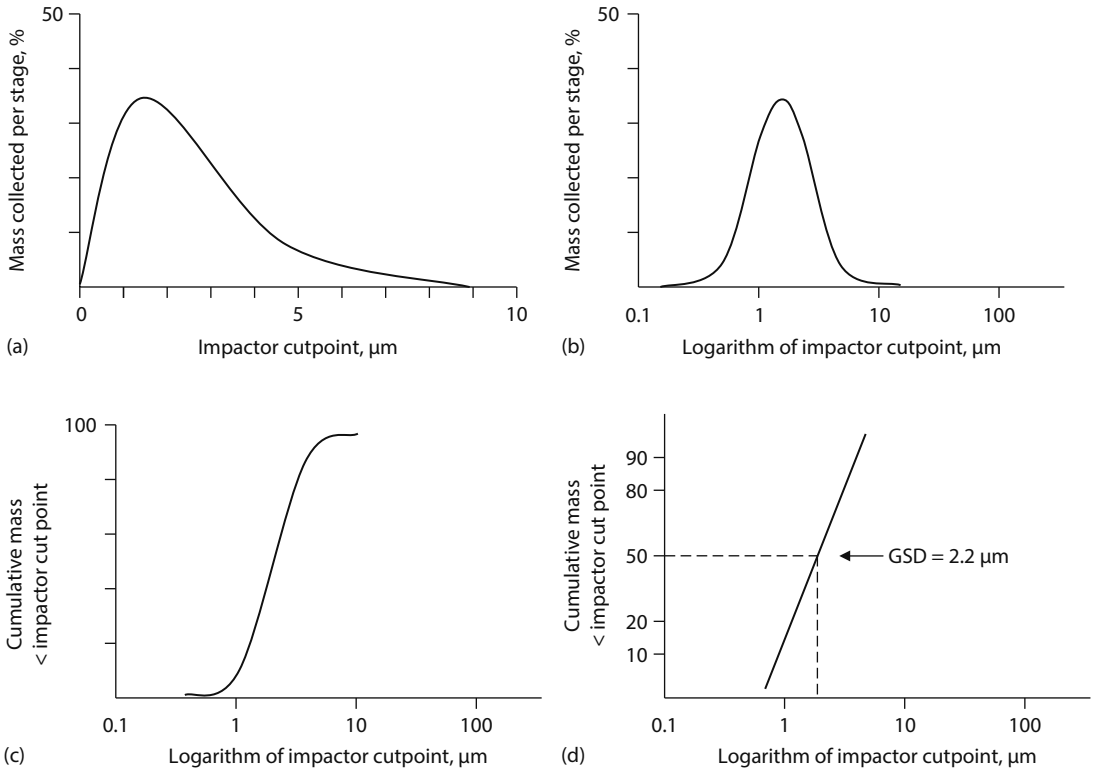

FIGURE 28.10 (a) Cascade impactor data from Table 23.3 plotted on arithmetic axes. (b) Size distribution data from (a), replotted using logarithmic scale for impactor cutpoint. (c) Size distribution data from (b), replotted using cumulative mass less than cutpoint diameter. (d) Size distribution data from (c), replotted using probability scale for cumulative mass. GSD is 2.2 µm.

stage overloading. Based on the same measurement principle as the QCM, the tapered element oscillating microbalance (TEOM) has been widely used for aerosol mass measurements as described in Baron and Willeke (2001). The instrument provides continuous and remote measurements of high sensitivity and can produce high-time-resolution data. The original TEOM has a recommended temperature setting of 50°C. There is evidence that semivolatile components of aerosols are lost from the TEOM measurements at this temperature. The most likely explanation is that semivolatile organic compounds, nitrates, and water are lost from the aerosols during sampling. In newer TEOM models, the operating temperature has been lowered to 30°C to reduce losses of the semivolatile components. New modifications have been made to dry the aerosol particles before measurement to reduce the effect of ambient relative humidity.

A different type of instrument for continuous mass measurements, the β-meter, has also been commercialized. It operates on the principle of β-ray attenuation by a layer of aerosol on a filter substrate. The β-ray source is usually ^{14}C or ^{85}Kr decay and the attenuation can be calibrated with a known mass. Sampling can be done with individual filters or filter tapes, and the β-ray that passes through the filter is continually monitored. Comparison with gravimetric methods usually produces reasonable agreement. Filter or filter tapes can be used for subsequent chemical analysis.

Particle number concentration is a useful adjunct to the determination of number size distributions, providing a direct measure of the integral over the size range of ambient aerosols. Particle number is a relatively easily measured variable that can be used to evaluate aerosol dynamics models that predict aerosol particle number size distributions. Condensation nuclei can be detected after the condensation of water or other condensable vapor (often an alcohol such as butanol) from a supersaturated atmosphere onto the particle. The supersaturation in condensation particle counters (CPCs) is typically quite large, around 150%, allowing detection of particles as small as a few nanometers in diameter. Optical methods are usually used to detect the resulting droplets including photometric observation of light scattered by a collective of droplets in the optical sensing volume. However, the most satisfactory method is individual counting of particles condensed in a continuous flow, as employed in the family of CPCs that have developed from the design of Bricard et al. (1976). This procedure is employed in most modern commercial CPCs. It has the advantage of giving a direct determination of particle number concentrations up to about 10^5 cm^3. Most modern CPCs utilize continuous flow with condensation of an alcohol (typically butan-1-ol) to achieve supersaturation and employ single-particle counting, although photometric detection is used in some. Flow rates, lower detection diameter, and upper concentration ranges vary between counters; the particular counter used needs to be selected to suit the application.

While the uncontrolled increase of size in the CPC during vapor condensation makes it impossible to size classify

the particle, the differential mobility particle sizer (DMPS; Liu and Pui 1974, Knutson and Whitby 1975) and the scanning mobility particle sizer (SMPS; Wang and Flagan 1990) are basically the same type of instrument with only different electronic control mechanisms. They consist of a differential mobility analyzer (DMA), a CPC, and some electronic controlling devices, everything controlled by a personal computer. The DMA enables selecting only a very narrow size fraction of particles that then enters the CPC. The CPC counts the number of particles as usual, and since the size is already determined, information of both concentration and size is obtained. By changing the setting of the DMA, scanning over the whole particle size interval (3 nm to 1 μm) is possible.

The aerosol time-of-flight mass spectrometry (ATOFMS) offers real-time measurement capabilities for characterizing aerosol particles by the aerodynamic size and chemical composition of individual aerosol particles sampled directly into the instrument (Noble and Prather 1996). Such measurements are made in situ by combining a unique dual-laser aerodynamic particle sizing system to size and track individual particles through the instrument and laser desorption/ionization time-of-flight mass spectrometry to obtain correlated single-particle composition data. The size and chemical composition of 50–100 particles/min can be measured (up to 600/min at high particle concentrations).

Recently, a small transmission electron microscopy (TEM) sampler was designed to collect UFPs on grids used for TEM. The sampler is based on electrostatic deposition (Fierz et al. 2007). Particles are collected directly on TEM grids and can be analyzed in the TEM without any further treatment. The sampler consists of three functional units: an aerosol charger, a deposition zone, and an aerosol electrometer. The aerosol is drawn through the sampler at a flow rate of 0.5 L/min with an integrated pump. The charger is a unipolar diffusion charger. In the charging zone, the particles acquire a positive charge. Once charged, the aerosol enters the sampling region through a metallic inlet pipe. The TEM grid is mounted opposite to the aerosol inlet of the sampling region on a conducting rod. Finally, the electrometer can be used to measure the charge of the collected particles on the TEM grid and thereby allowing an estimate of the number of deposited UFPs.

Recently, a new method to classify aerosol particles according to their mass-to-charge ratio was introduced. This method works by balancing the electrostatic and centrifugal forces that act on particles introduced into a thin annular space formed between rotating cylindrical electrodes. Particles having a mass-to-charge ratio lying in a certain narrow range are taken out continuously as an aerosol suspension. This technique involves the use of a DMA (Liu and Pui 1974, Knutson and Whitby 1975) to select particles of a given mobility size and an aerosol particle mass (APM) analyzer (Ehara et al. 1996) to measure the mass of those particles. The APM classifies particles according to mass, regardless of particle density or shape. By multiplying the number concentration downstream of the DMA by the particle mass, it is possible to determine the mass concentration size dependently at a given DMA classifying voltage. The total mass concentration is obtained by integrating across the measured mass size distribution. Because measurements are made on particles suspended in the air, this technique avoids sampling artifacts associated with volatilization or adsorption that occur with filter or impactor sampling. This system also allows the measurement of particle density (McMurry et al. 2002).

Exhaust/Scrubbing Systems

Exhaust systems are included in chamber design not only to assist in the flow of air through the exposure chambers but also to minimize discharge of test material into the environment. Federal, state, and local regulations on air emissions can be stringent in requiring that adequate abatement systems must be incorporated. Depending on the physical properties of the test material, liquid or solid absorbents (e.g., water for water-soluble solvents and activated charcoal for low-level organics) may be employed to remove gases or vapors, whereas high-efficiency particulate air (HEPA) filtering material may be appropriate for aerosols. Since liquid or solid absorbents become saturated with test material, they must be monitored with sufficient frequency to ensure that the adsorbent capacity has not been exceeded. In contrast, particle filters generally become more efficient as particle loading increases, but because airflow may be reduced concurrently when filters become loaded, the exposure chamber aerosol concentration may inadvertently increase. Thermal oxidation or incineration are other means of treating chamber effluent and can be applied to scrubbing combustible test material.

Exposure Modes: In Vivo, Instillation, and In Vitro

A variety of exposure modes have been used to evaluate the effect of substances following inhalation. The whole-body exposure mode is encountered frequently and allows the exposed test species to move freely in the atmosphere containing the test substance. Whole-body inhalation exposures will result in a portion of the substance being inhaled and deposited regardless of physical form by grooming. Gases or vapors can dissolve in the mucus fluid lining the respiratory tract and, via the mucociliary escalator, reach the pharynx, where they are swallowed. Droplets or solid particles also reach the gastrointestinal tract via this mechanism. Further, contributions to total absorbed dose can occur by dermal absorption of the test substance. The normal grooming and preening activities of rodents both during and after inhalation exposures can deliver the substance to the gastrointestinal tract. For example, rats were exposed to respirable zinc chromate dust either in conventional wire mesh cages or nose only in fiberglass tubes (so that the only exposure was through the external nares). The caged rats excreted 8.4 times as much fecal and 5.5 times as much urinary chromium as rats in the tubes, indicating that a significant amount of dust could be ingested or absorbed following whole-body exposure (Langard and Nordhagen 1980).

Dermal absorption may contribute to the total body burden from whole-body vapor exposures. The skin is a partial or incomplete barrier to inward or outward migration of most vapors. Because the surface area of the skin is large, a small flux of vapor across the entire skin surface can result in absorption of sufficient amounts of vapor through the skin to cause adverse health effects. McDougal et al. (1985) described a whole-body chamber in which rats were fitted with face masks and received positive-pressure, charcoal-filtered fresh air while being exposed to vapors of either dibromomethane (DBM) or bromochloromethane (BCM) (volatile organic chemicals for which their major metabolites could be easily measured in blood). In rats exposed to the vapor by inhalation for 4 h, the percent absorption through the skin for DBM was 5.8%, and for BCM was 4.2%. These uptakes were independent of inhalation exposure concentration. Determination of the relative rates of absorption of vapor through the skin and the respiratory tract can be determined by comparing dermal absorption in rats without inhalation contact and inhalation by the usual methods. When inhalation is the route of exposure, the rate-limiting process in absorption of substances with large blood–air partition coefficients, such as bromomethane, is the rate at which the vapor is transferred to the blood by alveolar ventilation (Andersen 1981). The rate-limiting process of absorption for dermal exposure is passage through the stratum cornea, which can be represented by the permeability constant. Blood concentration measurements allow the input of both vapor inhalation and dermal vapor absorption integration when relating total delivered dose to biologic effects (McDougal et al. 1986).

Because nose-only systems limit exposure of the test substances to the respiratory tract and not the entire body, the amount of test material needed is considerably reduced. This feature facilitates testing highly toxic and/or limited-availability test substances. In addition, nose-only exposure units simplify test substance containment and subsequent cleanup and allow the chamber concentrations to be more easily and readily altered. Disadvantages of this system may include loss of material to the fur of the head, difficulty in obtaining a proper seal at the neck without impairing circulation, the possibility of restraint and handling induced stress of the animals, and the relatively limited number of animals that can be tested simultaneously. All of the previously mentioned disadvantages can be dealt with in a manner that makes this type of testing useful in safety evaluation programs. To ensure uniformity of exposure conditions on an animal-to-animal basis, a relatively large airflow is needed. For larger animals such as the dog and monkey, helmet exposures can be conducted. Pressure fluctuations in these systems may be great, and records of breathing patterns during exposure may be needed to accurately measure the exposure doses. In addition, the fitting at the neck must be comfortable and easy to adjust. Designs including both inflatable collars and thin rubber membranes have been used successfully.

A typical nose-only exposure unit employs an animal exposure cylinder, generally constructed from rigid plastic (or glass). This tube is connected to an enclosure or exposure chamber that allows introduction and evacuation of test atmospheres and contains sampling probes to determine test concentrations, particle size distributions, temperature, and humidity. Rodent holders are usually fabricated of plastic (polymethylmethacrylate, polycarbonate), aluminum, or stainless steel cylinders to conform to the general shape of the animals and are fitted with conical headpieces projecting into the plenum of the exposure chamber. Some nose-only systems minimize rebreathing of test atmospheres by attempting to have a constant flow of fresh test atmosphere directed onto the face of the animal while having a local exhaust near the nose, as shown previously in Figure 28.8. The animal holder needs to be of various sizes to accommodate rodents of differing sizes to minimize stress, provide careful support to minimize discomfort to the animal, and adequately ventilated to minimize heat retention within the tubes.

Both physical restraint and inadequate temperature control within the chamber can subject the test animals to additional stress. This seems to be especially true for inhalation developmental and/or reproductive studies. Pathological effects on the male reproductive system, independent of the inhaled test substance, have been reported in restrained male rats where inadequate temperature control occurred, leading to excessive body temperature (Brock et al. 1996). Inhalation developmental toxicity studies conducted with restrained pregnant rodents have produced slight increases in maternal toxicity and either slight (Miller and Chernoff 1995) or no (Tyl et al. 1994) increases in fetal anomalies when dams were restrained during the period of organogenesis. To minimize confinement-induced stress in inhalation developmental toxicity studies in mice, Dorman et al. (1996) utilized a rat restrainer tube to expose an unrestrained pregnant mouse, making essentially a whole-body exposure chamber for individual mice. Adaptation to the stress induced in rats and mice in nose-only inhalation tubes was examined. In naïve animals during the first hour of restraint, heart rate increased by 58 beats/min (BPM) (18.6%) in rats and by 174 BPM (32.3%) in mice, as compared to cage controls. Temperature increased by 2°C in mice and was unchanged in rats, compared to cage controls. Heart rate and temperature values remained within normal physiologic values during restraint. In rats, the response to restraint in nose-only holders was the same after 4 days regardless of whether the duration of restraint was increased gradually to 4 h/day or kept constant at 4 h/day. In mice, the group that was gradually adapted had a statistically significantly higher heart rate and temperature after 4 days than the fixed-duration adapted group. Rats and mice restrained for 4 h/day every day showed a gradual decrease in heart rate and temperature over time. Full adaptation to restraint required 14 days of fixed-duration daily restraint (Narciso et al. 2003).

Overall, the selection of the nose-only exposure mode can affect the outcome of some inhalation toxicity studies and represents a decision that investigators should properly control for in experimental design. Nonetheless, high-capacity nose-only exposure systems, such as those described by Cannon et al. (1983), have been used for various acute and subchronic

inhalation studies. A thorough description of the components used in this nose-only inhalation system, with details on automated collection methods for airflow regulation and test atmosphere analysis, was presented by Pauluhn (1994).

Using a directed-flow nose-only exposure system at flow rates close to the minute ventilation of the animal, it was possible to maintain desired concentrations. At an airflow rate of 2.5 times the animal's minute ventilation, the concentration of test substance in inhaled air was reduced less than 10% (Moss et al. 2006).

Design considerations of head-only units are identical to those of nose-only units. Mask designs represent a unique kind of nose-only exposure and usually are limited to relatively large animals. Masks for dogs (Bair et al. 1969), monkeys (Greenberg et al. 1977), ponies (Mauderly 1974), donkeys (Albert et al. 1974), chickens (Battista et al. 1973), and rats (Stavert et al. 1991) have been used successfully.

The use of intratracheal instillation as an alternative to exposure of animals by inhalation deserves some comment. Conditions exist in which the pulmonary effects of a substance cannot be easily evaluated by inhalation. Although for not entirely valid reasons such as space, time, and/or economics there may be decisions not to use inhalation as the route of test material exposure, but rather to use intratracheal instillation techniques to get the material directly into the respiratory tract. The reason for choosing intratracheal instillation over inhalation can also rest on lack of sufficient quantities of test material or on safety issues (extreme toxicity, flammability, explosivity). Using this method, the actual dose delivered to the lung of the experimental animal can be directly and precisely measured. This technique is inexpensive in that very small amounts of substance are needed (while expensive chambers, generating apparatus, and support personnel are avoided). Also, because the technique is contained and uses relatively little material, exposure hazards to laboratory workers are greatly reduced compared to that of an inhalation study. Finally, materials that are not readily respirable in rodents can be introduced to the lungs with this technique; notably, long fibers that can be inhaled by man but not by rodents can be tested via this route. The problem that limits the usefulness of intratracheal instillation as an exposure technique relative to inhalation is that the dose to the respiratory tissues can be variable and highly artificial and does not accurately reflect the lung distribution of a substance following inhalation exposure. Additionally, intratracheal instillation techniques focus on the lower respiratory tract and, as such, cannot evaluate responses that would occur in the nasopharyngeal region by inhalation exposure.

Intratracheal instillation involves a suspension of particles in a carrier liquid that is injected directly into the lumen of the trachea. By gravity, the fluid and particles flow into the areas of the lungs that cannot be entirely controlled. The carrier liquid is then rapidly absorbed into the pulmonary circulation, leaving the particles on the internal surfaces of the lung. This technique permits the introduction of a wide range of doses and substances to the lung in a short period of time. In larger animals, localized exposures to specific areas or lobes of the lung are possible, often allowing the contralateral lung to serve as a control (for nonsystemically acting agents). This technique was first applied by Kimura (1923), using rabbits and guinea pigs to look at the response to various coal tars. Early works have described in detail the methodologies found to be useful in the mouse (Nettesheim and Hammons 1971, Kouri et al. 1976), in the rat (Blair 1974), and in the hamster (Saffiotti et al. 1968, Brain et al. 1976). The procedures used in these studies are quite similar, with the main difference being the maximal amount of total liquid that can be used to deliver the test substance to the lung without harming the animal.

In small rodents, intratracheal instillation is accomplished by inserting a catheter or needle transorally through the mouth and epiglottis into the tracheal lumen. In larger species (including human), a fiber-optic bronchoscope can be used to more precisely visualize the instillation site. Because the animal must not move during the procedure, the choice of anesthetic is important, with short-acting materials that suppress reflexes for a minimal period of time being preferred. Physiologic saline is the vehicle most frequently used to suspend or solubilize the test substance, although even this may evoke a mild transient inflammatory response. Surfactants can be used to improve the suspension properties for dusts, but the surfactant effect(s) on the lungs needs to be evaluated. In addition, dosage volumes need to be adjusted for the body weight of the animal.

Although intratracheal instillation enables administration of large amounts and nonrespirable sizes of particulate matter that would otherwise not be able to gain access to the lung, more localized deposition of particle results. Indeed, the major obstacle for routine use of intratracheal instillation as a replacement for inhalation bioassays lies in the fact that the patterns of particle distribution in the lung following instillation are uneven and are unlike those resulting from inhalation. Particle deposition by inhalation is focal, that is, inhaled particles deposit at selected sites in the lung depending upon their size and breathing pattern. Brain et al. (1976) showed that intratracheal instillation of particles produced nonuniform deposition patterns, largely dependent on gravity acting on the instilled fluid. These investigators studied the distribution of particles labeled with ^{99}Tc in both rats and hamsters following either intratracheal instillation or aerosol inhalation. Particle distribution patterns in the lung following inhalation were distributed evenly within a given lobe; among lung lobes, most of the dust deposited in the apical lobes (Brody and Roe 1983). Pritchard et al. (1985) found that variability in the deposition of cerium oxide particles in rats within a specific lobe was considerably greater following instillation than inhalation, with little penetration of instilled particles to the peripheral lung. Similarly, greater peripheral lung loading was seen following inhalation of ferric oxide particles than following instillation (Dorries and Valberg 1992). In contrast, the distribution of both short and long glass fibers in rats was reportedly similar using either inhalation or

instillation (Henderson et al. 1995). More recently, uneven and patchy distribution was confirmed after intratracheal instillation of suspensions containing either submicrometer titanium dioxide (TiO_2) particles or nanosized primary TiO_2 particles when compared to intratracheal inhalation (Osier et al. 1997). An important issue of intratracheal instillation is the optimized timing of the instillation coordinated with the inspiration of the animal, for example, during expiration, the instilled volume may be expired completely.

Recently, intratracheal aspiration as a derivative of intratracheal instillation was introduced in which a droplet of particle suspension was placed onto the glottis while the nose of the animal was closed. When opening the nostrils, the droplet was accelerated and partially dispersed into the trachea during a high flow, gasp-like breath of the animal. Aspiration was compared to inhalation and intratracheal instillation using ^{99m}Tc radiolabeled nanosized sulfur colloids followed by gamma camera imaging using a pinhole collimator (Foster et al. 2001). The authors observed higher particle doses and higher 24 h retention in the lungs after instillation and aspiration compared to inhalation. Only after inhalation was the particle distribution in the lungs rather homogeneous. Yet they showed that the aspirated suspension volume critically determines the deposited dose and retention. In addition, cytometric analysis of the bronchoalveolar lavage fluid showed increased numbers of eosinophil and neutrophil granulocytes suggesting an inflammatory response of the peripheral lungs to contaminants on the glottis, which were flushed with the droplet during the high airflow inspiration. Similarly, a higher delivered dose was reported for SWCNTs, but a less even and patchy distribution was observed when compared to inhalation of aerosolized small agglomerates of SWCNT (Shvedova et al. 2008). While the results seem to be promising in mice, we found highly variable deposits of radiolabeled nanoparticles on the thoracic airway epithelium. Mucociliary cleared particle fractions varied from 15% to as much as 80%, as determined by gamma camera imaging during the next 24 h, although emphasis was made to perform the aspiration procedure as standardized as possible. Hence, this highly variable dose to the airways results in an inverse but also highly variable dose to the lung periphery, which hampers the advantage of aspiration in rats considerably.

Another aerosol delivery method utilizes a pressurized air jet to nebulize particle suspension directly through a tiny nozzle within the trachea. Yet air velocities required to disperse the liquid volume led to immediate impaction of the dispersed droplets right behind the nozzle. A standardized method was developed using 2 μm monodisperse.[111] In radiolabeled polystyrene particles, which were dispersed through a nozzle at the end of a bronchoscope placed two-thirds down into the trachea of beagle dogs (Kreyling and Wohland 2007). In all cases without any intrasubject and intersubject variabilities, the deposited particle bolus at the carina and first main bronchus contained more than 95% of the administered particles. Since the main canine bronchus is 10 mm in diameter, the quantitative droplet deposition adjacent to the nozzle within rodent tracheas of less than 2 mm diameter is also close to 100%. Yet the deposited liquid volume may flow down into the lungs when the animal is in an upright position. Finally, some companies make devices similar to those described above. For example, Penn-Century makes a microsprayer that delivers liquid aerosols from the tip of a tube inserted into the airways, as well as a dry powder insufflator, which can be used for nonliquid aerosols.

In summary, pulmonary effects of test substances, for reasons of space, time, and economics, along with nonavailability of sufficient quantities and/or sample tissues, cannot always be tested following inhalation exposures. Uses of intratracheal administration involve methodology that is simple and uses relatively little material so that risk to laboratories is much reduced (from an inhalation study). One can deliver relatively large amounts of material to the lung in a short period of time and with less variability than by inhalation.

Against these advantages, however, is the primary fact that amounts and sizes of particulate that would otherwise not be able to gain access to the lung can be delivered. This requires recognition when designing intratracheal instillation studies as they may lead to doses that can only be inhaled during prolonged time periods of days, weeks, and months. In addition, the dose rate of intratracheally instilled particles is almost high and can never compare to inhaled dose rates, which are several orders of magnitude lower. Furthermore, the patterns of particle distribution in the lung following instillation are uneven. The nonuniformity is partially random but represents systematic and reproducible regional differences. Another difference is that the instillation technique bypasses the upper respiratory tract. Problems can result from altering dosing rates and the use of differing suspending agents. Frequently, an optimal strategy is to do preliminary experiments by direct delivery to the lungs and then having explored clearance rates and biological responses, use this information to design an appropriate inhalation study.

Chamber Selection and Operation

Most inhalation experiments utilize dynamic exposure conditions. That is, the test atmosphere, comprising test substance diluted with fresh air, is continually replenished. Thus, the test atmosphere flows through the exposure chamber and is then exhausted. Once the generation apparatus is turned on, the concentration in the chamber rises to a theoretical equilibrium value, which is the ratio of the flow of the substance to the total flow in the chamber. The equation describing the concentration time curve is

$$C_t = \frac{f}{F\left[1 - e^{-(F/V)t}\right]} \tag{28.1}$$

where

C_t is the concentration after t minutes
f is the flow of substance
F is the total flow through the chamber
V is the chamber volume

Usually, this equation is converted to an expression that defines the time required to attain a given percentage of the equilibrium concentration ultimately attained:

$$t_x = K \times \frac{V}{F} \tag{28.2}$$

where

t_x is the time required to attain $x\%$ of the equilibrium concentration

K is a constant whose value depends on x

Most frequently, the concentration–time relationship is described by t_{99}, the time needed to attain 99% of the theoretical equilibrium concentration. For determination of t_{99}, $K = 4.605$. At this time point, the concentration in the chamber may be considered constant, assuming no changes in air or test substance supply rates. The concentration–time characteristics of a given chamber are described by the values of V and F, which are more descriptive than the outdated practice of giving air changes per hour (Silver 1946, MacFarland 1981).

Airflow to a chamber can vary from approximately 10 to 60 changes/h. This is understood as the addition and withdrawal of a volume of air equal to the volume of the chamber. Because added air mixes with that already present, a complete change of air has not occurred. The term air change is misleading, and the dynamics of mixing in inhalation chambers has been described by the statistical considerations first put forward by Silver (1946). As already discussed, a more descriptive term for chamber airflow is the t_{99}.

The duration of exposure is defined as the interval from the start of flow of test substance to the point where delivery is discontinued. The exposure is terminated by stopping the flow of substance to the atmosphere generator, which leads to the decline in the chamber concentration on an exponential curve that is the inverse of the rising curve (Figure 28.11). Animals are not removed from the chamber, nor are the chamber doors opened to observe the animals, until at least t_{99} min to allow sufficient time for the chamber contents to be fully eliminated. For longer-term exposures, the rising and falling section of the curves can be neglected, and the exposure profile becomes a square wave form. For short exposures, where exposure duration is less than $13 \times t_{99}$, the system

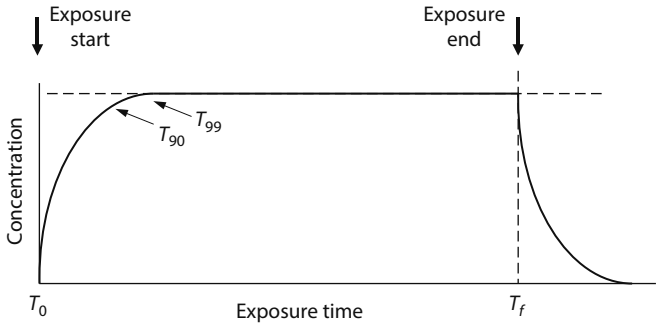

FIGURE 28.11 Concentration and time relationship in a dynamic exposure chamber.

should include an airlock mechanism or some other instant exposure/nonexposure mechanism (MacFarland 1976).

Chambers should be operated at a slight negative pressure (e.g., −20 to 200 Pa) with respect to room pressure to protect personnel against leaks in the system. Chamber pressure should be monitored continually, especially in older units to avoid the possibility of spatial or temporal concentration gradients. The distribution of the test substance within the exposure chamber is determined by taking repeated samples from different areas of the chamber in relation to a reference sampling location, using statistically valid sampling strategies. After homogeneous distribution of test substance within the chamber has been established, the actual concentration should be measured continuously or at least several times during the exposure. For exposures of 4–6 h duration, a common practice is to sample every 0.5 h (or at least hourly), although for regulatory purposes, much less frequent sampling (i.e., 2–4 times per exposure) may be acceptable; the frequency of sampling should be dictated by the temporal stability of the test atmosphere generation and airflow supply systems. Continuous readout monitoring and control instruments are especially useful in long-term studies, because they are capable of preventing concentration excursions when appropriate alarms are integrated into the system. Some type of alarm instrumentation can prevent many weeks or months of effort from being destroyed by sudden elevations in test substance concentration, which could even be lethal.

Inhalation chambers operated in a dynamic mode should have a reasonably uniform distribution of test substance to avoid differential animal exposures. Due to their comparatively higher diffusion rates, it is generally acknowledged that gas and vapor concentrations show less intrachamber variation than aerosols. Aerosols are also subject to size-dependent diffusion, impaction, and sedimentation losses, compounding the difficulty in maintaining uniform chamber concentrations. Furthermore, the uniformity of aerosol concentration may be reduced when animals are housed in the chamber since their body temperatures lead to convection flow, which may cause concentration variations. However, MacFarland (1983) calculated the percentage differences in concentrations between aerosols and gases/vapors and found little difference in the chamber uniformity between aerosols and gases/vapors. Examples from the literature concerning the percent variability from mean concentrations range from 0.7 to 18.0. Because variations exist in all chamber types and airflow control operating systems, the investigator is well advised to carefully characterize the exposure chamber spatial/temporal distribution of test substance under actual generation conditions to prevent concentration-gradient errors from complicating the study.

Control of the relative humidity, but more importantly the internal temperature, of inhalation chambers is crucial to the integrity and interpretation of animal inhalation studies. As already noted, elevated temperatures can alter animal physiology, affect metabolism and ventilation, and increase the rate and type of chemical interactions. Ideally, chamber loading (the total volume of animals in a chamber) should not

exceed 5% of the chamber volume to avoid heat-induced artifacts among test animals (Fraser et al. 1959). Coincidentally, chamber loading also affects the test substance equilibrium concentration in the exposure chamber. Silver (1946) reported, for example, that when the chamber loading exceeded 5%, excessive losses of test substance occurred, presumably by absorption to body surfaces. Temperature should be monitored continuously but at least regularly during the exposure. In some instances, such as when chamber loadings exceed this recommendation, chamber walls or inlet air may be cooled to maintain normal interior temperatures. Heat-balance studies with rats in stainless steel or glass chambers of equal size and using a room air intake of 100 L/min showed that the chamber walls were effective at removing approximately 90% of the animal body heat as compared with the heat loss through the airstream. If conducting studies in chambers with low airflow rates, heat transfer to the surrounding environment can be increased by painting the chambers, by attaching cooling coils to the chamber walls, or by directing air-conditioning ducts directly onto the chamber (Bernstein and Drew 1980). In addition, control of relative humidity is essential for proper heat balance in rodents. Although it is generally recommended that relative humidity should be controlled within relatively narrow limits (e.g., 40%–60%) to minimize adverse effects on feed consumption and behavior on study outcome (Rao 1986), recent work showed that rats tolerated humidity levels of either 3%, 40%, or 80% in repeated-exposure studies without effects on body weights, feed or water consumption, or respiratory tract histopathology (Pauluhn and Mohr 1999).

Administered Dose

The fundamental concept of dose (i.e., the amount of substance taken up by the test subjects) is not excluded from the domain of inhalation toxicology but continues to represent a major effort. Yet due to the difficulty in measuring individual respiratory parameters and the test substance's physical/chemical properties (two primary characteristics that affect the respiratory tract deposition of inhaled materials), the determination of the internalized dose is much more complex by inhalation compared to other exposure routes. Of the total amount of test material inhaled, a variable fraction may actually be deposited, absorbed, and reach target tissues. This absorbed fraction is, in turn, dependent on the local absorptive, metabolic, and clearance processes that may modify the chemical composition and/or its concentration of the inhaled substance along deposition sites in the respiratory tract. Although these processes are not easily determined and have restricted the ability of inhalation toxicologists to provide quantitative measures of inhaled dose, progress in the area of dose determination has evolved in the related areas of biomarkers and mathematical modeling. Increasingly, sophisticated models are being used to estimate toxicant concentrations in the blood and organs by treating the uptake, distribution, metabolism, and excretion of toxicants as a series of independent processes occurring at experimentally determined rates (Crapo et al. 1989).

The administered dose in inhalation studies is most often characterized in terms of the exposure conditions, that is, the exposure concentration and duration according to certain conventions. For example, "rats were exposed to x ppm (or x' mg/m^3) of substance y for 6 hours/day, 5 days/week for 13 weeks." For gases and vapors, concentration may be expressed on a volume basis (i.e., mole *x*/total moles, parts per million [ppm], or parts per billion [ppb]) or on a weight basis (milligrams per liter [mg/L] or milligrams per cubic meter [mg/m^3]). For solid or liquid aerosols, concentrations generally are expressed on a weight basis (milligrams per liter [mg/L]) or, for fibrous materials or nanosized UFP or ENP, on a number basis (number of fibers or particles per cubic centimeter [#/cm^3]). Furthermore, for fibrous aerosols, consideration should be given to expressing concentration on both a respirable mass (mg/m^3) and a particle count (fibers/cm^3) basis; with all UFP or ENP aerosols or some low-density fibers, the number count may be extremely high but the mass concentration may be very low.

A fundamental interrelationship exists for many inhaled substances between exposure concentration and time. Haber (1924), for example, determined that the response of an animal to a gas could be related to the product of the concentration and the exposure time. This relationship, known as Haber's law, states that the product of the concentration (C) and exposure time (T) required to produce a specific physiologic or toxicologic effect is equal to a constant, $C \times T = K$. The specific effect can be something other than death, but death is still a commonly applied end point. Haber's law has been used to predict response for exposure conditions that have not been described experimentally, for example, predicting the expected l h LC50 for a chemical substance where only 4 h data exist. There are short but finite time periods during which some end point may never be attained (i.e., death may not occur at practically attainable concentrations in short [e.g., 0.1, 1, or 10 min] time periods). At the other extreme, some exposure levels exist where a substance does not produce measurably adverse effects despite continuous, prolonged exposures. For these reasons, Haber's law does not apply to all substances but generally is applicable when extrapolated to $C \times T$ values that differ by a factor of about 3–4 for a given $C \times T$ product. Rinehart and Hatch (1964) and Kelly et al. (1981) have shown, for example, that Haber's law applies for phosgene gas and titanium tetrachloride aerosol when evaluated for respiratory rate and lethality end points, respectively. Gelzleichter et al. (1992) also showed that Haber's law applied for nitrogen dioxide or ozone in rats exposed for 3 days over a fourfold range of exposure concentrations. However, if rats were exposed to mixtures of ozone and nitrogen dioxide, lung damage was found to be related to the peak concentration rather than the cumulative dose of the gases: this observation was related to the synergistic, adverse effects of the mixture of these two gases. A quantitative assessment of the temporal and concentration relationship for lethality with several irritant and systematically active substances was evaluated by ten Berge et al. (1986). The authors reported that for the substances evaluated, the $C \times T$

relationship does not always represent the response data over the exposure periods reported. They reported that $C^n \times T$, where the exponent n may vary from 0.8 to 3.5, is often a better predictor of mortality. However, work by Pauluhn et al. (1996), which explains $C \times T$ relationships from acute inhalation testing for product classification purposes suggests that 4 h exposure data provide essentially the same projected acute lethality value as would 1 h testing; where only 4 h data were available, a default value of 4 should be used for conversion of 4 h LC50 values to 1 h LC50 values, independent of the physical state of the test substance.

With sensory irritation as the end point, varying the exposure concentration has a proportionately greater effect than does changing exposure duration. Thus, with the exception of calcium oxide, the other four test substances (chlorine, ammonia, formaldehyde, and 1-octane) did not follow Haber's law (Shusterman et al. 2006).

The internalized dose is related to the atmospheric concentration of the test substance, the duration of exposure, the individual's respiratory volume and frequency, and the deposition efficiency within the individual. These parameters are related according to the following general equation:

$$D = E_d V_m C T \tag{28.3}$$

where

D is the deposited dose (mg)
E_d is the deposition efficiency for the substance within the respiratory tract
V_m is the minute volume (L/min)
C is the concentration of the test substance (mg/L)
T is the time of exposure (min)

The use of this equation presupposes that the previously mentioned parameters are well characterized for the species and test substance under investigation and a given set of experimental conditions. In practice, such data are not generally available, especially for gases or vapors and other methods of dose determination have been used. For a series of vapors, the internalized dose has been determined by measuring the net decrease in test substance concentration in the exhaled air compared to the concentration in the inhaled air (Stott and McKenna 1984); similar methodology has been used to measure disposition of ultrafine aerosols in the human respiratory tract. In a system designed to validate a rat-based physiologically based pharmacokinetic (PBPK) model of nasal extraction and metabolism of vinyl acetate (a water-soluble, reactive vapor), Hinderliter et al. (2005) exposed human volunteers to radiolabeled vinyl acetate via nasal inhalation. Measurements of vinyl acetate and its metabolite, acetaldehyde, concentrations in nasal airways compared favorably with the model predictions providing support for human risk assessments conducted for workplace exposures.

Alternative methods to estimate the deposited dose have included chemical assays for the total amount of test substance or its metabolite in excreta, a useful technique for radioactively labeled materials. Measurement of metabolite levels and DNA or protein adducts in biologic fluids or tissues represents another means of assessing substance exposure and internalized dose (Lucier et al. 1989, Dahl et al. 1991, Bond et al. 1992). An advantage of measuring DNA adducts in evaluating dose–response relationships is the comparatively higher sensitivity associated with the measurement of such biomarkers. In contrast to the comparatively high exposure concentrations employed in cancer bioassay studies, changes in the amount of metabolite DNA adducts may be measured at much lower exposure concentrations compared to those associated with changes in tumor incidences. This method provides a better estimation of the shape of the dose–response curve at low concentrations, provided information is available to address the stability, extraction efficiency, and accuracy of the methods used to measure DNA adducts. For dose estimates of radiation-induced health effects, the International Commission for Radiological Protection (ICRP) provided a large compendium on the human respiratory tract model considering particle deposition of inhaled aerosols, subsequent biokinetics of radionuclides, and finally their effects in the organ of intake as well as target organs in the body (ICRP 1994).

The need for quantifying the internalized dose, particularly as it relates to human risk assessment, has encouraged research in the area of pharmacokinetic (PK) modeling (Andersen 1987). The use of PK or biokinetic modeling is finding wide acceptance among risk assessors and regulatory agencies for predicting the dose of a toxicant to a given tissue across species under various exposure conditions. This approach describes the kinetic relationships between physiologic factors (such as organ and body weights, respiratory rate, and blood flow), biochemical factors (such as substrate affinity for an enzyme and reaction velocity), and physicochemical factors (such as the extent of substance partitioning into air, blood, or tissue) on the disposition of that substance within the body. PK and biokinetic modeling is based on a thorough understanding of these processes in an experimental animal and then extrapolating these parameters, with appropriate validation in experimental animals, to predict target tissue doses and toxic effects in humans. Validated PK models, using accepted physiological values, may then be useful in predicting expected tissue levels of a substance (and thus organ toxicity) under various exposure regimens. Indeed, PK models have been developed to describe the expected tissue doses of substances in rodents or humans for vapors such as butadiene (Himmelstein et al. 1997), benzene (Roy and Georgopoulos 1998), halogenated hydrocarbons (Vinegar et al. 1998), butoxyethanol (Lee et al. 1998), as well as particulate matter such as powdered fire suppressants (Kimmell et al. 1998) or diesel soot, titanium dioxide (Stober et al. 1990), quartz dust (Stober 1999), formaldehyde (Kimbell 1994), vinyl acetate (Plowchalk et al. 1997), carbon tetrachloride (el-Masri et al. 1996), and radionuclides contained in aerosol particles (ICRP 1994). When PK modeling is employed, due caution must be exercised with respect to the applicability of the animal model and experimental techniques used to human values in order for the data to be useful in human health risk assessment.

In Vitro Toxicity Test Methods

The focus of toxicology has shifted somewhat, since the mid-1980s, from whole-animal toxicity tests to alternative in vitro toxicity methods as a result of reducing and even banning the use of experimental animals (Silbergeld 1998, Gad 2000, Bakand et al. 2005, European commission 2013). In vitro toxicology describes a field of study that applies to technology using isolated organs, tissues, and cell culture to study the toxic effects of substances (Hayes and Markovic 1999). The development of in vitro toxicity test methods has been influenced by a variety of socioeconomic factors.

The ethical/technical bases for continued use of animals in research balance a reduction in the number of experimental animals, refinement of test protocols to minimize suffering, and replacement of current animal tests with appropriate in vitro tests opposite to the need for valid, toxicological data (Verheugen 2006). The necessity of determination of the potential toxic effects of a large number of substances and formulations has sparked the need for rapid, sensitive, and specific test methods. At present, in vitro methods cover a broad range of techniques and models, and a standardized battery of in vitro tests can be used for assessing acute local and systemic toxicity as long as they are validated.

A practical strategy for incorporating the concepts of in vitro approaches for inhalation toxicity testing has been proposed by ECVAM (EURL ECVAM 2013). This strategy uses existing literature, evaluating the physicochemical characteristics of test substances, and predicting potential toxic effects based on structure–activity relationships (SARs). Physicochemical characteristics of substances, such as molecular structure, solubility, vapor pressure, pH sensitivity, electrophilicity, and chemical reactivity, are important properties that may provide critical information for hazard identification and toxicity prediction (Gad 2000, Faustman and Omenn 2001).

In vitro exposure techniques include direct exposure to the test substance itself, exposure to collected air samples containing the substance(s) of interest, submerged exposure conditions, intermittent exposure (gases only), or continuous direct exposure (gases).

In vitro screening tests may identify cellular responses allowing estimation of test substance toxic potency. Based on the obtained result, in vitro tests may be followed by a second phase using isolated cells specific to the target organs, for example, nasal olfactory cells, fibroblasts, or mesothelial cells (Lambre et al. 1996). Freshly isolated cells maintained in suitable culture media that can mimic biotransformation activities and cellular functions comparable to the in vivo environment are preferred to long-term cultures or cell lines that may differentiate, lose their organ-specific functions, and lack the enzyme systems required for biotransformation (Barile 1994, Lambre et al. 1996).

The effects of air contaminants have been studied using several in vitro exposure techniques. Most techniques have involved studies of isolated particulate substances added to culture medium (Nadeau et al. 1995, Becker et al. 2002). Although this exposure condition may be adequate for soluble test materials, this may not simulate in vivo exposure conditions of dose and dose rate from airborne aerosols. Moreover, such techniques may often ignore the effects of particle size, which is crucial for in vivo toxicity testing. Some in vitro research has investigated the effects of suspended and extracted particles from atmospheric aerosols (Hamers et al. 2000, Glowala et al. 2002) or cigarette smoke condensate (McKarns et al. 2000, Putnam et al. 2002).

The toxic effects of sulfuric acid aerosols on human airway epithelial cells (Chen et al. 1993) and the effects of ozone on cultures of primate bronchial epithelial cells (Jabbour et al. 1998) or human bronchial cells (Mogel et al. 1998) have been studied using cells grown on collagen-coated membranes. Toxic effects of individual airborne substances such as ozone and nitrogen dioxide, and complex mixtures such as diesel motor exhaust (Knebel et al. 2002) and cigarette smoke (Aufderheide et al. 2003), were studied using cultured human lung cells on porous membranes permeable to culture media. A direct exposure technique at the air–liquid interface offers a reproducible contact between chemically and physically unmodified airborne contaminants and target cells, and technically may reflect more closely inhalation exposure in vivo (Knebel et al. 2002, Paur 2011, Aufderheide et al. 2003). In fact, when studying the effects of deposited aerosol particles on the epithelia of the respiratory tract submersed cell test systems, those are limited as the cells may have undergone functional changes under submersion and, in addition, the submersed particles may have undergone substantial physicochemical changes in the cell media such that results obtained from submersed exposure systems may no longer be valid and comparable to the in vivo exposure as discussed recently (Paur et al. 2011). Hence, test systems utilizing an air–liquid interface of epithelial cells can be exposed under more physiological conditions and results are likely to provide better information.

An important principle of toxicology is that all materials are toxic, if exposure occurs in sufficient quantities (Timbrell 1998). Most commonly, this principle is quoted as phrased by Paracelsus (CH 1493–1541): "All things are poison and nothing is without poison, only the dose permits something not to be poisonous." This fundamental toxicology principle implies that substances with a low hazard generally pose a low risk. If, however, there is a high enough exposure of these low hazard substances, then even these substances can be harmful, or even fatal. And, of course, the opposite can also occur: high hazard but relative low exposure also results in a low risk.

A very important aspect is the identification of the most relevant dose metric for particle toxicity (Schmid et al. 2009, Grass et al. 2010). While the common dose metric for micron-sized particles is their mass, it becomes much more challenging in the cases of UFP or ENP. Hence, it is quite obvious that a single-dose parameter (e.g., number, mass, or surface area) is not likely to adequately describe the toxic effects of any type of nanoparticle. While particle mass is likely to be a reasonable dose metric for completely soluble or biodegradable nanoparticles, (partially) inert or nonbiodegradable nanoparticles reside for an extended period of time in the tissue as

particulate matter interacting with the biological environment via the surface–tissue interface. This is reflected in the mounting evidence that surface area, and not mass or number, best predicts the toxic response of numerous types of nanoparticles (Oberdorster et al. 2007, Stoeger et al. 2007, Donaldson et al. 2008, Stoeger et al. 2009). However, it is important to note that while surface area may be a very relevant contributor to the dosimetry for insoluble, nonbiodegradable particles, there are also other important properties for predicting the toxicity of nanoparticles; for example, frustrated phagocytosis is encountered for rigid, nonsoluble fibers larger than about 10 μm (e.g., asbestos and CNTs) in the lungs (Poland et al. 2008). A more detailed discussion of the effect of the physicochemical properties of nanoparticles on the dose definition has recently been presented by Grass et al. (2010).

The key point for nanotoxicologists is that for submerged in vitro cell exposure systems with standard media heights of one or more millimeters above the cell layer, the theoretical transport rates by diffusion, sedimentation, and thermal convection are sufficiently different between particles of different sizes and different densities in different test cavities and between materials with different agglomeration states, that large differences in the amount of particles reaching the cells in a given time period (cellular dose) are likely, and that there is no simple relationship between particle characteristics and the amount of particles reaching the cells. That is, the exposure–dose relationship is complex. For example, despite concluding that nominal media concentrations (NMCs) were an appropriate metric of dose in vitro, Lison et al. (2008) showed no clear relationship between NMC and cellular dose for 35 nm amorphous silica. More important, perhaps, is the fact that measures of exposure (media concentrations) may misrepresent dose to the cell by many orders of magnitude when viewed across particle size (Limbach et al. 2005, Teeguarden et al. 2007). This can clearly be established based on principles of particle motion in liquids that have been well understood for more than 100 years (Einstein 1905, Dusenberry 2009). In fact, based on theoretical work on the diffusion and sedimentation of particles in fluids by Mason and Weaver (1924), Hinderliter and coworkers (2010) developed an in vitro sedimentation, diffusion, and dosimetry model (ISDD) for nanoparticle transport in cell culture media. While this model had to make estimates on the gravitational density of the nanoparticles that may agglomerate or absorb proteins onto their surfaces, Cohen et al. (2013) developed a rather simple method to measure the apparent density of the tested nanoparticles in the culture medium which improved the prediction of nanoparticle transport in the culture medium considerably. Based on this model, Figure 28.12 shows the transport time (min) of solid, non-agglomerated gold nanospheres for a 1 mm distance.

Surprisingly, this fact has received little attention in the literature, where the focus seems to be predominantly on the suitability of assays, cell types, and methods of characterization (Doak et al. 2009, Donaldson 2009, Kroll et al. 2009, Marquis et al. 2009, Park et al. 2009, Stone et al. 2009).

As a result, for in vitro studies, a clear distinction should be made between exposure and dose metrics for nanomaterials. NMCs (μg/mL, SA/mL, #/mL) and their derivatives most commonly used in in vitro studies are *not* measures of dose; they are measures of exposure and do not accurately reflect the amount of material coming in contact with the cells. Processes such as diffusion, sedimentation, and perhaps convection, all of which are dependent on the size, shape, and density of the nanoparticles, influence the relationship between the nanoparticles in solution above cells and nanoparticles reaching the cells (Limbach et al. 2005, Teeguarden et al. 2007), the latter being a measure of nanoparticle dose. The general comfort in using these exposure metrics, rather than

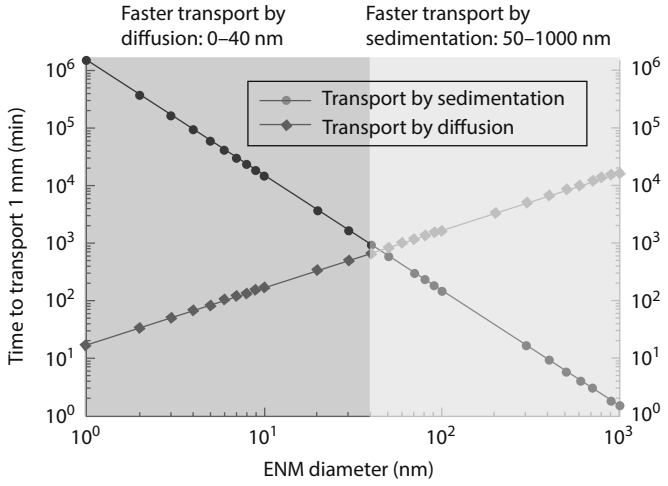

FIGURE 28.12 Impact of particle diameter on the transport in in vitro cell culture systems. The theoretical time to transport non-agglomerating gold nanospheres of known density over a distance of 1mm is plotted against particle diameter, demonstrating a clear size-dependency of diffusion and sedimentation. Nanoparticles ≤40 nm in diameter are primarily driven by diffusion, while nanoparticles >40 nm are primarily driven by sedimentation. (From Cohen, J. and Demokritou, P., Towards accurate and relevant dosimetry for in-vitro nanotoxicology, in *Nanoparticles in the Lung*, Tsuda, A. and Gehr, P. eds., in press.)

dose metrics, is misplaced because there is no justifiable expectation that in vitro exposure and dose metrics consistently have a direct relationship.

In this regard, it is instructive to consider dose levels in terms of nanoparticles per cell. For a number concentration of $3 \times 10^4/cm^3$ in ambient air, whereof almost 100% (80%–90%) can be considered nanoparticles (mobility diameter smaller than 100 nm [Kreyling et al. 2003]), about 2.3×10^{11} nanoparticles are deposited onto the alveolar epithelium per day. Assuming that the alveolar epithelium consists of 2×10^{10} epithelial type I cells, 3×10^{10} epithelial type II cells, and 6×10^9 alveolar macrophages (Stone et al. 1992), about 10 nanoparticles per cell will be deposited daily presuming a uniform deposition density per alveolar surface area (which is not unrealistic since nanoparticles deposit due to their diffusional properties). This rather low value needs to be kept in mind when considering doses of tens of μg per one million incubated cells, which is several orders of magnitude higher than a realistic daily dose of inhaled and deposited nanoparticles.

Air–Liquid Interface of Cell Culture Systems

Early versions of modern ALI cell culture techniques were introduced in the 1970s (Voisin et al. 1975, 1977a,b). As schematically shown in Figure 28.12, the essential aspects for in vitro exposure devices mimicking realistic inhalation exposure conditions as found in the lung are (1) complex pulmonary cell systems, which can be cultivated for at least several hours at the ALI, (2) direct contact between the cultivated cells and the inhalable substances without interfering medium, (3) uniform exposure of the entire cell layer, (4) temperature and humidity conditioning of the air to maintain cell integrity ($T \sim 37°C$; $RH > 85\%$), and (5) precise control of the pollutant levels (Rasmussen 1984, Aufderheide 2005, 2008) for accurate dosimetry.

A variety of such ALI cell exposure systems have been described in the literature (Massey et al. 1998, Aufderheide and Mohr 1999, 2000, Fukano et al. 2004, Aufderheide 2005, Bitterle et al. 2006, Mulhopt et al. 2008, BeruBe et al. 2009). While most of them rely on diffusion and/or gravitational settling as deposition mechanisms, some exposure systems have been introduced utilizing electrostatic deposition for bipolarly or unipolarly charged particles (Savi et al. 2008, Stevens et al. 2008).

The majority of studies are performed with monocultures; however, recent publications have shown that cocultures using a combination of different cell types have an influence on the observed cellular response (Lehmann et al. 2009). Recently, a triple cell coculture in vitro model of the human airway wall was developed to study the cellular interplay and the response of epithelial cells, human blood monocyte–derived macrophages, and dendritic cells to particles (Rothen-Rutishauser et al. 2005, 2008, Blank et al. 2007, Rothen-Rutishauser 2007). In this model, monolayers of two different epithelial cell lines, A549 (Lieber et al. 1976) and 16HBE14o epithelia (Forbes 2000), were grown on a microporous membrane in a two-chamber system. After isolation and differentiation of human blood–derived monocytes into macrophages and dendritic cells (see also paragraph *primary cell cultures*), they were added at the apical and basal sides of the epithelium, respectively. After the triple cell coculture was established, cell densities of macrophages and dendritic cells within the culture were quantified using the specific surface markers CD14 and CD86 for the labeling of macrophages and dendritic cells, respectively, and the quantitative occurrence of macrophages and dendritic cells resembled very closely the in vivo situation (Blank et al. 2007). After its thorough evaluation, this model was exposed to particles (either airborne or suspended in medium) of different materials (polystyrene, titanium dioxide, gold) and of different sizes (≤1μm) (Blank et al. 2007, Rothen-Rutishauser 2007, Rothen-Rutishauser et al. 2007, 2008, Brandenberger et al. 2010). Translocation and cellular localization of particles were studied, as well as the effects of particles on cellular interplay and signaling. Recent results showed that dendritic cells and macrophages collaborate as sentinels against fine polystyrene particles (1 μm in diameter) by building a transepithelial interdigitating network of cell processes (Blank et al. 2007). The translocation of nanoparticles into the different cell types, however, is different compared to their larger particle counterparts (Rothen-Rutishauser et al. 2007).

In that respect, the use of air–liquid exposure systems has the great advantage that such overdosing of cells of the respiratory epithelia is impossible since the nanoparticle deposition follows the same rules of aerosol physics as nanoparticle deposition in the alveolar region during normal breathing.

Regardless of the techniques employed, before in vitro methods can be formally used in lieu of traditional inhalation testing protocols for use in hazard classification, labeling, or risk assessment, validation efforts must be initiated to ensure the approaches, results, and conclusions reached from such techniques are applicable to and consistent with the objectives of international testing guidelines.

DEPOSITION, CLEARANCE, AND RETENTION OF INHALED SUBSTANCES

Gases and Vapors

Gases and vapors share a common physical property of interest to the inhalation toxicologist; both can be present in the gas phase under ambient testing conditions. Gases, by definition, exist in the gaseous state at standard temperature and pressure, while vapors represent the gas-phase components from substances that are solid or liquid at standard temperature and pressure. As the temperature increases, the amount of gas-phase components (represented by its vapor pressure) from a liquid will increase. Thus, liquids with higher vapor pressure will have a greater amount (concentration) of molecules in the gas phase relative to those with lower vapor pressure. For the inhalation toxicologist, liquid- or solid-phase test substances present a special challenge since test atmospheres may exist in both gas and solid/liquid forms, necessitating consideration of their different depositional, clearance, analytical, and biological response characteristics.

Inhaled gases and vapors can be absorbed throughout the respiratory tract to produce either local injury or systemic toxicity. Absorption depends largely on the physiochemical characteristics (e.g., concentration, water solubility, partition coefficient) of the inhaled substance and physiologic characteristics of the respiratory tract (e.g., airflow, respiratory rate, tissue perfusion, local metabolism).

The net driving force for absorption of inhaled gases or vapors is diffusion, the net movement of molecules down a concentration gradient, from regions of high molecule concentrations to low concentration. Substance absorption into tissues continues until the vapor concentrations in the air space and tissue are the same. At this point, the system is at equilibrium and no further transport of substance occurs. However, substance absorption into tissues can continue if the absorbed vapor is removed by transport into the bloodstream, metabolism to a different chemical or reaction with tissue macromolecules. These processes serve to lower the substance concentrations in the tissue so that further diffusion into the tissue may again proceed. Conversely, once the vapor concentration is higher in tissue than the inspired air, the net diffusive force is external, that is, from tissue to air. Passive diffusion is the mode by which carbon dioxide, a byproduct of aerobic metabolism, is conducted from the tissue, to the bloodstream, to the alveolar air space and ultimately removed in the expired breath.

Inhaled gases or vapors may be absorbed directly within the nasal cavity. While this spares the lung from direct exposure, the nose is consequently exposed to high, potentially injurious concentrations of inhaled substances. Vapors may be either physically absorbed within nasal tissues, a reversible process driven in part by the relative solubility of the vapor in air or tissue, or irreversibly bound to tissue components. Vapors highly soluble in aqueous solutions, such as the mucus covering the epithelial surface of the upper respiratory tract, generally tend to be absorbed within these regions. Highly water-soluble substances, such as acetone, methanol, hydrogen fluoride, and propylene glycol monomethylether, are essentially completely absorbed in the nose (Morris and Smith 1982, Stott and McKenna 1984, Morris and Cavanagh 1986). In contrast, gases or vapors with low water solubility, such as nitrogen dioxide or methylene chloride, are not well absorbed in the upper respiratory tract but are absorbed deeper in the respiratory tract (Chang et al. 1986, Morris 1990). These generalizations oversimplify nasal absorption of vapors, but improvements in regional airflow and physiologic parameter measurement can allow deposition to be extrapolated from animal to human results. Physiochemical and physiological parameters (diffusivity, tissue–air partition coefficient, chemical reactivity, metabolism) have been incorporated into computational fluid dynamic models of the rodent and human nose to predict species-dependent nasal deposition of vapor (Frederick et al. 2001); the results suggest that good agreement between rats and humans was attained with either one- or multi-tissue compartment models. Additional determinants of vapor absorption are the upper respiratory tract vascular blood flow and tissue metabolism or binding (Stott and McKenna 1984, Morris 1990). Irreversible binding to nasal tissues may occur if the inhaled vapor is reactive or is metabolized within nasal epithelium, with subsequent formation of covalent bonds with tissue macromolecules. Such reactivity reduces the substance concentration in tissues and facilitates further diffusion and absorption.

Vapors with low chemical reactivity and water solubility generally bypass the nasal cavity and are conducted into the conductive airways. Limited absorption occurs in the conducting airways, and since gas exchange does not occur here, the conducting airways represent a respiratory dead space amounting to approximately 30% of the resting tidal volume (i.e., about 150 mL in humans). Beyond the conducting airways, inhaled substances enter the alveolar (gas exchange) region and diffuse across very thin epithelial and endothelial membranes (typically 0.1 μm thick) into the tissue or blood.

Aerosols

Deposition is the process that determines the fraction of the inspired particles that is caught in the respiratory tract and thus fails to exit with expired air. Deposited dose equals the amount of material inhaled multiplied by the fraction of material that deposits. All particles that touch a surface are assumed to deposit at the site of initial contact. Distinct physical mechanisms operate on inspired particles to move them across streamlines of air and toward the surface of the respiratory tract; these are gravitational sedimentation, inertia, Brownian diffusion, and electrostatic forces. Which mechanisms contribute to the deposition of a specific particle depends on the particle's aerodynamic and physicochemical characteristics, the subject's breathing pattern, the geometry of the respiratory tract, and the flow and mixing pattern of the aerosol. For most aerosols, the first three mechanisms are most important. Detailed treatments on particle deposition are available (Lippmann 1977, Brain and Valberg 1979, Heyder et al. 1980, Raabe 1982, Agnew 1984, Stuart 1984, Stahlhofen et al. 1989, Gerrity 1990), as are comprehensive treatises on the physics of aerosol behavior (Davies 1966, Friedlander 1977, Hinds 1982, Reist 1984, Hesketh 1986, Fuchs 1989, Willeke 1993).

Aerodynamic equivalent diameter: The transport and deposition of a particle larger than 1 μm in the respiratory system are largely determined by aerodynamic characteristics: particle size, density, and shape plus gas velocity (Heyder 1982). A sphere of 3 μm diameter with a density of 4 g/cm^3 behaves aerodynamically the same as a 6 μm sphere with a density of 1 g/cm^3. Such particles may be compared by their aerodynamic equivalent diameter (d_{ae}). The aerodynamic equivalent diameter is the diameter of the unit-density (1 g/cm^3) sphere that has the same gravitational settling velocity in air as the particle in question. Aerodynamic diameter is proportional to $d_p\rho^{1/2}$, where d_p is the geometric particle diameter and ρ is the particle density.

Particle size and size distribution: As mentioned earlier, a major factor governing the effectiveness of deposition mechanisms is the size of the inspired particles. The importance

TABLE 28.4
Total and Regional Deposition Fraction of Stable Unit-Density Spheres in the Human Respiratory Tract during Oral Breathing from FRC at Two Flow Rates (Q) and Tidal Volumes (V)

Particle Diameter (μm)	Deposition Fraction (%)							
	Q = 0.25 L/s, V = 0.5 L				Q = 0.75 L/s, V = 1.5 L			
	Total	Larynx	Airways[a]	Acini[a]	Total	Larynx	Airways	Acini
0.005	67				87			
0.01	62				84			
0.05	33	0	0	33	45	0	0	45
0.1	21	0	0	21	25	0	0	25
0.2	13	0	0	13	14	0	0	14
0.4	11	0	0	11	11	0	0	11
0.7	12	0	0	12	12	0	0	12
1	15	0	0	15	15	0	0	15
2	28	2	1	25	39	1	1	37
3	44	8	4	32	63	8	5	50
4	56	16	7	33	77	21	11	45
5	65	24	11	30	86	40	17	29
6	72	34	15	23	90	52	20	18
7	78	43	18	17	93	61	22	10
8	82	52	20	10	95	69	21	5
9	84	59	19	6	96	77	17	2
10	86	65	17	4	97	82	14	1
12	87	74	12	1	98	89	9	0
15	89	81	8	0				

Source: Heyder, J. et al., *J. Aerosol. Sci*., 17, 811, 1986.

[a] The airway and acinar region designations are based on fast- and slow-clearing compartments, respectively.

of particle size is seen in Table 28.4, which lists the total and regional deposition fractions as a function of particle size in three healthy subjects. Regional deposition fraction is the fraction of the total inhaled mass that deposits in a defined region. For deposition studies, the typical respiratory tract regions are the oropharynx, nose, larynx, central airways, peripheral airways, and acini. Depending on particle size, total deposition fraction is similarly sensitive (Heyder et al. 1986). As discussed later, proper selection of particle size is one way to target particles to a desired lung region.

In Table 28.4, one can compare the particle size corresponding to the highest deposition fraction for each lung compartment. It can be seen how, during oral breathing, the larynx and airways act as sequential upstream filters for the acini. The larynx, which collects particles by impaction, consistently removes more particles from the airways. The laryngeal deposition fraction maximum is at a d_{ae} greater than 15 μm, since impaction efficiency increases with increasing d_{ae}. For the airways, which collect particles by impaction and sedimentation, the deposition maximum is at a d_{ae} of 7–8 μm. Larger particles are removed upstream in the larynx, while smaller particles have lower impaction and sedimentation efficiencies. For the acini, which collect particles by sedimentation and diffusion, there are actually two maxima. One maximum occurs at a d_{ae} of 3–4 μm. Again, larger particles are removed upstream in the larynx and airways, while smaller particles have lower sedimentation efficiencies. The other maximum occurs at d_{ae} less than 0.006 μm, since diffusional displacement increases with decreasing particle size. A deposition fraction minimum exists at a d_{ae} of 0.4 μm, the particle size with the lowest combined settling and diffusional displacements (see Table 28.5).

Our ability to estimate deposition fraction on the basis of particle size is confounded by the fact that most aerosols are polydisperse. Figure 28.13 shows typical size distributions for polydisperse particles produced by different mechanisms. Diu and Yu (1983) found that increasing polydispersity increases the deposition fraction for aerosols with diameters between 0.04 and 2 μm and decreases the deposition fraction for aerosols with diameters outside this range. Thus, the overall effect of increasing polydispersity is to decrease the dependency of deposition fraction on particle size.

Hygroscopicity and evaporation: Many types of particles undergo size changes with time. Water droplets shrink through evaporation, while hygroscopic droplets, such as NaCl particles, may undergo hygroscopic growth, especially as the relative humidity nears 100% (Cinkotai 1971, Ferron 1977, Ferron et al. 1988a). Since the relative humidity in the lungs beyond the upper airways is 99.5% (Ferron et al. 1988a), hygroscopicity can substantially affect particle size and deposition fraction.

TABLE 28.5
Root Mean Square Brownian Displacement in 1 s Compared with Distance Fallen in Air in 1 s for Unit-Density Particles of Different Diameters[a]

	Diameter (μm)	Brownian Displacement in 1 s (μm)	Distance Fallen in 1 s (μm)
Settling greater in 1 s	50	0.978	71,700
	20	1.55	11,500
	10	2.20	2910
	5	3.14	740
	2	5.10	124
	1	7.50	33.7
Diffusion greater in 1 s	0.5	11.4	9.71
	0.2	21.6	2.23
	0.1	38.1	0.871
	0.05	71.4	0.382
	0.02	172	1.41
	0.01	339	0.0689
	0.00037[b]	9060	0.00249

Source: Weiss, E.B. and Stein, M., eds., *Bronchial Asthma: Mechanisms and Therapeutics*, 3rd edn., Little, Brown, and Company, Boston, MA, 1993.

[a] Values are for inspired air at 37°C and 100% humidity; air viscosity = 1.906×10^{-5} Pa s; air density = 1.112 kg/m^3; Cunningham slip correction calculated according to Jennings (Heyder 1986).

[b] Diameter of typical air molecule (Gerrity 1990).

Fibers

A special type of aerosol, and one of particular significance to human health, is fibers, which are particles with an elongated shape. Because of the association of naturally occurring (e.g., asbestos and erionite) and man-made synthetic fibers with the development of pulmonary fibrosis and carcinogenesis in humans (IPCS 1986), interest in the hazards of inhaled fibers is heightened. Major differences in the nature and persistence of lung injury have been identified based on the fiber composition, fiber durability (a factor determining biologic persistence in the lung), fiber size (another factor governing fiber penetration and reactivity into the lung), and fiber exposure concentration.

For the purposes of describing the physical dimensions of fibers of toxicological concern, general characteristics of fibers relating size with respiratory tract toxicity have emerged from both animal and occupational exposure studies. From the animal studies, a variety of fiber types and dimensions have been instilled or injected into the pleura or abdominal cavities (Stanton and Wrench 1972, Wagner et al. 1973, Davis 1974, Pott 1978). Although these methods of fiber introduction into the body are not representative of normal exposure pathways both in terms of the rate and location of fiber deposition and quantitative aspects of the cellular response, this early research provided fundamental insights into the physical bases for the biological reactivity of fibers. Based on this work, a general relationship between the fiber dimension and lung response emerged. Notably, Stanton and Wrench found that intrapleural sarcomas were likely, especially if the implanted fibers were >8 μm in length and <1.5 μm diameter (Stanton and Wrench 1972). In contrast, nonfibrous preparations of these same materials evoked a much less severe pulmonary response (Wright and Kuschner 1977). Walton has suggested that asbestos fibers with sizes of >5–10 μm in length and diameters of <1.5–2 μm with an aspect ratio (i.e., length-to-width ratio) of >5 are most hazardous (Walton 1982). A considerable body of evidence has accumulated supporting the hypothesis that especially long and thin fibers present the greatest health hazard. Recognizing that there aren't necessarily clear distinctions between fiber dimension

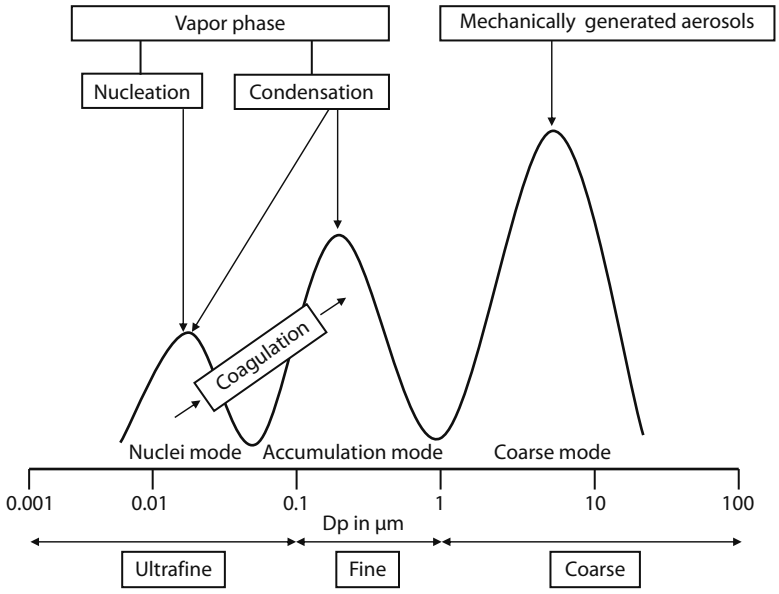

FIGURE 28.13 Particle size regimes and aerosol formation mechanisms. (Adapted from Whitby, 1978; Whitby et al., 1976.)

and pathogenicity, the U.S. Environmental Protection Agency (EPA) has defined a fiber as a particle with a length of >5 μm with an aspect ratio of at least 3 (EPA 1986). In recognition of the higher hazard of the smaller-diameter fibers, the World Health Organization has also stipulated a diameter of <3 μm (WHO 1985) in their fiber definition.

Fiber diameter, length, and shape all influence lung fiber deposition and thus potential tissue injury. Fiber diameter is recognized as the most important factor in determining fiber respirability; the thinner the fiber, the more respirable and penetrating it becomes for the lung. The probability of fiber impaction and sedimentation in the respiratory tract is governed by the aerodynamic diameters of the fibers, which are approximately three times their physical diameter (Timbrell 1965, Stober et al. 1970). Thus, a fiber with a physical diameter of about 3.5 μm will behave aerodynamically as a spherical particle of about 10 μm diameter, a size considered the upper limit of alveolar deposition. Fibers that are very thin are more likely to reach alveoli than larger-diameter fibers. In humans, the upper limit for aerosol deposition in the lung is considered around 10 μm in diameter, so that fibers with actual diameters exceeding about 3 μm are considered to be nonrespirable; conversely, fibers with diameters under 3 μm but that are very long (50–100 μm) are highly respirable. Timbrell and Skidmore (1970) showed that finite limits exist for fiber respirability so that fibers with lengths exceeding about 200 μm and diameters greater than about 3 μm would not penetrate the airways to deposit in distal alveoli to a significant amount. Empirical observations of fiber deposition are supported by modeled predictions of alveolar deposition in rats and humans (Dai and Yu 1998). In their studies, they found that alveolar deposition curves were similar in both species but maximal at an aerodynamic diameter of 2–3 μm in rats (15% alveolar deposition) and ~4 μm (about 30% alveolar deposition) in humans. Essentially no alveolar deposition would occur in humans at ~10 μm aerodynamic diameter, while for rats, it would be ~5 μm. As the fiber aspect ratio increased, the deposition maxima occurred at smaller aerodynamic diameters in both species and the extent of alveolar fiber deposition decreased. Lastly, fiber shape is another determinant of deposition. Timbrell and Skidmore (1970) found that long, straight fibers tended to align their long axis in the direction of airflow, facilitating penetration into the lung. Fibers with any curvature, bends, or twists can twist and rotate in the airstream, increasing the fiber's aerodynamic diameter and therefore reducing its respirability and deposition.

Fiber-containing test atmospheres generated for inhalation toxicity studies require assessment of both fibrous and nonfibrous components to fully characterize the test atmosphere. Large amounts of respirable nonfibrous dust may exacerbate the lung response by taxing the ability of alveolar macrophages to remove inhaled particles. To document exposure conditions, it is essential to minimally characterize fiber composition (including surface modifying agents, such as binding agents), nonfibrous and fibrous length and width distributions, and air sampling data describing fiber concentration (typically by count, i.e., fibers/cm^3); supplemental measurements can include morphology and aerodynamic particle size and mass. Fiber-sizing techniques involve fiber sample collection on an appropriate filter media and counting fiber numbers and/or length/width dimensions by using light electron microscopy, scanning electron microscopy, or transmission electron microscopy.

There are three general fiber categories based on their origin. These include naturally occurring fibers, man-made synthetic mineral fibers, and synthetic organic fibers (Figure 28.13). The most well-known and studied naturally occurring class of mineral fibers is asbestos, which is grouped into serpentine (e.g., chrysotile, a hydrated magnesium silicate) and amphibole asbestos (e.g., amosite, crocidolite, and tremolite, which are hydrated metal silicates). The surface morphology of the two is very distinct; while chrysotile is a curly fiber comprised of parallel fibrillar subunits, amosite fibers have a straight, rodlike structure. These are important distinctions that relate to the durability and pathogenicity of these fibers in the lung. Amphibole asbestos has less water solubility than chrysotile, and chrysotile is more likely to fragment axially, releasing long thin fibrils, which are more reactive, while amphiboles are more likely to fragment longitudinally, releasing shorter fiber fragments. Of the man-made mineral fibers, fibrous glass is perhaps the most studied. Unlike the naturally occurring mineral fibers, fibrous glass is amorphous and does not possess a crystalline structure. Glass fibers are produced by drawing molten glass through a small orifice producing very-small-diameter fibers, typically 3–10 μm in diameter but some as fine as 1 μm. The synthetic organic fibers are useful due to their high tensile strength and chemical or flame resistance. Para-aramid fibers, for example, have been used as asbestos replacement, in automotive and aerospace applications and, more visibly, in ballistic armor. The para-aramid fibers exist as either a continuous filament or as pulped material. As-produced, para-aramid fibers are too thick to be deposited in alveoli, but fiber processing operations can physically break interfibril bonds, literally peeling away respirable-sized, curly fibrils (Lee et al. 1988).

Fibers possess broad differences in biological reactivity in lung tissue that can be related to three general properties: fiber dimension, fiber durability (or biopersistence), and fiber dose.

The physical dimensions of fibers determine how and where fibers will deposit (just as with other aerosols, the deposition site will impact bioavailability of the fiber due to site-dependent processes responsible for facilitating fiber clearance). An early clue to the importance of fiber length in the pathogenicity of inhaled fibers was the observation by Stanton and Wrench (1972) that fibers, regardless of composition, longer than about 8 μm were more potent in the development of pulmonary mesothelial tumors than shorter fibers. For fibers to have any significant lung deposition, fiber diameters must be less than about 3 μm, so the fibers most likely to be pathogenic will have diameters less than ~1–2 μm. Similarly, there is an upper boundary on length, as fibers with lengths >~200 μm do not readily penetrate to peripheral alveoli in humans. Besides the ability of very fine diameter, high aspect ratio fibers to penetrate deeply into the respiratory

tract, long fiber pathogenicity may relate to whether alveolar macrophages are capable of phagocytizing the fibers. Once fibers are longer than about 10–20 μm, they can no longer be readily engulfed and affected by intracellular hydrolysases. The longer fibers are thus less likely to be cleared by macrophages, thereby enhancing fiber retention, interaction with lung epithelium, and translocation to the pleura. Collectively, the results from various in vivo and in vitro studies of fiber toxicity indicate that long thin fibers (i.e., fiber lengths longer than 20 μm and fiber diameters less than 1 μm) are the most pathogenic in causing lung tumors (Miller et al. 1999).

Fiber durability is another critical determinant of ultimate fiber toxicity since fibers that resist dissolution or degradation (i.e., are biopersistent) in the lung or other organs of the body increase the likelihood for adverse effects. Fiber properties that impart chemical or mechanical resistance to fiber disintegration or fragmentation in the lung will prolong fiber presence in tissue. This property of fibers to disintegrate in tissues was first noted when natural and man-made fibers of different compositions were found to have different toxicological properties and lung retention characteristics (Morgan et al. 1982, Johnson et al. 1984, Morgan 1992). Investigators found lower burdens of some fibers in exposed lungs than expected based on the fiber exposure concentrations, and in general, this correlated with a less severe spectrum of pulmonary pathologic effects (Hesterberg et al. 1998). Indeed, a sizeable body of data has accumulated showing that the biological effects of inhaled fibers were not so much dependent on their composition but rather to their durability and resistance to disintegration in the lung (Lippmann 1990). Studies show that biopersistence of fibers longer than 20 μm are good predictors of pathological responses following inhalation (Hesterberg et al. 1998, Bernstein et al. 2001).

There are two general pathways by which fiber degradation may proceed. In the first, the fiber dissolves and is completely solubilized within the lung milieu. In the second, the physical dimensions of the fiber (either diameter or length) may be altered by the action of lung fluids on the fiber surface. If solubilization alters the mechanical integrity of the fiber such that the fiber breaks along its longitudinal axis, both the dimensions and number of subsequent fibers change. If a fiber breaks transversely, an additional fiber or particle is produced (depending upon the dimensions of the resulting fragments), and the overall length of the fiber is reduced; this can result in facilitated clearance, especially if the fiber fragments are now of a size that may be readily engulfed and removed from the lung by macrophages. Conversely, some fiber types are especially prone to mechanical disruption, releasing more fibers. Chrysotile asbestos deposited in the lung, for example, may split longitudinally, releasing numerous fine fibrils and increasing the lung fiber burden, as demonstrated by Bellmann et al. (1986). Similarly, para-aramid fibers can be disrupted transversely at fibril defect sites by enzymatic attack to release shorter fibers (Kelly et al. 1993, Warheit et al. 2006). Dissolution processes that affect fiber length are more likely to reduce potential adverse lung effects by allowing phagocytosis of fiber fragments and alveolar clearance. The various factors influencing fiber durability have been reviewed by Searl (1994), and experimental methods for the evaluation of fiber solubility in vitro and in vivo have been reviewed by Hesterberg et al. (1996, 1998).

Experimental demonstration of fiber degradation in vivo has been reported by measuring dimensions of fibers recovered from the lungs of animals. Techniques vary, but common lung digestion methods utilize either thermal or microwave ashing techniques, chemical digestion with strong alkali, or oxidizing agents (Kelly et al. 1985, Warheit et al. 1991) followed by standard fiber size/count techniques. It is imperative to determine the effects of the lung digestion method on the fibers alone to ensure that artifactual reductions in fiber size or number do not occur. This consideration may be bypassed with in situ observations of fiber dimensions by using histological techniques and scanning electron microscopy/TEM (Brody et al. 1981).

Particle Size and Mass Distributions

To estimate total and regional lung doses, it is necessary to characterize the aerosol in terms of the particle size distribution and the mass concentration. Particle size is a prime factor governing the fraction of inhaled particles that penetrates past the oropharynx and enters the lungs and the particle fraction that deposits in each lung region. The mass concentration is proportional to the actual amount of particles that deposits in each region. Size distributions can be described in terms of particle number, surface area, or mass; most dose estimates use mass. Particle mass distributions are often characterized by two values: the MMAD ($d_{ae,mm}$) and the GSD (σ_g) (Heyder 1991). The $d_{ae,mm}$ denotes the particle size at which half of the total aerosol mass is contained in larger particles and half in smaller particles. Since the $d_{ae,mm}$ is expressed as an aerodynamic diameter, it describes how the overall aerosol behaves in the respired air and can be used to estimate where and by what processes particles deposit in the respiratory tract. Another frequently encountered particle size parameter is the mass median diameter (d_{mm} or MMD), which is the same as the $d_{ae,mm}$ except that the particle size is expressed in terms of the particle's actual density (Figure 28.15).

The σ_g denotes the spread of particle sizes. Most aerosols have particle sizes that are distributed lognormally; that is, on a plot of frequency distribution versus log particle diameter, the distribution looks Gaussian. An aerosol with all identically sized particles has a σ_g of 1.0. An aerosol with a $\sigma_g \leq 1.22$ is considered monodisperse (Fuchs and Sutugin 1981): to a first approximation, all the particles behave aerodynamically alike. An aerosol with a $\sigma_g > 1.22$ is polydisperse: there are appreciable differences in aerodynamic behavior among the particles. Most therapeutic aerosols are polydisperse. An aerosol with a $d_{ae,mm}$ of 2 μm and a σ_g of 2 has ±1 GSD or 68% of its mass contained between particles of 1 and 4 μm. An important implication of a lognormal distribution is that most of the aerosol mass is contained in the large particles, since mass is proportional to the cube of diameter. Where these large particles deposit in the respiratory tract governs where much of the dose deposits.

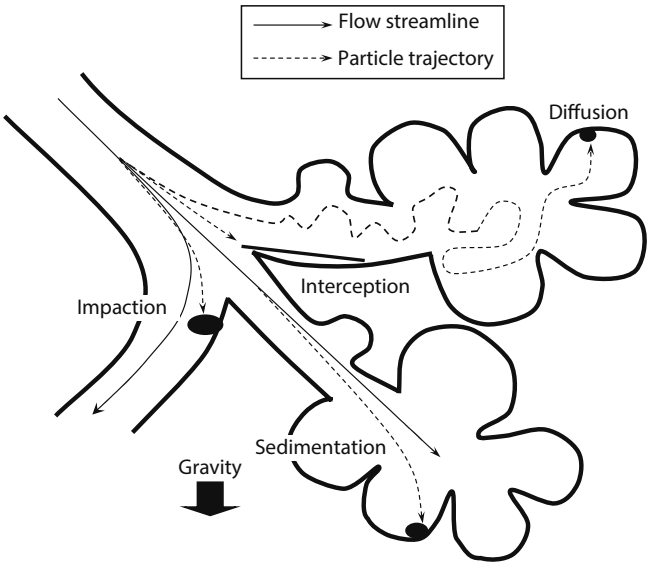

FIGURE 28.14 Mechanisms of particle deposition.

Aerosol Deposition Mechanisms

The major mechanisms of particle deposition are shown in Figure 28.14.

Gravitational Settling

Gravity accelerates falling bodies downward, and a particle reaches its terminal settling velocity when the gravitational force equals the opposing viscous resistive forces of the air. Particles small enough to enter the lungs reach their terminal settling velocity in less than 0.1 ms. Particles deposit when they have enough time to settle onto airway walls or alveolar surfaces. Thus, the probability that a particle deposits by gravitational settling increases with increasing $d_{ae}2t$, where t is the residence time in the respiratory tract (Heyder 1982). Breath holding enhances deposition by sedimentation. Sedimentation is important for particles larger than about 0.1 μm that enter the peripheral airways and alveoli, where airflow rates are slow, residence times are long, and distances to lung surfaces are short (Heyder et al. 1986).

Inertial Impaction

Inertia is the tendency of a moving particle to resist changes in speed and direction. It is related to momentum, the product of the particle's mass and velocity. High air velocities and abrupt changes in airflow direction occur in the nose and oropharynx and at central airway bifurcations. Inertia causes a particle entering bends at these sites to continue in its original direction instead of following the curvature of the airflow. If the particle has sufficient mass and velocity, it crosses airflow streamlines and impacts on the airway wall. The probability that a particle deposits by inertial impaction increases with increasing product of $d_{ae^2}Q$, where Q is the respired flow rate (Lippmann and Albert 1969, Heyder 1982). Impaction probably also increases with increasing angle of airstream deflection. Inertial impaction is an important deposition mechanism for particles with a a_{ee} larger than 2 μm (Heyder 1982) and may occur during both inspiration and expiration in the extrathoracic airways (oropharynx, nasopharynx, and larynx) and central airways. In obstructed airways, the enhanced linear velocities at narrowed sites may cause smaller particles to deposit by impaction as well.

Diffusion

Aerosol particles also undergo Brownian diffusion, a random motion in three dimensions caused by their collisions with gas molecules; this motion can lead to contact and deposition on respiratory surfaces. Diffusion is significant for particles with diameters less than 1 μm, so that their size is similar to the mean free path of gas molecules. Unlike inertial or gravitational displacement, diffusion is independent of airflow rate and particle density; however, it is affected by particle shape and size (Heyder and Scheuch 1983). For diffusing particles that measure 0.4 μm and smaller, size can be expressed in terms of the thermodynamic equivalent diameter (d_{te}), the diameter of a sphere that has the same average diffusional velocity in air as the actual particle (Heyder 1991). The probability that a particle deposits by diffusion increases with decreasing particle size and decreasing values of $(t/d_{te})^{1/2}$ (Heyder 1982); thus, deposition is dependent on the square root of residence time. Diffusion, like sedimentation, is usually most important in the peripheral airways and alveoli (Heyder et al. 1986) but can also occur in the nose and mouth (Gradon and Yu 1989).

As particle size decreases, inertia and sedimentation become less important, but diffusion increases in importance. For example, a particle with a d_{ae} of 2 μm moves by diffusion (Brownian displacement) only about 9 μm in 1 s; it settles by gravity about 125 μm in the same period. However, as particle size drops to 0.2 μm, the diffusional displacement increases to 37 μm, whereas gravitational displacement decreases to only 2.1 μm. Table 28.5 shows a comparison of settling and diffusion displacements for a range of particle sizes. Generally, gravity and inertia dominate the transport and deposition of particles larger than 1.0 μm in diameter, and diffusion dominates the transport of particles smaller than 0.1 μm. For particles between 0.1 and 1.0 μm, sedimentation and diffusion are both important (Heyder and Scheuch 1983, Heyder 1991) (Table 28.5).

Electrostatic Attraction

Electrical forces may cause charged particles to deposit in the respiratory tract. The surfaces of the respiratory tract are uncharged but electrically conducting. When an electrically charged particle approaches the lung surface, the particle induces on that surface an image charge of the opposite polarity that attracts the particle. This attraction may cause the particle to deposit, and thus the deposition of charged particles relative to that of neutral particles is increased. Experimental (Melandri et al. 1983) and theoretical (Yu 1985) studies show that electrostatic attraction may be an important deposition mechanism in the lung periphery for particles (especially fibers) that are charged above a certain threshold and have a diameter of 0.1–1.0 μm (Vincent 1985).

Melandri et al. (1983) established that the probability of electrostatic deposition is proportional to $(q^2/d)^{1/3}$, where q is the electrical charge on the particle. For charged 1 μm particles, deposition is increased over that of uncharged particles once there are about 40 charges on the particle; for 0.3 μm particles, only 10 charges are needed.

Interception and Other Forces

Deposition can also occur in the lungs when particles have dimensions that are significant relative to those of the air spaces. As particles move into smaller and smaller air spaces, some may reach a point where the distance to a surface from the center of a particle is less than the particle size. The resulting contact is called interception. Interception is particularly important for the deposition of fibers (Timbrell 1965, Harris and Fraser 1976). Other forces acting to affect deposition, such as acoustic forces, magnetic forces, or thermal forces, are normally not significant in the respiratory tract.

The physical mechanisms responsible for deposition have already been discussed. The effectiveness of these mechanisms depends on various factors such as particle size, breathing pattern, airway geometry, and disease. Many aspects of particle deposition in the respiratory tract have captured the energy and imagination of investigators; these studies are reviewed in many papers and books (Lippmann 1977, Brain and Valberg 1979, Heyder et al. 1982, 1986, Raabe 1982, Agnew 1984, Stuart 1984, Stahlhofen et al. 1989, Gerrity 1990, Vincent 1990). Other excellent sources of information on deposition are published works from the International Symposia on Inhaled Particles (Davies 1961, 1967, Stuart 1971, Wright 1977, Dodgson 1985, Dodgson et al. 1988). Some major influences on the fraction of the inspired particles that deposit within the respiratory tract and the anatomical distribution of retained particles are now discussed (Figure 28.15).

Effectiveness of Deposition Mechanisms

Anatomy of the Respiratory Tract

The configuration of the lungs and airways is important because the efficiency of deposition depends in part on the diameters of the airways, their angles of branching, and the average distances of particles to lung surfaces in the acini. Along with the volumetric flow rate, airway anatomy specifies the local linear velocity of the airstream and thus determines whether the flow is laminar or turbulent. For example, at the laryngeal aperture, an air jet is formed that creates turbulent and unstable airflows that enhance particle impaction in the trachea (Lippmann and Altshuler 1976, Chan et al. 1980). There are interspecies and intraspecies differences in lung morphometry (Soong et al. 1979, Schlesinger and McFadden 1981, Nikiforov and Schlesinger 1985); even within the same individual, the dimensions of the respiratory tract change with lung volume, age, and pathological processes. Among normal subjects breathing in the same manner, total deposition fraction has a coefficient of variation as large as 27%. This is largely due to intersubject differences in airway geometry (Heyder et al. 1982, 1988, Yu and Diu 1982, Blanchard et al. 1991). Gender differences in laryngeal and airway geometries may cause women to have greater upper airway deposition by impaction as compared with men (Pritchard et al. 1986, Svartengren et al. 1991). For any person, decreasing lung volume not only increases the deposition fraction but also causes the major site of particle deposition within the airways to shift from the lung periphery to more central airways (Agnew 1984). At low lung volumes, airways have smaller cross-sectional areas and higher linear velocities and thus enhanced deposition by impaction in central airways for a given flow rate. As a person ages, anatomical changes of the respiratory tract also affect deposition fraction (Phalen et al. 1991). Children under 8 years old who are breathing at rest have higher total, head, and tracheobronchial deposition fractions and lower alveolar deposition fraction than adults (Xu and Yu 1986, Yu and Xu 1987, Hofmann et al. 1989, Becquemin et al. 1991b). The higher airway deposition may help explain why some children have a greater response than adults to a given dose of a provocation agent (LeSouef 1992).

Choice of Pathway

Most people breath nasally at rest. As ventilation rate rises above 35–40 L/min, the high-resistance nasal pathway starts to be combined with the low-resistance oral pathway (Saibene

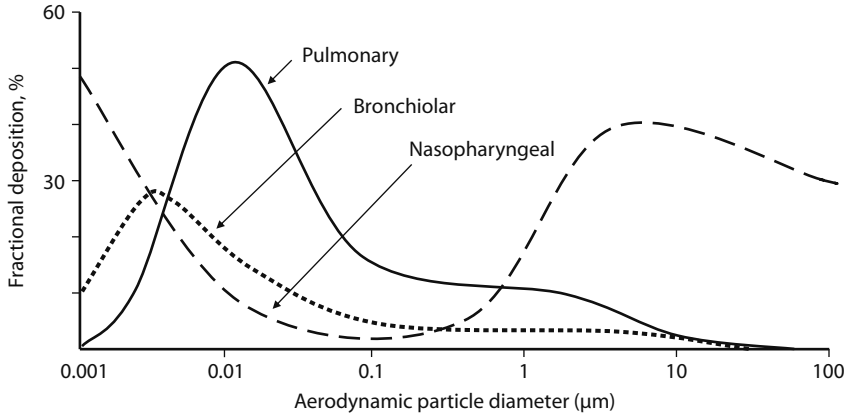

FIGURE 28.15 ICRP model of regional aerosol deposition in humans.

et al. 1978, Niinimaa et al. 1980, Chadha et al. 1987). With continuing increases in ventilation rate, the oral path takes on a greater percentage of the total ventilation, but this percentage varies considerably among subjects, ranging from only 10% to as much as 80% (Niinimaa et al. 1981, Chadha 1987, Wheatley et al. 1991).

A highly significant change in the effective anatomy of the respiratory tract occurs when air is inhaled through the mouth instead of the nose or when the nose is bypassed because of a tracheostomy or an endotracheal tube. The nose has a major role as an upstream filter that prevents inhaled particles from penetrating into the lungs. The smallest total cross-sectional area of the respiratory tract also occurs in the nose. High velocities, sharp curves, nasal hairs, and secondary flows all combine to collect inhaled particles in the nose. Excellent reviews of particle inhalability and deposition in the nose are available (e.g., Hounam and Morgan [1977], Lippmann [1977], Yu et al. [1981], Sato [1988], Stahlhofen et al. [1989], Vincent [1990], Martonen and Zhang [1992]). The inhalability, deposition, and clearance of particles in the head during nasal breathing have been studied by several investigators (e.g., Pattle [1961], Lippmann [1970], Hounam [1971], Heyder and Rudolf [1977], Yu et al. [1981], Heyder et al. [1986], Schiller et al. [1988], Breysse and Swift [1990], Rasmussen et al. [1990], Becquemin et al. [1991a]). Collectively, these reports indicate that the anterior portion of the nose collects particles larger than 1 μm by impaction, with the probability of impaction increasing with increasing $d_{ae^2}Q$, though there is some evidence that impaction probability is better described by $d_{ae^2}p^{2/3}$, where p is the pressure drop across the nose (Hounam 1971, Stahlhofen et al. 1989). Most studies show that particles larger than 10 μm are completely trapped in the nose. The actual fraction of inhaled material trapped in the nose varies considerably among individuals and is more variable than the deposition fraction in the lungs (Rasmussen et al. 1990). These intersubject differences may be due to variations in nasal distensibility and geometry, including the number and shape of nasal hairs.

Besides being an upstream filter, the nose also humidifies the inspired air; it is a more effective humidifier than the mouth because the inhaled air passes by the large surface area of the highly vascular nasal turbinates. Because of this humidification, hygroscopic particles ($d_{ae} > 1$ μm) grow more rapidly when inhaled through the nose than when inhaled through the mouth and thus have enhanced deposition fraction (Ferron et al. 1988b).

The nose is an efficient filter for large particles, but the mouth, because of its simpler geometry, is not. The shift from a higher filtering efficiency during nasal breathing to lower filtering efficiency during oronasal breathing may potentiate asthma attacks during exercise. During exercise, large volumes of air are inhaled by the mouth, so that more and more antigenic particles, such as pollens, penetrate to and deposit in the lungs. This increased exposure of airways increases the risk of allergic responses. With exercise, the amount of particles depositing in the lungs may increase more than predicted by the increased ventilation.

Breathing Pattern: Flow Rate and Lung Volume

When a subject changes breathing pattern, deposition fraction may change profoundly. Minute volume determines the total number of particles entering the lungs. Flow rate affects the probability of particle impaction and the extent of turbulent flow, which enhances particle deposition in the upper airways. The combination of flow rate, tidal volume, breath-holding time, and lung volume affects particle residence time in each lung region and hence the probability of deposition by gravitational and diffusional forces (Heyder 1982). Tidal volume and flow rate also influence the motion of the larynx and affect particle deposition there (Rudolf et al. 1986). Changing lung volume also alters the dimensions of the airways and parenchyma. High levels of ventilation and breath holding represent extremes of breathing patterns; they give rise to markedly different deposition patterns.

Generally, when airflow velocities increase, as during physical exertion or labored respiration consequent to an asthma attack, inertial deposition increases. During slow, relaxed breathing, particle sedimentation and diffusion are the primary deposition processes, and particle collection in the acini increases because of the proximity of lung surfaces there.

Species Differences

Human responses to inhaled gases and particles are often predicted from experiments done in other species, such as rodents or dogs (Brain et al. 1988). In addition to obvious differences in body mass, anatomy, and life-span, differences in metabolism among species are often substantial. For example, the oxygen consumption in rats per kilogram of body weight is 5.5 times greater than that of humans (Badeer 1974). Other differences, such as nocturnal activity or coprophagy, may be important and may influence responses to inhaled toxic agents. Despite these differences, it is often necessary to extrapolate experimental results from animals to humans (Table 28.1).

All aspects of this chapter are influenced by species differences. This includes deposition, clearance, and the magnitude and type of biologic responses. A major difference, which is sometimes not recognized, is that rodents and dogs are obligatory nose breathers, while humans, particularly with increased exercise and ventilation, switch from nose breathing to mouth breathing. This has a profound influence on the concentration of particles and gases reaching airways.

Particle Clearance Mechanisms in Airways and Parenchyma

The lungs' response to particles depends not only on the amount of particles deposited but also on the amount retained over time. Retention is the amount of material present in the lungs at any time and equals deposited mass minus cleared mass. During continuous aerosol exposure, the lungs reach a steady state of retention when the rate of deposition equals the rate of clearance. It is both the amount of particles retained over time within a specific lung region and the physicochemical properties of the retained particles that determine the magnitude of the lungs' response.

As discussed previously, such factors as particle size, hygroscopicity, and breathing pattern affect the site of deposition within the respiratory tract. In turn, where particles deposit in the lungs determines which mechanisms are used to clear them, how fast they are cleared, and the amount retained over time. The implications of particle characteristics for integrated retention are described by Brain and Valberg (1974) on the basis of the model developed by the Task Group on Lung Dynamics (Bates et al. 1966). They show that the total amount and the distribution of retained dose among the nose and pharynx, trachea and bronchi, and pulmonary and lymphatic compartments are dramatically altered by particle size and solubility.

Particles that deposit on the ciliated airways are cleared primarily by the mucociliary escalator. Those particles that penetrate to and deposit in the peripheral, nonciliated areas of the lungs can be cleared by many mechanisms, including phagocytosis by alveolar macrophages, particle dissolution, or movement of free particles or particle-containing cells directly into the interstitium or the lymphatics. Excellent reviews discuss these clearance processes in detail (Lippmann et al. 1980, Raabe 1982, Jones 1984, Lippmann and Schlesinger 1984, Pavia 1984, Stuart 1984, Schlesinger 1985, Cuddihy and Yeh 1988, Oberdorster 1988, Sleigh et al. 1988, Byron and Phillips 1990, King 1990, Lehnert 1990, Vincent 1990).

Mucociliary Transport

Physiology

The ciliated epithelial cells of the airways and nasal passages are covered by a two-layered mucous blanket: a top high-viscosity gel layer and a bottom low-viscosity serous layer. The gel–mucous blanket, along with any less soluble particles that deposit on it, is moved toward the pharynx by the cilia. Also present in this moving mucous carpet are cells and particles that have been transported from the nonciliated alveoli to the ciliated airways. Mucus, cells, and debris coming from the nasal cavities and the lungs meet at the pharynx, mix with salivary secretions, are swallowed, and enter the gastrointestinal tract. In humans, the ciliated epithelium extends from the trachea down to the terminal bronchioles. The particles are removed with half-times of minutes to hours; the rate depends on the speed of the mucous blanket. The speed is faster in the trachea than in the small airways (Serafini et al. 1976) and is affected by factors influencing either the cilia or the amount and quality of mucus. In the airways, there is little time for solubilization of slowly dissolving materials. In contrast, particles deposited in the nonciliated compartments have much longer residence times; there, small differences in solubility can result in significantly different clearance rates.

Ciliary action may be affected by the number of strokes per minute, the amplitude of each stroke, the time course and form of each stroke, the length of the cilia, the ratio of ciliated to nonciliated area, and the susceptibility of the cilia to intrinsic and extrinsic agents that modify their rate and quality of motion. The characteristics of the mucous layer are critically important. The thickness of the mucous layer and its rheological properties may vary widely. In asthmatic subjects, tracheobronchial clearance can be retarded in comparison with normal subjects, possibly because of a combination of ciliary dysfunction and altered mucous rheological properties (Mezey et al. 1978). In the peripheral bronchioles, for example, the gel layer is less evident. Another factor influencing mucociliary clearance in asthmatics is the presence or absence of flow-limiting segments during tidal breathing (O'Riordan et al. 1992). Flow-limiting segments may mechanically disturb the local mucociliary layer, thus slowing clearance (Foster et al. 1988). Asthmatics without flow-limiting segments may have normal or even faster-than-normal mucociliary clearance rates (O'Riordan et al. 1992). Many of the factors that influence mucociliary clearance, and their clinical implications, have been reviewed (Wanner 1977, Camner and Mossberg 1988, Satir and Sleigh 1991).

Measuring Mucociliary Transport

Mucociliary transport has been studied by a variety of techniques, such as monitoring the movement of inert or radiolabeled particles deposited on the tracheal mucus via either a bronchoscope or an inhaled bolus. One can estimate tracheal mucous velocity from the distance the particles move over time, as observed either with movies filmed through the bronchoscope or with a gamma camera (Sackner et al. 1973, Yeates et al. 1975). Bronchoscopic techniques yield higher numbers for tracheal mucous velocity (15–21 mm/min) than do the noninvasive bolus techniques (4.4 mm/min). These values are characteristic only for the trachea and large central airways. Transport in the terminal bronchioles is about 0.1–0.6 mm/min (Morrow et al. 1967).

Many investigators have estimated mucociliary transport from whole-lung clearance curves. These curves are generated by first having a subject inhale a radiolabeled aerosol and then monitoring the amount of radioactivity in the lungs over time (hours to days). Albert and Arnett (1955) first used this method and noted that the clearance curve can be divided into two phases, a fast and a slow phase. The fast phase is complete within 24–48 h and has generally been attributed to tracheobronchial clearance; the slow phase has been attributed to alveolar clearance (Booker et al. 1967, Morrow et al. 1967, Lippmann and Albert 1969). This approach has been used to study clearance in normal and abnormal subjects (Lourenco et al. 1971, Sanchis et al. 1972, Poe et al. 1977, Camner and Philipson 1978, Stahlhofen et al. 1980).

There is now evidence that clearance from the airways may be incomplete in the first 24 h; this may be even more pronounced in patients with lung disease. Gore and Patrick (1982) noted that particles instilled into the trachea can be sequestered in epithelial cells. Geiser et al. (1990) found latex particles trapped in the periciliary sol fluid below the gel–mucous blanket. The particles may have been displaced there by surface tension forces created by surfactant (Schurch et al. 1990, Gehr et al. 1991). Stahlhofen et al. (1986) noted both fast and slow phases of clearance even in humans given a bolus of particles delivered only 45 mL beyond the larynx. Wolff et al. (1989) delivered radiolabeled particles with a bronchoscope to airway generations 6–10 in dogs and found that about 20% of the particles were cleared slowly.

Another approach to measuring mucociliary clearance is to examine the deposition and clearance of particles in central versus peripheral lung regions (Smaldone et al. 1988), which is referred to as the aerosol penetrance or the airways penetration index (Dolovich et al. 1976, Agnew 1991). The appropriate interpretation of such clearance curves bas been noted (Foster 1988, Agnew 1991).

More studies are needed to elucidate the best methodology to model mucociliary clearance and to understand its role and importance in patients with pulmonary disease. Clearly it is also essential to understand the initial deposition pattern of inhaled particles to determine the importance of mucociliary clearance for the disappearance of particles from the lungs.

Nonciliated Regions: Alveolar Macrophages

Particles that deposit in the nonciliated portion of the lungs are cleared mechanically, by dissolution, or by the combined action of these processes. During mechanical clearance, most particles move toward the ciliated region within alveolar macrophages. It is possible that particles also move as a result of a surface tension gradient due to surfactant that extends from the alveoli to the airways (Rensch et al. 1983). Other particles may enter the interstitial tissues, particularly when deposited levels are high (Lehnert 1990). Macrophages are credited with keeping the alveolar surfaces clean and sterile. These cells rest on the continuous epithelial layer of the lung. It is their phagocytic and lytic potentials that provide most of the bactericidal properties of the lungs. Rapid endocytosis prevents particle penetration through the alveolar epithelia and facilitates alveolar–bronchiolar transport. Particle clearance by alveolar macrophages was the focus of international symposium (Morgan 1992). In addition, the biology of lung macrophages has been reviewed (Brain 1985, Herscowitz 1985, Du Bois 1986, Fels and Cohn 1986, Bowden 1987, Sibelle and Reynolds 1990, Reynolds 1991, Valberg and Blanchard 1991), and the various kinds of lung macrophages have been described (Brain 1988). These include alveolar, airway, connective tissue, pleural, and intravascular macrophages.

Particles enter the lung connective tissue by translocation through type I epithelial cells (Adamson and Bowden 1981), though there are some data indicating that alveolar macrophages carry particles into the interstitium as well (Harmsen et al. 1985). Once in the interstitium, most particles are phagocytozed by interstitial macrophages, though some particles may temporarily remain free. Interstitial particles are cleared eventually through either dissolution (Kreyling et al. 1991) or transportation to regional lymph nodes through lymphatic pathways. In tissues, particles are cleared with half-times ranging from a few days to thousands of days, depending on their solubility. In contrast, particles remaining on alveolar surfaces in humans are cleared with biological half-times estimated to be hours to months (Table 28.6).

TABLE 28.6
Particle Clearance

Anatomic Region	Clearance Mechanism	Approximate Clearance Half Time
Nasopharynx	Mucociliary transport	Minutes
Tracheobronchial	Mucociliary transport	Minutes to hours
Alveolar	Macrophages	Days to weeks
Alveolar	Interstitial migration	Months
Alveolar	Dissolution	Hours to months

Measuring Deposition, Clearance, and Retention

A concept essential to inhalation toxicology is the idea of a dose–response curve. Implicit in this concept is quantifying retention (the amount of an aerosol present at any time). It should be remembered that retention is different from deposition (initial attachment of suspended particles to a surface in the respiratory tract). As discussed elsewhere, retention but not deposition is influenced by clearance and translocation. In the next paragraph, we briefly mention strategies to measure retention in animals and humans.

There are obvious advantages to using nondestructive, noninvasive methods for quantifying particle retention. Commonly used technologies include gamma cameras, magnetometry, and PET scans. An advantage is that repeated measurements in the same human or animal is possible. A disadvantage is that there is a lack of spatial resolution, which can be better obtained by killing and dissecting animals. It is then possible to divide the respiratory tract into pieces and analyze the particle content of each piece. In addition to using particles that are radioactive, fluorescent, radio opaque, or magnetic, other particles have a characteristic visual appearance that allows them to be identified in light electron microscopy. Iron oxide and welding fume are good examples.

It is also possible to estimate initial deposited dose based on measurements of the size and concentration of the aerosol, as well as breathing pattern. Numerous models for predicting deposition have been described. Another approach is simply to measure the difference in the aerosol content between inspired and expired gas, that is, it's disappearance as well as ventilation. Then one can determine the total amount of material initially deposited in the respiratory tract. However, no information is obtained regarding the anatomic location.

INHALATION TOXICOLOGY: BIOLOGICAL RESPONSES

Biological Mechanisms of Particle and Gas Injury

There is a growing body of evidence that fine particles are inherently more toxic than larger particles. Proposed hypotheses for fine particle-induced injury include (1) reductions in the integrity of the pulmonary epithelial and/or endothelial barriers, (2) impaired pulmonary host defense mechanisms, (3) release of inflammatory mediators to produce either local or systemic effects, (4) impaired particle clearance with airway hypersecretion of mucus, (5) aggravation of preexisting airway occlusion, and (6) direct or indirect effects on

cardiovascular function (Castranova 1998). While all appear to be involved to varying degrees, particle-mediated inflammation may well be the predominant mechanism for respiratory tract injury and cardiovascular effects.

Inhaled particles can be directly cytotoxic, effecting transudation of serum proteins from alveolar capillaries. This may involve peroxidation of membrane lipids, resulting in defects in membrane integrity, function, and, subsequently, cell death; if injury is sufficiently severe, the airways and alveoli fill with fluid, causing pulmonary edema and ultimately death. The source of these radicals may be membrane-based oxidases (Vallyathan et al. 1992).

Some metal-containing particles, especially those that either contain or can complex the ferric form (Fe^{+3}) of iron, are particularly active in inducing membrane peroxidation through a reduction–oxidation pathway, producing hydroxyl radical ($OH^{\bullet}$) (Ghio et al. 1992b); inclusion of chelators markedly reduces the oxidant-generating capacity of the particles (Ghio et al. 1992a). This pathway may be especially important for nanoscale materials as these particles may have a disproportionately larger number of catalytic sites on the surface relative to larger particles (Tran et al. 2000, Duffin et al. 2002). By a related mechanism, particles, either by themselves (Wilson et al. 2002) or by interaction with pulmonary alveolar macrophages or alveolar type II cells (Kanj et al. 2005), pulmonary epithelium (Shukla et al. 2000), or polymorphonuclear leukocytes (Prahalad et al. 1999), can stimulate generation of reactive oxidant species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}), or hydroxyl radical (Gurgueira et al. 2002, Gonzalez-Flecha 2004, Ghelfi et al. 2008, Rhoden et al. 2008). These radicals may cause either direct tissue injury or genetic damage. A variety of metal-containing dusts have been shown to stimulate production of ROS from macrophages (Berg et al. 1993), with iron being among the most active metals (Fubini et al. 1995, Rice et al. 2001), while other metal dusts, such as titanium dioxide, are either inactive or minimally active (Goodglick and Kane 1986, Nyberg and Klockars 1990, Blackford et al. 1994).

Oxidant production, whether caused by fine or UFPs, may also oxidize and deplete glutathione, a cellular antioxidant (Stone et al. 1998). The relative levels of reduced and oxidized glutathione appear to play a pivotal role in determining the lung's inflammatory response to oxidant stresses. Alterations in the redox levels of glutathione can affect the degree of acetylation/deacetylation of histone proteins on DNA and allow transcription to occur (Rahman et al. 2002). Conformational changes in DNA allow access of the nuclear transcription factors (nuclear factor-kappa B [NF-κB]) and the associated activation protein (Galter et al. 1994, Haddad et al. 2000), permitting gene transcription. Studies with SWNT in vitro, for example, show cells undergoing oxidant stress with a decreased ratio of reduced/oxidized glutathione, thereby triggering activation of the AP-1 and NF-κB nuclear transcription factors (Dick et al. 2003, Manna et al. 2005). Gene transcription results in the production of various proinflammatory cytokines, a class of chemical messengers that regulate cellular homeostasis and cell proliferation. Notably, the initiating or proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), can induce production of other cytokines that are responsible for the recruitment of inflammatory cells such as neutrophils to sites of particle deposition (Driscoll 1996) with subsequent activation of those recruited cells to produce ROS, providing further toxic insult to airway epithelium integrity.

Noncytotoxic, poorly soluble particles can lead to tissue injury from the prolonged inflammatory response induced by high particle concentrations (either from high exposure levels or inability to remove inhaled particles) in the lung (Morrow et al. 1996). This phenomenon, known as lung overload, can produce similar pathologic lesions in the lung as more cytotoxic dusts such as quartz (Oberdorster 1988), but requires much higher lung burdens than with quartz. Lung overload arises when the normal clearance processes become less and less effective with high particle exposures, leading to accumulation of particles within the lungs (Morrow and Mermelstein 1988). Pathologically, overload appears initially as an increased number of particles in the interstitium in macrophages in air spaces; with time, chronic inflammation may ensue, resulting in alveolar cell hyperplasia, granulomas, fibrosis, and potentially lung tumors (Morrow and Mermelstein 1988). Lung tumors have not been reported if particle clearance and pulmonary inflammation do not occur, suggesting a threshold-related phenomenon for overload-related lung tumors.

The functional decrements in clearance due to dust overloading are apparent if the clearance kinetics is measured; overloaded lungs exhibited a marked increase in particle retention compared to normal lungs. The mechanism for the overload phenomenon is believed to be due directly to a saturation of the capability of alveolar macrophages to ingest particles. The data suggest that alveolar macrophages become overloaded not by the total mass of ingested particles but by the cumulative volume of particles within macrophages. Morrow and Mermelstein (1988) estimated that overload may occur when the average volumetric load of particles exceeds 60 μm^3/macrophage and that macrophage-mediated clearance ceases when the volumetric load exceeds 600 μm^3/macrophage. Once the phagocytic activity of macrophages is reduced by large numbers of particles within alveoli, the mobility of macrophages also diminishes even if the particles are not inherently cytotoxic. The immobilization of alveolar macrophages within the lung results in two detrimental effects: first, subsequent clearance is greatly slowed, and second, the macrophages may continue to elaborate bioactive substances (e.g., proteases, cytokines, growth factors, oxidants, and immunomodulating agents) within the lungs. The overall effect is to increase the likelihood for a prolonged inflammatory response with attendant lung injury and/or tumor development.

The impetus for the overload theory lies, in part, from rodent chronic inhalation bioassays with diesel soot (McClellan et al. 1985, Mauderly et al. 1987), carbon black (Heinrich et al. 1994, Nikula et al. 1995), antimony trioxide (Drew et al. 1986), toner (Muhle et al. 1991), titanium

dioxide (Lee et al. 1985), and talc (Program 1993), particles that previously had been considered benign and do not ordinarily cause pathologic changes if lung clearance processes are not overwhelmed. As a class, these particles have low solubility, low cytotoxicity, and with the exception of diesel soot, are nongenotoxic, although genotoxicity per se is not a rigid requirement for tumor development with these particles (Heinrich et al. 1995). Consistent with all, however, is the observation that high levels of dust exposure were associated with an increasing lung particle burden. At some burden above the capacity of alveolar macrophages to remove particles, lung overload occurs (Warheit et al. 1997).

There are several features of the lung overload phenomenon that impact risk assessment. First, species differences are known in the levels at which overloading and the pathologic sequelae occur, complicating extrapolation of results in test animals to humans. For example, rats have developed lung tumors whereby mice or hamsters, exposed to similar particle concentrations and producing impaired alveolar macrophage clearance, did not (Brightwell et al. 1989, Heinrich et al. 1995). Rats had more severe inflammation after repeated inhalation of carbon black or ultrafine TiO_2 compared to hamsters or mice (Bermudez et al. 2004, Elder et al. 2005). These authors concluded that hamsters had elevated rates of particle clearance relative to the other strains, and while mice and rats experienced lung overload with similar but high TiO_2 lung burdens, mice did not exhibit the progressive fibrosis observed in rats. Elder et al. (2005) also noted that the lung injury and particle burdens with TiO_2 tended to increase as the surface area of the particles increased. Therefore a second consideration is that local tissue dose and subsequent injury from inhaled particles may also be related to particle surface area. While other metrics of exposure concentration may include particle mass, count, volume, or surface area for overload to occur, surface area appears to be the particle characteristic most closely associated with pulmonary inflammation, fibrosis, and lung tumor development (Oberdorster et al. 1994, Castranova 1998, Brown et al. 2001).

By whatever metric, the most important outcome of overload is the persistent inflammation produced by the particles. The consequences of this have an important bearing on the mechanism of tumor development. Marked impairment of particle clearance occurred in rats at 7.1 mg/m^3 or greater with carbon black and was associated with lung injury, chronic inflammation, epithelial hyperplasia, and fibrosis (Driscoll et al. 1996); such changes were not seen in comparably exposed rats at 1.1 mg/m^3, suggesting a threshold phenomenon. In the overloaded rat lung, the investigators reported a significant, dose-dependent increase of HPRT mutations in alveolar epithelial cells at inflammatory concentrations. Inflammatory cell products, including ROS and cytokines, were thought to provide the environment, including induction of cellular mutations and cell proliferation that are necessary for tumor development from oxidant-induced genotoxicity. The observation that particle-elicited rat inflammatory lung cells can be directly mutagenic suggests that there will be an exposure level (or lung burden) below which it does not induce persistent inflammatory changes and therefore mutations should not occur for nongenotoxic particles. However, at inflammatory concentrations, both the inherent genotoxicity of the particle and the resultant genotoxicity ensuing from the particle-elicited proinflammatory milieu may drive tumor development. Overall, the overload theory provides a plausible mechanism into the development of lung tumors from low cytotoxicity, low-solubility nongenotoxic dusts.

Animal Models

The selection of an animal species for an inhalation study is an important one for practical, technical, and ethical reasons. It is apparent that there is no one ideal human surrogate but that each species presents its own advantages and disadvantages. Most commonly, rodents, including the rat, mouse, guinea pig, and hamster (probably in that order), are used. As a consequence of the extensive use of rodents in inhalation toxicology, investigators have generated a large body of information on their responses. Rodents have been used in toxicological tests as models for vapor particle deposition and clearance, mechanisms of toxicant-induced lung and airway injury, and as models for infectivity and immunologic function. Due to their relatively short life-span, the use of rodents represents a reasonable approach for studying chronic toxicity and carcinogenesis. When extrapolating from experimental animal data to humans, factors that need to be considered include the comparative anatomy of the respiratory tract, presence or absence of concurrent diseases or infections, and similarities of the biochemical and physiologic responses in the intended species.

The use of animals in product development and safety assessment needs to continually reflect the welfare and ethical treatment of animals. Intelligent strategies to minimize animal testing by using in vitro or tiered approaches to data collection should be practiced, where possible, recognizing that in vitro tests for inhalation-related end points are only recently being explored; before they can be developed for regulatory acceptance, harmonization and validation activities must occur. In practice, inhalation testing should be conducted at laboratories that possess appropriate facilities and have a trained, competent staff capable of humane animal care and that have institutional review committees to review their testing protocols for compliance with animal welfare policies. All inhalation studies should be scientifically and ethically justified, and every study should be designed to avoid or minimize pain, discomfort, and stress. Care needs to be taken to avoid unnecessary or duplicative testing. Finally, the use of animal enrichment measures, wherever practical, should be encouraged.

In practice, however, selection of the test species is more often based on criteria such as the size and availability of the test animals, the number of animals needed to differentiate chemically induced changes from background or incidental changes, and the expense involved in obtaining and maintaining the requisite number of animals needed for statistical

validity opposite animal use/welfare considerations. Indeed, techniques to reduce the use of animals, without compromising the study, are an important consideration in study design.

In the selection of an appropriate animal species, one must consider the objective of the study, the available toxicological information, the unique functional or structural characteristics, if any, of the species that make it a good (or bad) animal model, what the anticipated response might be that would enable the investigator to most accurately determine the number of animals needed for the duration of the study, and appropriate controls. Historically, the use of small rodents predominates because their size allows testing of larger groups, their relatively short lifetime allows testing over the entire life span (a large body of data already exists on these species), and, finally, the cost of their acquisition and upkeep is relatively low.

The guinea pig has been used in studies of immune function and respiratory sensitization and has been particularly useful in determining the relative potencies of isocyanates (Wong and Alarie 1982, Weyel and Schaffer 1985). The guinea pig's unusually abundant bronchial smooth muscle makes this species a useful model to study airway hyperresponsiveness or bronchoconstriction in asthma models.

The hamster, having a relatively low spontaneous lung tumor rate and resistance to pulmonary infections, has been used in respiratory tract cancer studies. Some investigators feel that the hamster is the best animal model for the study of experimental lung cancer (Saffiotti 1970).

There are several disadvantages in using rodents to predict effects in humans. Because the nasal/pharyngeal anatomy of rodents is unlike that of humans, the amounts and sites of particle deposition may be quite different. Rodents are also obligate nose breathers, with superior nasal filtering efficiency compared to humans. Assessment of pulmonary function in nonanesthetized rodents is difficult, although recent miniaturization of probes and detectors has allowed some success here (Costa 1985). The most serious problems with rodents, especially rats, are those involving spontaneous respiratory infections and their sequelae. Rats appear to be uniquely susceptible to chronic inflammation, pulmonary fibrosis, and cancer from insoluble, noncytotoxic particles by overwhelming lung clearance mechanisms (particle overload). Because rats appear most susceptible to particle overloading, their selection for some types of particle inhalation studies often complicates extrapolation of the results to humans (Oberdorster 1995). In oncogenicity studies, rats appear to be less susceptible to fiber-induced mesothelioma, a malignant tumor of the pleural lung surface. Conversely, hamsters appear to be more susceptible to the development of fiber-induced mesothelioma but less sensitive to induction of lung tumors than rats. Despite these considerations, rats remain the most favored animal model for both short- and long-term inhalation studies.

Inhalation toxicity studies have also been conducted on dogs. The dog is a convenient size for a number of laboratory measurements including evaluation of pulmonary and cardiac function (Godleski et al. 2000, Wellenius et al. 2003, Bartoli et al. 2009a,b). Several natural disease states exist in the dog, making it a good model for evaluating the impact of the substance on conditions such as asthma. The cost and facilities needed to properly care for dogs are a disadvantage relative to rodents. The dog also may be relatively insensitive to certain inhaled gases such as ozone (Stokinger 1957). On the advantage side, extensive use of the dog as an experimental model occurred in studying the effects of radioactive materials following inhalation. There is a wealth of information concerning long-term inhalation of radionuclides.

Because the nasal anatomy of monkeys is similar to humans, monkeys are sometimes used in inhalation experiments. Lung function can be determined. However, considerable cost is involved in obtaining and maintaining monkey colonies, and their lack of general availability is a problem limiting their use. Note that within the species identified as monkey, intraspecies differences do exist. Also, the subgross pulmonary anatomy differs between types of monkeys and humans (McLaughlin et al. 1961).

Other species, such as the ferret, horse, donkey, sheep, cat, rabbit, and pig, have been suggested for use in inhalation studies. Each of these species has been used for special applications, which may take advantage of a particular anatomical feature, chemical sensitivity, or research curiosity. In some instances, the end point of concern drives the animal model chosen. The brown Norway rat has been used as an asthma model in a study design including analysis of cellular infiltrate in the lung, inflammatory factors in bronchoalveolar lavage, immunoglobulin E product in the serum, and changes in delayed-onset respiratory reaction upon inhalation challenges (demonstrated with diphenylmethane diisocyanate [Pauluhn 2005]).

Specific Respiratory Tract Targets for Deleterious Responses and Their Toxicological Assessment

Nose and Nasopharyngeal Region

The nasopharyngeal region plays an important role in the physiological responses to inhaled irritants. There are three basic types of irritants (Alarie 1973): (1) sensory irritants that act on the trigeminal nerve, (2) pulmonary irritants that act on irritant receptors of the airways, and (3) mixed sensory and pulmonary irritants. The initial response to an inhaled irritant may involve an immediate burning or stinging sensation in the eyes, nose, and/or throat. Such stimuli may range from unpleasant to extreme pain and are mediated by interaction of irritants with chemoreceptive nerve endings from the trigeminal nerve in the cornea, nose, tongue, oral cavity, and upper respiratory tract. Once stimulated, these nerve endings can cause systemic responses, resulting not only in a burning sensation of the nose and eyes but also a reflex reduction in respiratory rate. This response occurs once a threshold concentration at the irritant receptor is exceeded and develops immediately or within a few minutes of exposure and is both time- and concentration-dependent. Irritant responses are generally protective in nature, limiting further exposure of

TABLE 28.7
Effects of Inhaled Materials on the Nose

Effect	Examples
Restrict airflow	Temperature changes (cold air), irritants (acids, bases)
Mucociliary flow	Slowing by sulfur dioxide, formaldehyde, methyl amines
Cellular	
Olfactory degeneration	Chloroform, aliphatic esters, methyl bromide
Respiratory/olfactory irritation	Chlorine, sulfur dioxide, formaldehyde
Nasal tumors	Hexamethylphosphoramide, acetaldehyde, formaldehyde, dimethyl sulfate

the offending substance, especially to the lower respiratory tract. Although the effects of sensory irritants per se are not usually life threatening, irritants have the capacity to cause changes in the respiratory tract ranging from minor epithelial injury to fatal lung edema hours or days after exposure.

In our environment, there are many different types of respiratory tract irritants. Chlorine, for example, has been used as a war gas, and an accidental release of methyl isocyanate caused massive human exposures in India. Chemical irritants act on specific targets. Formaldehyde acts primarily on respiratory epithelial cells, chlorine acts on the nasal cilia and bronchial functions, dimethylamine acts on olfactory sensory cells, and cigarette smoke affects the laryngeal epithelium. Furthermore, a number of disease states, including asthma, asphyxiation, chronic bronchitis, emphysema, pulmonary fibrosis, and pneumoconiosis, can be induced or exacerbated by exposure to irritants. Some of the effects of inhaled materials on the nose are shown in Table 28.7.

As a portal of entry, the nasal passages are a target site for a wide range of inhaled substances. For the epithelial mucosa in the nose, squamous tissue is the target of glutaraldehyde toxicity (Gross et al. 1994), transitional epithelial tissue may be damaged by ozone (Morgan 1991), respiratory mucosal tissue by formaldehyde (Morgan et al. 1991), and the olfactory region by β,β-iminodipropionitrile (Genter et al. 1992). The effects produced at these locations may be attributable to the local dose of the substances reaching the site, site-specific tissue susceptibility, or a combination of these factors.

It should be emphasized that recording the lesion distribution is only one step in the process of identifying patterns of nasal toxicity. Because the distribution of lesions results from the interplay of local dose and tissue susceptibility, the relative influence and relevance of such responses to humans are the most important. For example, the airflow-driven local dose of highly reactive water-soluble substances, such as formaldehyde, can dictate the lesion distribution, but airflow is not likely to be a major factor in the lesions produced by less water-soluble substances, such as ozone, chloroform, and methyl bromide (Kimbell et al. 2001).

It has been shown that nasal enzymes acting at localized sites are responsible for the conversion of chemicals, such as dibasic acid esters and hexamethylphosphoramide, to reactive intermediates that cause injury at enzyme-rich cellular locations (Bogdanffy 1990, Dahl and Hadley 1991). The role of local tissue metabolism has important implications in the risk assessment of inhaled materials that cause injury solely in the nose. For example, Bogdanffy and Valentine (2003) described the role of olfactory cell carboxylesterases in the metabolism of vinyl acetate in the development of nasal tumors in rats. Intracellular acidification from the liberation of acetic acid and protons from the metabolism of vinyl acetate and attendant cytotoxicity and regenerative hyperplasia of olfactory epithelium at high doses is thought to be a threshold-related phenomenon in the mode of action for vinyl acetate and perhaps other related esters. For most substances, however, further study is needed to elucidate the dosimetry, susceptibility, and mechanisms of action for inhaled substances on nasal tissue, particularly for use in extrapolating animal data to humans.

Larynx

The laryngeal region, located between the upper respiratory tract and the trachea, is also a potential target tissue of inhaled toxicants, as a variety of substances have produced epithelial injury to this area. The ventral laryngeal epithelium of rats and mice is highly responsive to inhaled materials such as cobalt sulfate (Bucher et al. 1990), tobacco smoke (Sagartz et al. 1992), and a wide variety of industrial chemicals, pharmaceuticals, and aerosol propellants (Gopinath et al. 1987).

Laryngeal lesions commonly involve degeneration of the epithelium with subsequent regeneration, hyperplasia, and squamous metaplasia. In more severe reactions, the larynx may have epithelial ulceration with exudation. Recovery or regression from induced changes is variable and dependent upon the time scale involved and severity of type of initial lesion (Lewis 1991). Specific areas of the rodent laryngeal mucosa appear to be sensitive to inhaled materials. These sites include the epithelium covering the base of the epiglottis, ventral pouches, and the medial surfaces of the vocal processes of the arytenoid cartilage. Therefore, the detection of induced changes requires a consistent, thorough, and detailed histological examination.

Tracheobronchial Airways

The tracheobronchial airways may be the target of a number of inhaled gases, particles, and fibers. Toxicity may be directed at the mucous layer, the mucociliary apparatus of the airway epithelial cells, the epithelial cells, the sensory nerve fibers at the epithelial surface, the basement membrane, or the serous and mucous glands in the lamina propria beneath the epithelium. An example of airway toxicity at all of these levels can be appreciated in animal models of chronic bronchitis using very high levels of sulfur dioxide (250 ppm over several weeks of exposure) first described by Reid in 1963. The chronic bronchitis model in the rat is very similar to the human disease. It includes mucus hypersecretion, mucous plugging of small airways, mucous gland hyperplasia, goblet

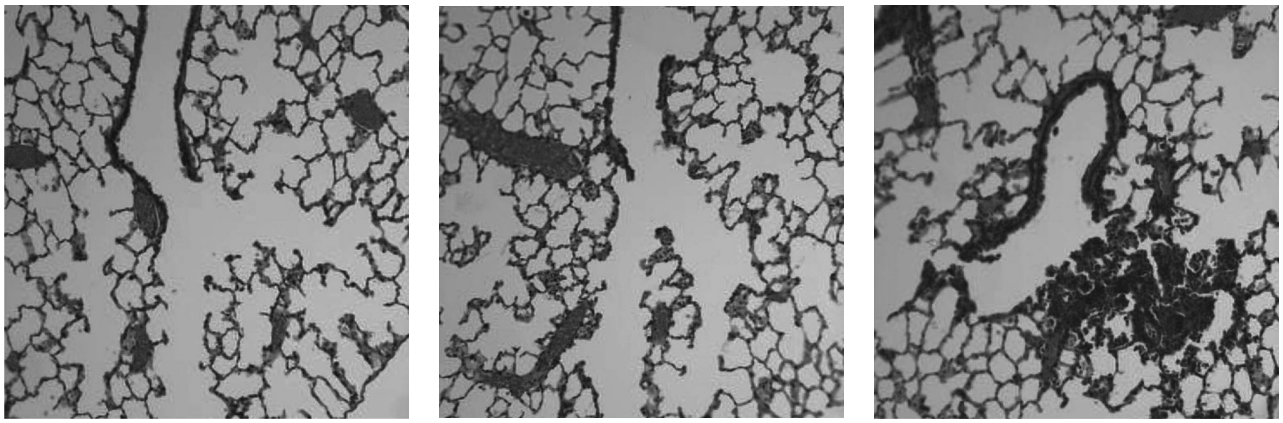

FIGURE 28.16 Bronchoalveolar junction of the rats: Bar at 50 μm in all pictures. (a) Normal rat bronchoalveolar junction. (b) Bronchoalveolar junction of a rat exposed to secondary power plant emissions with minimal inflammation. (c) Bronchoalveolar junction of a rat exposed to vehicular emissions. Note both epithelial proliferation and inflammatory cells in the alveolar.

cell metaplasia (Reid 1963), change in particle deposition patterns (Sweeney et al. 1995), airway hyperresponsiveness (Shore et al. 1995), and significant acute and chronic inflammation (Farone et al. 1995). Early studies with this model showed reversibility of these changes within a few weeks of cessation of SO_2 exposure (Reid 1963). Similar reversibility of changes of chronic bronchitis associated with smoking and its cessation has been described in people.

Pulmonary Region (Bronchioles and Alveoli)

The bronchiolar–alveolar junction is a particularly sensitive site of injury and inflammation with exposure to both particles and fibers. Figure 28.16 shows how this region is altered in rats exposed to either paper or plant or vehicular emissions. The deposition of fibers and particles has been demonstrated at this site as well as the increase in inflammation at this site (Brody and Hill 1981, Brody et al. 1981, Brody and Roe 1983, Saldiva et al. 2002). Demonstration that there is indeed an increase in responding neutrophils at this site often requires morphometric studies of the tissues.

The function of macrophages can be affected by cytotoxic airborne substances, such as asbestos, quartz, and heavy metals. This response is not limited to cytotoxic particles; for example, the instillation of 4 mg of carbon particles (considered a nuisance dust by its relative lack of cytotoxicity) into the lung of a mouse induces an acute macrophage response, with the number of macrophages increasing 10-fold (Bowden and Adamson 1978). A spectrum of responses, resulting in alterations in macrophage numbers, viability, morphology, and changes in the phagocytic capacity, can be produced by chemical insult (Gardner 1984). The effects of inhaled gases and particles on macrophage functions are important because of the function of these cells in homeostasis. Some of the studies of macrophage function describe the injurious effects of gases on macrophage bactericidal activity. In these models, animals first preexposed to ozone had higher mortality if subsequently exposed to pathogenic bacteria, ostensibly by impairing macrophage phagocytosis and lysosomal enzyme activity (Coffin et al. 1968, Goldstein et al. 1978, Gardner 1984).

Patterns of response include acute inflammation with infiltration of neutrophils and acute lung injury. Acute lung injury at the alveolar level includes damage to pulmonary epithelial cells resulting in atelectasis and the eventual formation of hyaline membranes at the level of small airways and alveoli. Hyaline membranes include fibrin, serum proteins, and cell debris from the injury to epithelial cells, alveolar capillary endothelial cells, and interstitial cells. In regeneration of the alveolar epithelium, type 2 epithelial cells, which produce surfactant, serve as the stem cell population that proliferates to cover any areas of denuded basement membrane and injured type 1 epithelial cells. Figure 28.17 illustrates the microscopic appearance of acute lung injury.

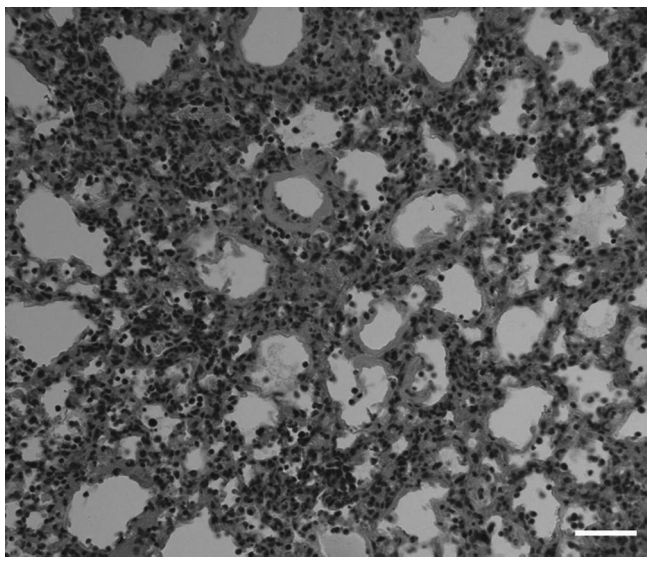

FIGURE 28.17 Acute lung injury in a mouse lung. Hyaline membranes line most alveoli, and the interstitium is thickened with inflammatory cells. Increased numbers of alveolar macrophages are seen in most alveoli. Bar = 50 μm.

Pulmonary Vasculature

The pulmonary vasculature may be the target of injury from ingested or inhaled materials (Batalha et al. 2002). The monocrotaline model of acute lung injury affects the pulmonary vasculature and ultimately results in pulmonary hypertension (White and Rothe 1989, Killingsworth et al. 1997).

Evolving Approaches to the Assessment of Pulmonary Responses

Analytic methods available for assessing the toxicology of materials reaching the respiratory tract are evolving. The continuing increase in the creativity and capabilities of science and engineering have given rise to an ever-growing number of chemicals, particles, and other reagents that have never been experienced by the billions of individuals living on our planet. For new materials, there can be no epidemiological studies in humans. Although considerable information can be learned from lifetime studies in animals, these experimental approaches are expensive and take many years. Thus, there is increasing demand for the development and calibration of high-throughput assays, most of which involve biological measurements in cultured cells. While many reagents can be tested, these assays fail to fully recapitulate the physical and humoral interactions provided by the extracellular matrix, cell–cell interactions, and the diverse cellular components of the actual respiratory tract.

One approach to filling the gap between simple cell assays and intact organisms is by using precision-cut lung slices. Slices can be made from both animal and human lungs, and they function for 7–10 days. This ex vivo approach comes far closer to reality than the cell culture systems in wide use. A recent description of this technique has been published by Rosner et al. (2013). Tice et al. (2013) have reviewed an approach called Tox21, which integrates data from a variety of technologies and multiple endpoints utilizing techniques of systems biology and applying them to toxicology. Geenen et al. (2012) review how systems biology can incorporate multiple sources of information and produce in silico systems where systems biology can explore network-mediated toxicity and make predictions about likely toxic responses as well as predict candidate biomarkers for injury to the respiratory tract.

Finally, as is true for all organizational levels of toxicology from isolated cells to humans, there is increased interest in genomics. We need to identify susceptibility genes and to quantify gene expression elicited by exposures to inhaled chemicals and particles. The emphasis inevitably is on gene–environment interactions. Then, Van Hummelen and Sadaki (2010) review the broad field of toxicogenomics and how it has been used in environmental toxicology. Also see McHale et al. (2010), who give specific examples as to how metals and organic chemicals can be profiled and used to produce risk assessments.

Supporting these approaches to hazard identification and risk quantification are the fields of proteomics, epigenetics or epigenomics, and metabolomics (van Ravenzwaay 2012). The number of genes, proteins, or metabolites is large and "big data" analyses are needed. Interpreting the significance of single cytokines is difficult. It is essential to identify signal-transduction pathways and to provide an integrated story for multiple proteins or metabolites so that we characterize the pathophysiologic story. It is difficult to distinguish cytokines that are an epiphenomenon common to many kinds of injury, such as healing and repair, from cytokines that actually control whether injury leads to progressive and nonreversible damage, such as fibrosis or cancer, or not. We need to go beyond the quantification of individual growth factors, chemokines, and other cytokines that are expressed after exposures to toxins. Instead, we need to delineate the relative intensity and the temporal and spatial relationships among cytokines (Brain 1996). In toto, there is considerable potential in these new approaches. How best to use them will be the subject of future studies.

CONCLUSION

This chapter has discussed the methods and the scientific basis of inhalation toxicology. It describes devices and facilities needed to expose animals to inhaled particles and gases, as well as approaches to estimating or measuring the fraction of the inhaled material that is retained in the respiratory tract. We review the ever-growing repertoire of techniques that can be used to measure the responses elicited by exposures to toxic materials in inspired air. Consequences of exposure to inhaled particles and gases can be studied at many different organizational levels. An understanding of the chemical reactions that take place is important, so are cellular effects and a growing number of in vitro assays. Controlled exposures, such as intratracheal instillation and insufflation, can be valuable, and so can acute and chronic exposures of whole animals. Data from humans are useful, both through experimental clinical studies and observational epidemiologic studies. A challenge is to integrate information from these varied sources.

Inhalation toxicology is of growing importance in the United States and globally. Increases in population and especially energy consumption per capita have increased the number of individuals being exposed to toxic particles and gases, as well as the consequences of those exposures. In poor, rural areas, there continues to be major concerns about indoor air pollution, especially from poorly ventilated sources of heat for warmth and cooking. This is particularly a problem for women and children. Moreover, the massive migration from rural to urban areas has increased the numbers of individuals exposed to significant levels of air pollution from mobile and rural sources. We see more clearly increases in morbidity and mortality, which are attributable to poor air quality.

Toxic exposures persist in a variety of work environments. In the United States, chronic obstructive pulmonary disease increases in importance. Much of the rising incidence in respiratory diseases is attributable to the inhalation of particles and gases in urban and work environments, and especially to the use of tobacco products. Pulmonary diseases are of concern because they are difficult to treat and because they typically manifest themselves only after decades of apparently innocuous exposure. Emphysema and lung cancer are two compelling examples. In their early stages, diagnosis is difficult. In their

end stages, they stand out as diseases that are difficult, sometimes impossible, to treat. This makes primary prevention of toxic exposures critical, and the science of inhalation toxicology in both experimental and observational studies essential.

The study of the toxic effects of inhaled chemicals is usually more challenging compared to dermal or oral routes of exposure due to the high level of technical expertise necessary for the safe generation, control, and characterization of test atmospheres. This chapter provided insights into the toxicological properties of inhaled materials and respiratory tract responses, as well as descriptions of the basic technology needed to properly conduct inhalation exposures. Textbook guidance (such as that provided by this chapter) is valuable, but is no substitute for hands-on experience. Inhalation studies are both art and science.

Despite the introduction of such novel materials as nanoparticles, a foundation in basic inhalation toxicology concepts is necessary to evaluate the hazards that these and all other new materials pose. The mark of a well-conducted inhalation study is clarity. Details of the test atmosphere generation, composition, concentration, and uniformity must be addressed so that the investigator can focus on the significance of the experimental results. Progress creates new materials, new opportunities for exposure, and a deeper understanding of biologic responses. Nevertheless, careful planning and execution of experimental studies will inevitably produce more valuable data.

QUESTIONS

28.1 For animals or humans, how do we relate exposure concentrations of inhaled aerosols to deposited dose? What are the key factors?

28.2 Why are there concerns about the potential consequences of nanomaterials, especially particles smaller than 20 nm and fibrous materials like CNTs?

28.3 Discuss polydisperse aerosols and explain why this distribution of size will be different depending on whether particles are counted, weighed, or characterized by their surface area? For a sphere, how is particle size related to mass and surface area?

28.4 What factors determine where inhaled gases are absorbed? For example, discuss the site of uptake and the site of injury for formaldehyde, sulfur dioxide, ozone, and carbon monoxide.

28.5 What considerations should be given to assess the inhalation hazard of a fibrous material intended to replace asbestos? What properties are associated with increased risk?

28.6 Discuss the relative merits of (1) longitudinal epidemiologic studies in humans exposed to particles, (2) chronic inhalation studies in animals, (3) acute in vivo bioassays in animals, and (4) an increasing array of in vitro tests.

28.7 Imagine marching down the respiratory tract from the mouth to the large airways to the small airways, and finally to the parenchyma. Now describe how geometry and linear velocities will help determine the relative efficiency of different deposition mechanisms. What are the major deposition mechanisms and which will dominate in these different anatomic regions?

28.8 Discuss major ways of generating aerosols for inhalation toxicologic experiments.

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29 Detection and Evaluation of Chemically Induced Liver Injury

Gabriel L. Plaa, Michel Charbonneau, and Isabelle Plante

CONTENTS

INTRODUCTION

Liver injury induced by chemicals has been recognized as a toxicological problem for more than 100 years. During the late 1800s, scientists were concerned about the mechanisms involved in the hepatic deposition of lipids following exposure to yellow phosphorus. Also, hepatic lesions produced by arsphenamine, carbon tetrachloride, and chloroform were studied in laboratory animals during the first 40 years of the twentieth century. During the same period, the correlation between hepatic cirrhosis and excessive ethanol consumption was recognized.

Liver injury is not a single entity; the lesion observed depends not only on the chemical agent involved but also on the duration of exposure. After acute exposure, one usually finds lipid accumulation in the hepatocytes, cellular necrosis, or hepatobiliary dysfunction, whereas cirrhotic or neoplastic changes are usually considered to be the result of chronic exposures. Different biochemical alterations may lead to the same endpoint; no single mechanism governs the appearance of hepatocellular degenerative changes or alterations in function. Some forms of liver injury are reversible, whereas others result in a permanently deranged organ. The mortality associated with various forms of liver injury is variable. The incidence of injury can differ among species, and the presence of a dose-dependent relation may not always be apparent.

The marked vulnerability of the liver to chemically induced damage is a function of (1) its anatomical proximity to the blood supply from the digestive tract and high blood flow rate, (2) its ability to concentrate and biotransform foreign chemicals, and (3) its role in the excretion of xenobiotics or their metabolites into the bile. The diverse nature of the functional activity of the liver and its varied response to injury makes the selection of appropriate testing procedures a difficult task.

This chapter discusses the major methods and models that are useful in the detection and evaluation of liver injury in laboratory animals.

CLASSIFICATION OF CHEMICALLY INDUCED LIVER INJURY

The morphological changes observed following hepatic injury produced by chemical and biological agents can be classified according to two parameters: location and type of lesion produced.

Location within the Hepatic Parenchyma

An early system of describing pathological lesions of the liver originated from the concept of the hexagonal lobule introduced by F. Kiernan in 1833 (Figure 29.1). This configuration, the classical manner of presenting the relations between the hepatic cell, its vascular supply, and the biliary system, was considered to represent the functional unit of the liver. The terminal hepatic venule (central vein) is found in the center of the lobule; and the portal space, containing a branch of the portal vein, a hepatic arteriole, and a bile duct, is located at the periphery of the lobule. Based on this

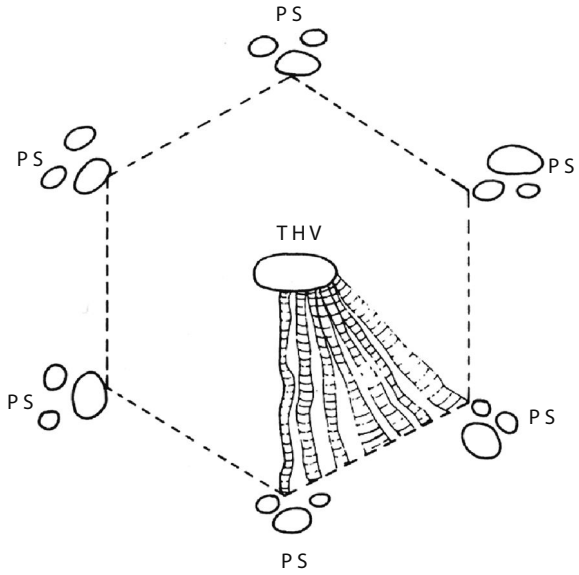

FIGURE 29.1 Schematic representation of the traditional hexagonal lobule. (*PS*) portal space, consisting of a branch of the portal vein, hepatic arteriole, and a bile duct; (*THV*) terminal hepatic venule (central vein). (From Plaa, G.L., *Toxic Responses of the Liver*, M.O. Amdur, C.D. Klaassen, and J. Doull, eds., Pergamon Press, New York, 1991, pp. 334–353. With permission.)

configuration, lesions of the hepatic parenchyma have been classified as centrilobular, midzonal, or periportal.

The hexagonal lobule configuration does not correspond to the functional unit of the liver. The hexagonal lobule is not conspicuous under microscopic examination. Injection of colored gelatin mixtures in the portal vein or hepatic artery shows that terminal afferent vessels supply blood only to sectors of adjacent hepatic lobules. These sectors are situated around terminal portal branches and extend from the terminal hepatic venule of one hexagon to the terminal hepatic venule of an adjacent hexagon. Rappaport defined the parenchymal mass in terms of functional units called the liver acini [397]. A simple liver acinus consists of a small parenchymal mass that is irregular in size and shape and is arranged around an axis consisting of a terminal portal venule, a hepatic arteriole, a bile ductule, lymph vessels, and nerves (Figure 29.2). This acinus lies between two or more terminal hepatic venules with which its vascular and biliary axis interdigitates. There is no physical separation between two liver acini. The hepatic cells of the simple acini are in cellular and sinusoidal contact with the cells of adjacent or overlapping acini. Even with this extensive communication, the hepatic cells of one particular acinus are preferentially supplied by their parent vessels. Three relatively discrete circulatory zones appear within each acinus (Figure 29.2). Hepatocytes in close juxtaposition to the terminal afferent vessel constitute zone 1. These cells are the first to be supplied with fresh blood, rich in oxygen and nutrients. The higher order of zones 2 and 3 is indicative of the greater distance between the cells comprising these zones and the supply of fresh blood.

One of the interesting correlates of the concept of zonal acinar circulation is the growing realization that not all hepatic

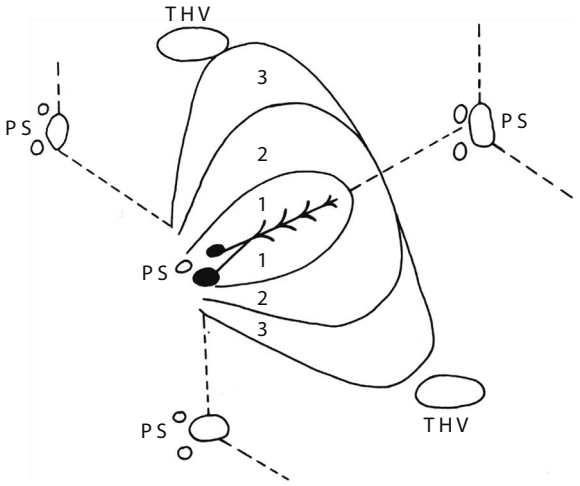

FIGURE 29.2 Schematic representation of a simple hepatic acinus, according to A. M. Rappaport. (*PS*) portal space, consisting of a branch of the portal vein, hepatic arteriole, and a bile duct; (*THV*) terminal hepatic venule (central vein); (1, 2, 3) zones draining off the terminal afferent vessel (in *black*). (From Plaa, G.L., *Toxic Responses of the Liver*, M.O. Amdur, C.D. Klaassen, and J. Doull, eds., Pergamon Press, New York, 1991, pp. 334–353. With permission.)

parenchymal cells within the liver lobule have the same kind of functional specificity. Rappaport's acinar concept has been modulated by others [152,277] to account for differences in enzyme distribution and redox state. Areas of differing metabolic activity exist within the liver [233]. Respiratory enzyme activity is particularly high in the zone closest to the terminal afferent vessel (zone 1) (Figure 29.2), whereas the most distant zone (zone 3) is particularly rich in cytochrome P450-dependent enzyme systems. The perivenous (zone 3) cells are relatively rich in some nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzymes, and periportal cells (zone 1) are relatively poor [152]. The concept of *metabolic zonation* is based on differences observed between enzyme activities in periportal and perivenous regions [233]. Thurman and Kauffmann [467] reported on the lobular distribution of maximal enzyme activities measured by immunohistochemical or microchemical techniques; these parameters do not always correlate with metabolic flux rates as measured by microfluorometry and miniature O_2 electrodes. Periportal-perivenous gradients are described for cytochrome P450s, sulfatation, glucuronidation, and glutathione-S-transferases [151]. Functional gradients are also reported for hepatobiliary activity [2]. The implications of such findings are not well understood. However, zonal and cellular enzymatic specificity and metabolic heterogeneity may permit the rationalization of differing mechanisms of action in the development of hepatic lesions associated with hepatotoxic agents.

The classical hexagonal descriptions of focal, midzonal, periportal, and centrilobular lesions, although functionally incorrect, are compatible with Rappaport's zonal acinar configuration. Centrilobular necrosis, for example, occurs in cells located in the distal acinar zone (zone 3) (Figure 29.2). When several such zones are affected, a concentric lesion can be visualized. Regeneration is said to occur from cells located in the midzonal region of the hexagonal representation, which corresponds to the acinar zone closest to the terminal afferent vessel (zone 1), a zone shown to be particularly high in cytogenic activity. Therefore, it appears that the acinal circulatory concept of the hepatic lobule does not come into serious conflict with the earlier descriptions of pathologic lesions.

Morphologic Classification

Morphologically, liver injury can manifest itself in different ways [378]. The acute effects can consist of an accumulation of lipids (steatosis) and the appearance of degenerative processes, leading to cell death (necrosis). The necrotic process can affect small groups of isolated parenchymal cells (focal necrosis), groups of cells located in zones (centrilobular, midzonal, or periportal necrosis), or virtually all of the cells within a hepatic lobule (massive necrosis). The accumulation of lipids can also be zonal or more diffuse in nature. Although acute injury may consist in both necrosis and fat accumulation, it is not necessary that both features be present. The cholestatic type of lesion, resulting in diminution or cessation of bile flow with retention of bile salts and bilirubin, is also an important form of liver injury [372,383]; this lesion leads to the appearance of jaundice. A type of massive necrosis that resembles a viral infection [528] is produced by certain chemicals. A number of drugs are also associated with a mixed type of lesion, that is, one that possesses both cholestatic and viral-like hepatic components [528].

Chemically induced liver injury resulting from chronic exposure can produce marked alteration of the entire liver structure with degenerative and proliferative changes observed in the various forms of cirrhosis. Through the years, a number of classification systems have evolved to describe the chemicals involved. The schemes are beyond the scope of this chapter. In brief, however, some are based on morphological changes [378], and others deal with the postulated mechanisms of action or the circumstances of exposure [528]. In the morphological classifications, one finds those chemicals (e.g., carbon tetrachloride [CCl_4], chloroform [$CHCl_3$], phosphorus, tannic acid, ethionine, ethanol) that produce zonal hepatocellular alterations (necrosis, steatosis). Intrahepatic cholestasis is a lesion produced by a number of drugs (e.g., phenothiazine derivatives, antimicrobial agents, anabolic steroids, oral hypoglycemics) and is characterized by biliary dysfunction. In addition, massive hepatocellular necrosis is produced by other drugs (e.g., iproniazid, monoamine oxidase inhibitors, halothane).

Regarding mechanisms of action involved [237,455,528], one finds a variety of possible effects. At least six different sites of action have been described [284], including bleb formation on the cell membrane, transport pumps in the canaliculus, enzyme-drug adduct formation in the endoplasmic reticulum, vesicle movement to the cell surface, apoptotic processes, and inhibition of β-oxidation and/or respiration in mitochondria. Predictability of the appearance of the injury and production of the lesion in laboratory animals

are important considerations. Some forms of drug-induced liver injury in humans are due to hypersensitivity (allergic reactions) or an expression of individual susceptibility (idiosyncratic reactions) [237,455,528]. Reactive metabolites and immunological mechanisms appear as important components in the elucidation of such lesions [351].

From these classifications, one sees that there are a variety of pathological processes involved in what is called, in general terms, *liver injury*. Furthermore, many different substances can cause injury. Although classification schemes assist in conceptualizing what is occurring, it should be understood that with additional knowledge of the events actually involved in the elaboration of the biochemical lesion changes in the classifications certainly occur. Regardless of this fact, the pathological types of injury produced by hepatotoxicants largely determine the biochemical and functional manifestation of injury and thus the battery of toxicological tests needed to detect and evaluate *liver injury*.

Neoplastic changes may be another endpoint of chemical liver injury. Liver cancer is one of the leading cause of cancer worldwide, being responsible for over 500,000 deaths a year [123]. Hepatocellular carcinoma (HCC), the most common type of liver cancer, arises from hepatocytes [473]. Chronic liver diseases are at the origin of the vast majority of HCC, particularly viral hepatitis B and C infections. HCC is a progressive disease, divided in three distinct phases: molecular, preclinical, and clinical [473]. Mutations or changes in gene expression occurring in the molecular phase are thought to provide a growth advantage to differentiated cells by promoting proliferation and inhibiting apoptosis [473]. Decreased cell–cell interactions are commonly observed in chemically induced HCC and can be considered as an early marker of hepatocarcinogenesis [475,476].

MODELS AND METHODS IN HEPATOTOXICITY

Many laboratory procedures have been performed to diagnose and monitor liver diseases in human clinical medicine. The performance characteristics of common tests used in humans were evaluated in 1999 and guidelines were published by the National Academy of Clinical Biochemistry [115]. Over the years, many techniques and procedures used in humans [143] were applied to laboratory animals. Ex vivo and in vitro models were also developed, allowing more mechanistic evaluation of hepatotoxicity of compounds. This chapter gives an overview of some of the common models and established methods used in hepatotoxicology, as well as more recent avenues. Some approaches, such as necrosis or hepatic function, are not discussed, and the reader is referred to good reviews for additional information [227,396].

Models Used in Hepatotoxicity Studies

Hepatotoxicological studies have been carried on virtually all common in vivo models generally used in medical sciences [147]. Other than the usual considerations in choosing an animal model (i.e., cost, caging, number of animals, and strain), it is crucial to consider the interspecies differences in the metabolism of xenobiotics in hepatic studies [530]. Indeed, the same compounds can be biotransformed at different rates and produce diverse metabolites or various amounts of metabolites in a given species [287]. Thus, when using an animal model to study human toxicity, one must choose the model that will reflect the most close human toxicity and/or biotransformation of the compound [147].

In complement to in vivo models, the approaches available to assess hepatotoxic properties of chemical agents encompass different levels of organization such as precision-cut liver slices and isolated livers (ex vivo) and isolated hepatocytes and cell cultures (in vitro). Liver cell organization is not disrupted in these four systems, such that the injuries caused by a chemical result from the overall effects of Phases I and II biotransformation reactions, defense and repair systems, and cellular processes. Zonal architecture of the liver, normal polarity of hepatocytes (biliary and plasmatic poles) and presence of all liver cell types are present only with the liver slice system and the isolated perfused liver. Isolated hepatocytes, however, offer many advantages: (1) they are relatively easy to prepare without sophisticated equipment; (2) a large number of experiments with the liver of one animal, serving as its own control, can be performed; and (3) sampling throughout the experiment is feasible. Selection of a particular approach depends on specific research needs; xenobiotic biotransformation is generally comparable from one system to another as exemplified by similar metabolic profiles for caffeine biotransformation in liver slices, hepatocyte cultures, and microsomes, with a rate of metabolite formation close to that calculated from in vivo caffeine elimination [40]. While in vivo model is required for an integrated evaluation of toxic effects of compounds, ex vivo and in vitro systems offer the possibility of assessing liver injury in the absence of extrahepatic factors, such as the absorption, distribution, and extrahepatic metabolism of the chemical, humoral factors, and toxic effects caused at other sites. For this reason, they are especially valuable for studying specific mechanisms involved in chemically induced liver injury. In the present chapter, however, we focus on the use of these systems to detect and evaluate hepatotoxic properties of chemical agents.

Ex Vivo Models

Isolated Perfused Liver

Studying the entire organ is probably what resembles the in vivo situation the most closely. In this model, the freshly isolated organ remains intact and is nourished with media via the ligation of the hepatic artery [168], thus retaining tissue architecture, hemodynamic parameters, bile production, tissue function, and cell–cell interactions [168,177,178]. On the other hand, perfused isolated livers are expensive and complex, can be maintained for only a few hours, and many animals are required as a particular liver can be used for only one experiment [176]. Technical aspects and applications of the isolated perfused liver system will not be discussed in the present section. For additional reading on the use of the isolated perfused liver as a model for studying drug- and chemical-induced hepatotoxicity, the reader can consult monographs and reviews [25,42,176,458].

Liver Slices

To overcome limitations of the isolated perfused liver model, methods were developed to preserve precision-cut liver slices in culture. Precision-cut liver slice technology was pioneered at the University of Arizona (reviewed by Brendel et al. [54]). Their dynamic organ culture system allows both the upper and lower surfaces of the cultured slice to be exposed to the gas phase during the course of incubation to overcome disintegration of the slice-medium interface in long-term incubation of tissue slices. The liver slice procedure appears to be more efficient for the collection of serial data than the perfused liver preparation. In a discussion of techniques for studying drug metabolism in vitro, Gillette [156] presented the liver slice system in the following terms: "Since the circulation of nutrients and oxygen through the capillaries is lost, the transfer of these substances from the medium to the innermost cells must depend on passive diffusion. It is therefore imperative that the slices be thin because the rate of diffusion is inversely related to the square of the distance the substances must traverse. Even with slices as thin as 0.5 mm, the concentrations of the nutrients and other substrates that are rapidly utilized by cells in the slice may not be identical throughout the slice." In recent years, technological improvements in the preparation of precision-cut rat liver slices have allowed this system to assess liver injury in a reproducible fashion. About 200–250 μm thick liver slices (~8 cell layers) allow multiple experimentations on the liver of a single animal [286,308,332,526]. Recent research showed that slices as thin as 100 μm can also be used successfully [103]. Three different tissue slicers (Krumidieck/Alabama Research and Development Tissue Slicer, Brendel/Vitron Slicer, Leica VT1200 S microtome) have been described [149,363,529]. All slicers appear to readily produce liver slices that are adequate for biotransformation or toxicity studies [391,529]. Produced liver slices can then be cultured in a continuously submerged culture system in which they are floating within the culture medium in plates or flasks that are gently shaken or placed onto a grid in magnetically stirred culture medium [484]. Liver slices can also be placed on inserts in a rolling glass vial or in rocking six-well plates and exposed to the gas phase and the culture medium in dynamic culture systems. In both continuously submerged and dynamic culture systems, tissues are cultured at 37°C in a humidified incubator in the presence of oxygen (5%–20%) and CO_2 (5%). One issue with these two systems is the accumulation of waste products and the depletion of substrates in the medium that is thought to result in decreased metabolic activity and protein expression over time [241,431,486]. Different systems have recently been designed to overcome these limitations that represent promising models for toxicological studies [195,241,431,486].

The slicing buffer medium should be similar in inorganic composition to the culture medium used in liver cultures; HEPES is recommended. Waymouth's medium is recommended as the culture medium, and a viability of 120 h is claimed for liver slices. A roller culture system is claimed to be most effective for the extended viability of precision-cut tissue slices. The parameters for assessing viability of liver slices originally described by the Arizona group include enzyme (lactic dehydrogenase [LDH]) leakage, intracellular potassium content, nonprotein sulfhydril content, lipid peroxidation, protein synthesis, gluconeogenesis, Phase I and II biotransformation, and histological evaluation. Olinga et al. [354] compared different methods (magnetically stirred 24-well, full immersed system; shaker-stirred, 6-well, fully immersed system; rocker platform, 6-well culture plate system; roller incubation vial, partially immersed system) for incubation of liver slices for periods of 1.5–24.5 h. In terms of cell viability, the stirred systems, generally, fared the least, whereas the rocker and roller methods appeared to be superior. In another study [471], where precision-cut liver slices were incubated in either a shaking platform or a roller incubation system for 72 h, the roller system was reported as the better one for preserving histological and functional integrity of the slices.

Behrsing et al. [32] took advantage of refinements in procedure that prevent necrosis at the contact point with the support mesh to demonstrate that rat liver slices can retain their integrity and viability for at least 10 days in culture. Refinements included (1) flushing the liver and slicing the cores in cold University of Wisconsin (UW) solution, (2) loading the slices onto cellulose–ester filters placed on the mesh inside the inserts in a dynamic roller culture system rather than directly in contact with the titanium mesh or Teflon screen, and (3) culturing in a defined medium based on Waymouth's MB 752/1 under a high O_2 content atmosphere. The known hepatobiliary toxicant, geldanamycin, produced biochemical and morphological deficits in both hepatocytes and biliary cells in this model system, consistent with its target in vivo. Subsequent studies showed that in rat liver slices it is possible to detect compound-, concentration-, and cell-specific toxicities in response to geldanamycin and 17-allylaminogeldanamycin [31] and that species-related differences in toxicity seen in vivo are replicated using dog liver slices [5].

Liver slices maintained for short periods of time have been used with success in biochemical and pharmacological research for over 50 years. For hepatotoxicity, Weldon [502] reported in 1965 a clear dose-dependent reduction in the metabolism of L-leucine by liver slices incubated with CCl_4. Smuckler [448] subsequently observed a marked reduction in amino acid incorporation into proteins by liver slices from animals that had received CCl_4, as well as in control slices incubated with CCl_4. Marsh and Bizzi [306] concluded that the rat liver slice was a valid and useful system for the study of the action of drugs on lipid and lipoprotein metabolism. The parameters measured to evaluate liver injury include LDH leakage, decreased protein synthesis, decreased intracellular potassium content and histological alterations. Pig and human liver slices have also been cryopreserved with success and used for subsequent toxicological and metabolic studies [134]. High viability of rat liver slices has been observed when tissues were rapidly frozen after preincubation with 18% Me_2SO or VS_4 (a 7.5 M mixture of Me_2SO, 1,2-propanediol, and formamide with a weight ratio of 21.5:15:2.4) [104]. Fisher et al. [132] observed that three

dichlorobenzene isomers were more toxic to human liver slices when the incubation medium was Krebs–Henseleit buffer compared to Waymouth's medium.

Assessing the relative toxicities of bromobenzene, *o*-bromobenzonitrile, *o*-bromobenzene, *o*-bromoanisole, *o*-bromotoluene, and *o*-bromo-benzomethyltrifluoride, Fisher et al. [133] concluded that results obtained with precision-cut liver slices appear to be more representative of those observed in vivo than do results obtained from isolated hepatocytes. Wormser et al. [512] observed species and age differences in toxicity and reported that the relative toxicities of various chemicals in the liver slice system were similar to those reported in vivo. The hepatotoxic properties of CCl_4 and $CHCl_3$ in precision-cut liver slices suggested that this system is a useful tool for the investigation of site-specific toxicants [16,17]. In rats, bromobenzene produces centrilobular necrosis in vivo, whereas allyl alcohol produces periportal lesions. In liver slices, centrilobular lesions following bromobenzene exposure were observed, but the expected periportal lesion with allyl alcohol was not observed [149,446]. The lack of the expected site-specific response with allyl alcohol is attributed to the absence of circulatory influences in the tissue slice system. Indications of oxidative stress (glutathione (GSH):glutathione disulfide (GSSG) ratio), as a mechanism of action to explain the hepatotoxic properties of atractyloside, was reported in studies using precision-cut liver slices obtained from rats and domestic pigs [350]. The hepatotoxic properties of coumarin, menadione, and allyl alcohol on protein synthesis, potassium content, and mitochondrial dimethylthiazoldiphenyl tetrazolium–formazan production (MTT assay) were assessed in liver slices prepared from different species (rats, guinea pigs, Cynomolgus monkeys, and humans) and incubated for 24 h periods [391]. Menadione toxicity was evident in all four species, while allyl alcohol toxicity was less evident in rats; coumarin concentration-dependent toxicity was evident in guinea pigs and rats, but less effective in monkeys or humans. These interesting species-dependent observations demonstrate the utility of the liver slice technique for evaluating species differences in chemical-induced hepatotoxicity.

The application of the precision-cut slice technique to the biotransformation of xenobiotics in liver, including the coupling of Phases I and II metabolic pathways, has been extensive [3,94,149,300,363,465]. The utility of cultured human liver slices for assessing the effects of chemicals on P450 enzymes has been demonstrated [121]. Liver slices from several species (rats, dogs, guinea pigs, and humans) have been employed with success. Liver slices also were shown to respond to several known different metabolic situations: peroxisome proliferation, hormone-regulated glucose metabolism, unscheduled DNA synthesis, and formation of neoantigens after halothane exposure. Other applications of the technique have been demonstrated. The live-time evaluation of cell toxicity by following production of specific fluorescent dyes in liver slices by confocal microscopy was studied after exposing precision-cut slices to 1,1-dichloroethylene and then examining them microscopically; very few dead cells were observed at 2 h, progressively more were seen after 3–7 h [93]. Apoptosis (detected by DNA fragmentation, amplification of the Bax gene, and histology) in liver slices was assessed after incubating slices in the presence of staurosporine, a protein kinase C inhibitor [81]; features of apoptosis were successfully detected even in the presence of necrosis. Histopathological changes, DNA fragmentation, caspase-3 activation, and release of cytochrome *c* induced by thioacetamide in both rat liver slices and in vivo treated rats indicated that precision-cut liver slices provide a valuable system to study chemical-induced apoptosis [328]. Chemical–DNA adduct formation has been observed in benzo[a]pyrene-exposed rat liver slices [194]. Precision-cut liver slices from two transgenic mouse strains, one of which couples the promoting region of CYP 1A1 to ß-galactosidase, and another that couples two forward and two backward 12-otetradecanoyl phorbol-13-acetate [12] repeat elements (TRE) to luciferase (termed AP-1/luciferase), showed promotional effects in the presence of β-napthoflavone and TPA, respectively, indicating that precision-cut liver slices from transgenic mice offer a novel in vitro method for toxicity evaluation, while maintaining normal cell heterogeneity [66]. Precision-cut liver slices from neonatal rats showed fewer morphological alterations and increased viability compared to adult rats and excellent induction of cytochrome P450 isoforms [300]. Finally, precision-cut liver slices have been reported to test specificity and efficiency of gene transfer as well as of viral replication using human tissue [244].

The diverse utility of precision-cut liver slice technique for evaluating liver function and injury is now well established, since its original introduction in 1985 by Smith et al. [447]. Furthermore, it is readily adaptable to the evaluation of human tissue. One advantage the system offers over isolated or cultured hepatocytes is the fact that tissue architecture is preserved, all cell types remain present, and multiple analyses coupled with morphological examination are possible.

In Vitro Models

Isolated Hepatocytes

The liver is constituted of six different cell populations: the hepatocytes, biliary epithelial cells, endothelial cells, Kupffer cells, Ito cells (lipocytes, fat-storing cells), and pit cells. The cells other than the hepatocytes are designated as the nonparenchymal cells. The hepatocytes (or parenchymal cells) constitute 60% of the liver cell population and occupy 80% of the total liver volume. Much of the effort in isolation of liver cells has focused on viable hepatocytes, since they are the main metabolic unit of the liver. Procedures, however, are also available for isolating nonparenchymal cells [140].

The literature on preparation, properties, and application of isolated hepatocytes is abundant, and excellent monographs and reviews have been published [37,180,312,313,458]. The in situ two-step procedure is the most commonly used technique to isolate hepatocytes. It is derived from the pioneering work of Berry and Friend [37], who introduced in situ liver perfusion with digestive enzymes, and from the subsequent work of Seglen [434,435], who enhanced the recovery of isolated viable cells by initially perfusing the liver with a calcium-free

buffer and then placing the digestive enzymes in a calcium-supplemented buffer. In current techniques, buffers containing a chelating agent and collagenase as the digestive enzyme are used for the first and second steps; suspensions exhibiting greater than 90% viability are usually obtained. Nonviable hepatocytes can be separated from viable ones using a dibutyl phthalate separation technique [128].

Hepatocytes are most commonly isolated from rat liver; the two-step procedure, however, has been successfully adapted to other species such as the mouse, rabbit, and guinea pig [312,313]. Specialized techniques are also available for preparing suspensions enriched with periportal or perivenous hepatocytes [38]. Increasing interests in toxicological risk assessment have prompted the development of procedures, such as the biopsy perfusion methods [4], to isolate hepatocytes from human liver samples. Successful approaches have also been achieved to optimize cryopreservation procedures for human hepatocytes [77,281,298].

Isolated hepatocytes have been extensively employed for studies on chemical (including drugs) biotransformation and toxicity. Over the years, the bulk of the reports published on chemical metabolism in isolated hepatocytes have defined the capabilities and limits of this system. There is no doubt that isolated cells have proved valuable for investigating biotransformation [37,38,182]. The activity of Phases I or II metabolizing enzymes in viable cells is maintained for a few hours [180]. However, in standard metabolic/toxicological studies, the incubation period of the cells with the chemical is relatively short (<3 h), such that the losses in activity do not generally invalidate the experiments. The substrate metabolic rates are often similar or slightly slower in isolated hepatocytes than in corresponding 9000 g supernatant or microsomes [180]. The parameters measured to evaluate liver injury include LDH leakage, decreased protein synthesis, decreased intracellular potassium content, and histological alterations.

Cytotoxicity of chemicals in freshly isolated hepatocyte suspensions is a valuable tool when screening xenobiotics for hepatotoxic properties. As with the in vivo tests, the major cytotoxicity parameters studied are based on the structural integrity of the cell membrane. The uptake of normally non-permeable dyes, such as trypan blue and neutral red, is one of the most common tests; the percentage of nonviable colored cells requires a cell counting analysis under a light microscope. Leakage into the medium (separated from the cells) of the cytosolic enzyme LDH is a biochemical test that is as frequently common as the dye exclusion assay. The former, however, is more convenient to perform than the dye assay, and it also offers the advantage of summing up the release of all damaged cells, including disintegrated hepatocytes. For some chemicals, decreases in intracellular concentrations of LDH have been reported at dosages in which LDH leakage into the medium could not be detected [303]. Measurement of intracellular potassium, sodium, or calcium content is a more sensitive marker of cell membrane integrity. Fariss et al. [128] observed a decrease in potassium level 3 h prior to LDH leakage in cells treated with adriamycin in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea or ethyl methanesulfonate. Indices of cellular metabolic competence are also sensitive markers; modifications of glycogen deposits and protein synthesis have been shown to detect early changes in isolated cells treated with chlorpromazine, promethazine, bromobenzene, acetaminophen, and isoniazid [160]. Frazier [139] reported that cellular potassium concentration was the most sensitive toxicity index, with inhibition of protein synthesis second, and trypan blue staining least, to evaluate cadmium, copper, and zinc toxicity. Finally, morphological changes in isolated cells can be assessed by electron microscopy.

The isolation procedure inevitably introduces changes and renders cells more susceptible to the effect of chemicals compared to the intact liver. When studying the hepatotoxic properties of organic solvents, care should be given to the selection of concentrations used, since these agents are thought to exert direct solvent effects [37]. In addition, it is preferable to use equilibration techniques in stoppered flasks to study volatile chemicals with low water solubility.

Studies have shown that known in vivo hepatotoxicants tested in isolated cell suspensions are cytotoxic in vitro. Individual chemicals such as acetaminophen, ethanol, methotrexate, fentanyl, bromobenzene, and chlorinated aliphatic compounds [182,479,494], as well as mixtures like CCl_4/$CHCl_3$ [349] and combinations of trichloroethylene, tetrachloroethylene, and 1,1,1-trichloroethane [451] are hepatotoxicants in vivo and in vitro. There are also examples of species and strain differences observed in vivo that also occur in vitro [326,511]. Differences between in vivo and in vitro hepatotoxic potency, however, were observed in several occasions. Dimethylnitrosamide and thioacetamide are not as potent in the isolated cell system as expected from their in vivo hepatotoxicity [453]. Rankings of relative toxicity for different haloalkanes or bile salts in isolated hepatocytes differ from those observed in vivo [98,169,451]. Finally, the ability of isolated cell suspensions to accurately detect the hepatotoxic properties for chemicals with unknown in vivo effects remains to be fully demonstrated, in particular for chemicals found to be weak or moderate hepatotoxicants.

Cholestatic responses per se cannot be seen in isolated hepatocytes. It is possible, however, to evaluate chemical interference with processes involved in the transport of endogenous substances into and out of hepatocytes. Such approaches have been successful for thioacetamides and cyclosporin A [55,273]. Hepatocyte couplets (two adjacent hepatocytes surrounding a lumen or vacuole) represent the primary bile secretory unit and are thought to be the equivalent of the bile canaliculus [49]. Secretory polarity is retained in hepatocyte couplets and methods that permit the isolation of these couplets, as well as their utility for assessing canalicular function, have been perfected. The functional aspects include (1) the excretion of fluid, organic anions and cations, lipids, and proteins; (2) the regulation of cellular and canalicular pH; (3) transcytosis and protein transport; (4) cytoskeletal function and canalicular contractility; (5) signal transduction, calcium signaling, and paracellular permeability; and (6) electrophysiological events [49]. The effects of cholestatic agents on tight junctional permeability in isolated

rat hepatocyte couplets have been assessed by monitoring the retention of a fluorescent bile acid analogue (cholyl-lysyl-fluoresein) or the penetration of horseradish peroxidase [417]. Incubation of couplets in the presence of taurolithocholic acid, cyclosporin A, estradiol 17β-glucuronide, menadione, or *t*-butyl hydroperoxide (substances known to affect hepatobiliary function in vivo) resulted in a quantifiable dose-dependent decrease in canalicular retention.

Hepatocyte Cultures

Hepatocyte cell cultures represent an extension of the isolated hepatocyte assay that permits longer test periods by attaching cells to a matrix placed in a periodically refreshed medium to keep them viable. Hepatocytes can be cultured in monolayers, in *sandwich* between two layers of collagen or in speroids, the two latest allowing induces the formation of distinct apical and lateral membrane domains, and enhanced hepatic functions. The comprehensive review prepared by Grisham [175] in 1979 is one indicator of the long-standing popularity of hepatic cell cultures to detect and evaluate the mechanisms of actions of toxic chemicals. Also, Berry et al. [37] reviewed the subject in their excellent monograph on the use of isolated hepatocytes for in vitro studies, while technical aspects of culturing hepatocytes are presented in other monographs [102,179,312]. Factors such as matrix used for cell attachment, culture medium, addition of hormones, oxygen tension, cell density, and the presence of other cell types determine enzyme and gene expression and affect cell viability in monolayer cultures of hepatocytes [312].

Biochemical parameters used to measure liver injury in isolated cells also apply to hepatocyte cultures. CCl_4-induced biochemical changes in cultured hepatocytes followed nearly the same continuum as observed in vivo, although the progression was much more rapid in vitro [297]; leakage of alanine aminotransferase (ALT) was increased and G6Pase activity was decreased in intact liver and cultured cells, whereas 5′-nucleotidase activity was unaffected in either preparation. In hepatocyte cultures incubated with tetracycline or norethindrone, Anuforo et al. [7] found that the leakage of arginosuccinate lyase (ALT), and LDH into the medium was more pronounced than that of alkaline phosphatase (AP) and aspartate aminotransferase (AST). Chao et al. [71] reported that intracellular LDH content is a better indicator of the number of viable hepatocytes in contrast to LDH released into the medium.

Cultures of viable hepatocytes can be maintained for several days. Xenobiotic biotransformation enzyme activities decrease, however, after 1 or 2 days in culture [180,313,444]. For this reason, assessment of cytotoxicity in hepatocyte cultures is routinely performed during the first 2 days of culturing. Hepatocytes cultured in hormone-supplemented, serum-free medium maintain, however, high biotransformation enzyme activities for several days [110,183,313]. Long-term cultures of functional hepatocytes can be achieved by coculturing hepatocytes with another rat liver cell type and represent a promising tool to investigate chronic liver toxicity [183]. Human hepatocytes, particularly when mixed with rat liver epithelial cells, may provide a valuable tool for predicting hepatotoxicity of new drugs in humans [181]. Finally, there is evidence for the reconstruction of functionally intact biliary polarity in hepatocytes in culture, indicating the possible utility of this system for studies on intrahepatic cholestasis [151].

Toxicity studies involving cultured hepatocytes usually assess three types of effects: cytotoxicity, genotoxicity, and enzyme induction [288]. For cytotoxicity, one can follow morphology, dye permeation (trypan blue), as well as release of cytoplasmic enzymes (ALT, AST, LDH). Promutagen activation and induction of unscheduled DNA synthesis can be used to assess genotoxicity. Finally, enzyme induction effects can be discerned by following peroxisomal induction or cytochrome P450 induction. Cultured hepatocytes are considered useful for the study of peroxisome proliferation, since the cells can be used for screening new chemicals, for evaluating the sequence of events involved in their mechanism of action, and for assessing the potential for producing such effects in humans [136].

Methods Used in Hepatotoxicity Studies

Traditional approaches used for evaluation of experimental hepatic injuries in laboratory animals can be divided into six main categories: (1) serum enzyme tests, (2) histological analysis of the liver injury, (3) hepatic excretory tests, (4) alterations in mitochondrial functions, (5) lipid peroxidation and oxidative stress analysis, and (6) formation of binding metabolites. This chapter covers major tests that have proved useful to detect hepatic dysfunction or injury over the years. While additional biochemical, metabolomic, genomic, and proteomic approaches are also employed to define the different mechanisms involved, they will not be covered in this chapter. The reader is referred to reviews and monographies for further information [30,253,487].

Serum Enzyme Techniques

Determination of the activity of hepatic enzymes released into the blood by the damaged liver is still one of the most useful tools in the study of hepatotoxicity. The application of serum enzyme methodology to the detection of liver injury was introduced during the 1930s and 1940s with the demonstration of abnormal serum activities of AP [414] and cholinesterase (ChE) [53]. However, the discovery during the 1950s that the activity of several serum aminotransferases was increased by tissue destruction represents the true advent of the serum enzyme methods. Subsequently, a number of other enzymes were identified in blood, several of which demonstrate abnormal activity in the presence of liver injury.

Zimmerman [528] identified four major categories of serum enzymes based on their specificity for and sensitivity to different types of liver injury. The first group contains enzymes such as AP, 5′-nucleotidase (5′-NT), and γ-glutamyltranspeptidase (γ-GT). Elevated serum activities of these enzymes appear to reflect cholestatic injury more effectively than necrogenic injury. In contrast, the second group of enzymes includes those that are more sensitive to cytotoxic hepatic injury. This group has been further subdivided into

(1) enzymes that are somewhat nonspecific and can reflect injury to extrahepatic tissue, for example, AST, LDH, malic dehydrogenase (MDH), and aldolase (ALD); (2) enzymes found mainly in the liver, for example, ALT, isocitrate dehydrogenase (ICDH), and glutamate dehydrogenase (GDH); and (3) enzymes that are almost exclusively located in the liver, for example, ornithine carbamyl transferase (OCT), sorbitol dehydrogenase (SDH), LDH, guanase, and fructose-1-phosphate ALD. Assay of the more hepatospecific subgroup of enzymes may be particularly useful when studying agents with unknown hepatotoxic potential. Although elevated serum activity of the aminotransferases may reflect injury to extrahepatic organs such as the heart, skeletal muscle, or kidney, elevated activities of OCT and SDH are reliable reflections of hepatic injury. The third and fourth serum enzyme categories contain, respectively, enzymes that are relatively insensitive to hepatic injury but are elevated with extrahepatic diseases, for example, creatine phosphokinase (CPK), and enzymes that demonstrate a depressed serum activity in liver disease, for example, ChE.

Aminotransferases, Ornithine Carbamyl Transferase, and Sorbitol Dehydrogenase

The selection of a battery of enzymes for evaluating the hepatotoxic potential of an unknown chemical in laboratory animals is complicated by the varying sensitivity of the enzymes to different types of lesion. In an early series of experiments, Molander et al. [325] found that the measurement of serum AST provided a more sensitive index of hepatocellular injury in rats treated with CCl_4 than did the measurement of either ChE or AP. A number of experimentally induced necrotic states are also detectable by an elevation in the serum activity of ALT, a liver cytoplasmic enzyme. Balazs et al. [23] assessed serum ALT as a liver test in rats after treatment with ethionine, CCl_4, thioacetamide, dimethylnitrosamine, or allyl alcohol. Serum ALT elevation occurred following the acute administration of all of these agents, but with ethionine the elevation was not pronounced. This finding is understandable in that ethionine does not produce extensive centrilobular necrosis but usually results in fatty infiltration.

Other investigators also found that ALT is an insensitive measure of hepatic steatosis [9,154]. On the other hand, those agents that are associated with severe necrotic lesions produce pronounced elevation of serum ALT. Balazs et al. [22,23] found that when the gross pathological changes or the severity of the histopathology were compared to the elevation in ALT, there was a good correlation between the elevation in serum activity and the severity of the lesion. Others showed [381] that there was an excellent correlation between the severity of quantified histologic damage produced by $CHCl_3$ and the elevation in serum ALT activity in rats. Therefore, it seems with ALT that not only is it possible to detect the presence of liver injury, but under some circumstances, the severity of the lesion can be estimated by the elevation in serum enzyme activity. In chemical interaction studies, in which the severity of the hepatic lesion of interest may be enhanced or diminished because of the presence of a second or third chemical [381], measurement of serum ALT activity is a useful investigative tool.

Aminotransferase activity in different tissues varies and distinct species differences occur [513]. In most instances, AST activity is greater than ALT. High AST activity occurs in skeletal muscle, diaphragm, heart muscle, and liver tissue. ALT is not as widely distributed; in humans, the greatest activity is found in the liver. Cornelius [87] studied the hepatic distribution of ALT in various animals and found that a relation exists between body size and the amount of hepatic ALT: generally speaking, the smaller the animal is within the weight range studied, the greater the hepatic ALT activity [88]. Low activity of ALT was found in horses, cattle, and pigs, and, when these species were subjected to CCl_4 intoxication, significant elevation of serum ALT occurred only in the dog. On the other hand, when serum AST activity was used for measuring hepatotoxicity, all species exhibited an increase in serum enzyme activity after CCl_4. In the rat, both serum AST and ALT activities are markedly elevated after experimental injury; either enzyme could probably be used for detecting injury in this species. Hemolysis, however, has a marked effect on serum AST in the rat, whereas its effect is practically negligible in the case of serum ALT [376]. This fact should be kept in mind when one is using rats for assessing liver function. In addition, since the hepatic specificity of ALT is greater than AST, measurement of serum ALT activity, rather than AST, might be preferable for determining the status of the liver.

OCT is found predominantly in the mitochondrial fraction of liver cells [96,403,404] and normally occurs only in minute amounts in serum. The mucosa of the small intestine contains a small amount, 1%–2% that of the liver, and tissue such as brain and kidney contains only trace amounts [405–407]. OCT serum activity is markedly elevated in both acute and chronic liver disease in humans [331,407]. With experimentally induced hepatotoxicity, Reichard [404] found that serum activity of OCT increased considerably more than those of the aminotransferases but followed a similar temporal phase. Serum OCT activity is a useful monitor of liver injury in rats treated with various hepatotoxicants [96,113,114,265]. Indeed, Drotman [113] described a dose-dependent relation between the amount of CCl_4 administered to rats and serum OCT activity. In addition, a sixfold increase in OCT activity was found at a CCl_4 dose that did not produce distinctive liver damage upon light microscopic examination of the tissue, suggesting that OCT may be as sensitive an index of liver injury as histopathological examination [114]. The correlation between elevation in serum OCT activity and quantified histological changes following $CHCl_3$ administration is good [381].

SDH, a cytoplasmic enzyme, is also relatively specific for liver [10,96], and an increase in the serum activity of this enzyme is a relatively sensitive index of hepatocellular damage. In an elegant series of experiments, Korsrud et al. [264] determined the serum activity of nine hepatic enzymes in CCl_4-poisoned rats in an attempt to identify those enzymes that would respond quantitatively to varying CCl_4 doses and would indicate minimal liver damage. They placed the

enzymes in three groups based on the lowest dose of CCl_4 required to elevate serum activity and concluded that SDH was the most sensitive enzymic index of liver injury. A group of four enzymes (ICDH, fructose-1,6-aldolase [F-1,6-ALD], ALT, and AST) were less sensitive to CCl_4 than SDH but were more responsive to liver injury than were alcohol dehydrogenase (ADH), 6-phosphodigluconase (6-PDG), LDH, and MDH. However, histological alterations were observed at CCl_4 doses that did not elevate serum enzyme activity. Subsequently, Korsrud et al. [265] studied thioacetamide and dimethylnitrosamine. As before, serum SDH was the most sensitive enzymic index of liver necrosis.

SDH, however, was not a preferentially sensitive index of liver injury in diethanolamine-treated rats. Six enzymes (SDH, ICDH, F-1,6-ALD, ALT, AST, and MDH) were equally responsive to diethanolamine hepatotoxicity, whereas a higher dose of this compound was required to elevate the serum activity of OCT, GDH, and LDH. Based on these observations, Korsrud et al. [265] suggested that SDH was the best enzymic index of liver injury when minimal damage or minimal changes are being assessed. However, histological changes characteristic of each hepatotoxicant were noted at doses that did not result in an elevation of serum activity. Thus, the serum enzyme assays were less sensitive than histopathological examination for detecting liver damage. Later work by Travlos et al. [472] compared the relative sensitivities of SDH, ALT, and histopathology as indices of liver injury in rats. When increases in both enzymes occurred simultaneously, terminal histopathological changes were very highly predictable (75%–100%). They concluded that clinical chemistry evaluations could be useful for detecting potential treatment effects throughout a study, although histopathological evaluation can only be performed on termination.

Serum ALT activity is probably the most frequently used enzymic parameter to assess hepatic injury in laboratory animals. Because of the high sensitivity of OCT and SDH, however, it appears reasonable that one of these two enzymes could be used in conjunction with ALT when examining the hepatotoxic potential of an unknown chemical. In this manner, the battery of tests might better reflect the range of sensitivity encompassed by light microscopy. When the effects of several hepatotoxicants on plasma ALT and GDH values were compared in rats, the latter enzyme was reported to be a more effective marker than ALT, based on plasma elevations following injury, persistence following injury, and sensitivity [348]. Also, plasma ICDH/ALT ratios appear useful for differentiating mild-to-moderate degrees of centrilobular hepatic necrosis from periportal necrosis in rats [249].

Analytical determination of aminotransferase activity is mainly achieved by two techniques. For AST, one measures the conversion of aspartic acid and α-ketoglutaric acid to glutamic acid and oxaloacetic acid; for ALT, one measures the conversion of alanine and α-ketoglutaric acid to glutamic acid and pyruvic acid. With the ultraviolet method of analysis, the enzyme processes are coupled with ones in which nicotinamide adenine dinucleotide (NAD) is converted from its reduced form (NADH) to the oxidized form (NAD). The course of the reaction is followed by the decrease in absorbance at 340 nm produced by the oxidation of NADH. The colorimetric procedure involves the reaction of the product (oxaloacetic or pyruvic acid) with dinitrophenylhydrazine to form a colored hydrazone. This product can be determined by its absorbance in the visible range. The principal advantages of the colorimetric method are that an ultraviolet spectrophotometer is not required and temperature control of the enzymic reaction is more easily attained. Many kits are available commercially.

Similarly, as OCT catalyzes the transfer of the carbamyl group from carbamyl phosphate to ornithine, which results in the formation of citrulline, OCT activity may be measured directly by following the appearance of citrulline. Citrulline is determined colorimetrically with diacetyl monoxime after destruction of serum urea with urease [67,68,489]. Alternatively, OCT activity can be monitored indirectly by arsenolysis, in which the enzyme catalyzes the reverse reaction of citrulline to ammonia, carbon dioxide, and ornithine. OCT activity is then determined by the production of $^{14}CO_2$ from (^{14}C-ureido)-L-citrulline [113,265,408] or by the production of ammonia. The formation of ammonia can be analyzed by conversion to indophenol [262] or by a microdiffusion procedure in a Conway cell [403].

Enzymes Useful for Detecting Obstructive Disorders

Most of the preceding discussion concerns the use of serum enzymes to detect necrotic or degenerative processes following the administration of toxicants. In general, these enzymes are not as useful for detecting those types of hepatic alteration that are associated with diminution or cessation of bile flow. The degree of change in serum enzyme activities that one can obtain by the induction of experimental hepatotoxicity in mice is demonstrated in Table 29.1. Three hepatotoxic procedures were employed in this study [376]. One group of animals received α-naphthylisothiocyanate (ANIT), another received CCl_4, and the third group had their bile ducts ligated. Serum enzyme activities (ALT and AP) were determined 24 h later. For comparative purposes, sulfobromophthalein (BSP) retention and serum bilirubin concentrations were also measured in these animals. It is evident (Table 29.1) that a necrotizing agent such as CCl_4 produces sufficient parenchymal injury to cause a large increase in serum ALT activity, whereas those experimental procedures that markedly impair biliary excretion (ANIT treatment, bile duct ligation) causes only a mild increase in ALT activity. The reciprocal relation is obtained when serum AP activity is assessed; obstruction of biliary flow (bile duct ligation) markedly increases serum AP activity, whereas the necrotizing challenge (CCl4) produces only a mild elevation.

Alkaline phosphatase is the prototype of those enzymes (Zimmerman's group 1) that reflect pathological reductions in biliary flow. In the rat, this enzyme is found in the liver and the intestine. Alkaline phosphatase exerts a role in the downregulation of the secretory activities of the intrahepatic biliary epithelium [4]. After bile duct ligation, the activity in the liver increases due to de novo synthesis of the membranous form of the enzyme [433]. The use of this enzyme

TABLE 29.1
Effect of Various Hepatotoxic Procedures on Four Liver Function Tests in Mice[a]

Hepatotoxic Procedure[b]	BSP Retention (mg/dL)	Alkaline Phosphatase (Units)	Bilirubin Concentration (mg/dL)	ALT Activity (Units/mL)
Control (no treatment)	0.3 ± 0.3	3.0 ± 0.5	0.2 ± 0.1	25 ± 5
ANIT (150 mg/kg p.o.)	45.0 ± 23	5.6 ± 2.6	1.1 ± 0.4	282 ± 126
CCl_4 (1 mL/kg p.o.)	13.0 ± 7	5.3 ± 1.3	0.4 ± 0.2	8510 ± 1930
Bile duct ligation	26.0 ± 3	19.0 ± 10	3.8 ± 0.8	655 ± 132

Source: Data obtained from Plaa, G.L., *Evaluation of Liver Function Methodology*, A. Burger, ed., Marcel Dekker, New York, 1968, pp. 255–288.

[a] Values are expressed as mean ± SE; each group contained 10 mice.

[b] Hepatotoxic procedure was performed 24 h before assessing function.

in chemically induced liver dysfunction has been fairly extensively investigated. In the dog, the enzyme is useful for detecting biliary dysfunction. In the cat, however, ligation of the common bile duct results in only a slight increase in serum AP activity. The normal level of serum AP in the rat is exceptionally high, independent of growth, and unusually susceptible to variations in diet [184]. Increases in serum AP activity were not remarkable in a comparative study of clinical chemistry and liver histopathology performed in a subchronic study in rats, whereas increases in serum total bile acid concentration were [472]. Thus, serum AP activity may not be useful for detecting cholestatic changes in the rat.

In addition to AP, other enzymes, for example, 5′N, γ-GT, and leucine aminopeptidase (LAP), may be of use in assessing obstructive liver injury. The serum activity of these enzymes, which are localized in the membranes of hepatocytes and bile duct cells, is increased during extrahepatic cholestasis in humans [28,507]. Kryszewski et al. [271] found a significant elevation in serum activity of AP, 5′N, and γ-GT 12 h after bile duct ligation in the rat; AP and 5′N peaked at 24 h and then gradually decreased, and γ-GT peaked at 48 h and remained elevated even 192 h after bile duct ligation. Fujii [143] found serum AP and γ-GT useful indicators of cholestasis in dogs. Thus, changes in the serum activities of these enzymes are useful for detecting toxicant-induced cholestatic changes in laboratory animals. A simplified electrophoretic method was developed to separate and quantify multiple forms of human serum 5′N [362]; three isoforms were identified in normal subjects and hepatobiliary dysfunction resulted in the increased activity of only one form of serum 5′-nucleotidase. To the best of our knowledge, comparable studies have not been performed in animals.

Of interest is the observation of Moritz and Snodgrass [96] that acute obstruction of the bile duct in the rat produced a rapid rise in the serum activity of SDH and OCT. From 1 to 24 h following bile duct ligation, the activities of these two enzymes increased in serum to levels approximating those found after a single dose of CCl_4, even though the histological degree of hepatic necrosis was substantially less with obstruction than with CCl_4 poisoning. This finding confirmed and extended the observation of Hallberg et al. [188] that bile duct obstruction in dogs resulted in increased serum OCT activity. These observations, if confirmed in other models of experimentally induced obstructive disorders, suggest that OCT and SDH could serve to identify hepatic alterations associated with a diminution or cessation of bile flow.

Lactate Dehydrogenase Isoenzymes

In addition to serum enzyme activities, serum isoenzyme patterns have been utilized for the detection of organ damage in humans and laboratory animals [89,90,174,508]. Isoenzymes are enzymatically active proteins that catalyze the same reactions and occur in the same species but differ in their physicochemical properties. The isoenzymes of LDH are used as diagnostic agents in clinical medicine [90] and in some instances have been evaluated for use in experimentally induced organ damage in laboratory animals. Cornish et al. [90] utilized LDH isoenzymes to detect specific organ damage in rats; they found that the serum isoenzyme patterns resulting from liver or kidney damage differed markedly and concluded that these differences could be utilized to distinguish the damaged organ. Liver damage resulted primarily in an increase in serum LDH-5 isoenzyme activity, whereas the activity of the LDH-1 and LDH-2 isoenzymes was elevated in rats with kidney injury. Grice et al. [174] treated rats with CCl_4, mercuric chloride, thioacetamide, or diethanolamine at doses that would produce either minimal or pronounced tissue damage. Although AST activity was a more sensitive indicator of organ damage than LDH, it did not provide isoenzyme patterns that could identify the specific target organ. In contrast, LDH isoenzyme patterns were capable of identifying the specific target organ; the LDH-5 bands indicative of liver injury were increased in rats poisoned with CCl_4, diethanolamine, and thioacetamide, whereas mercuric chloride, a potent nephrotoxicant, increased the activity of LDH-1 and LDH-2. Morphologic damage generally occurred at dosage levels considerably below those producing detectable serum enzyme alterations. Thus, these authors concluded that serum enzyme activities and isoenzyme patterns are an important supplement to, but not a substitute for, histopathological examination of tissues.

Histological Evaluation

Analysis of the hepatotoxic potential of a chemical agent is incomplete without a histological description of the lesion produced. Quantification of the degree of injury observed

by light microscopy can be achieved using the method of Chalkley [69] essentially as described by Mitchell et al. [320]. One ocular of a microscope is fitted with a micrometer eyepiece containing a grid on which 16 points of reference are chosen. A section of suitably stained liver tissue is selected from each animal and examined at 400× magnification. In a study of acetaminophen hepatotoxicity in mice, Mitchell et al. [320] found that a single section of liver could be considered to be representative of the entire organ. Each section is evaluated by scanning a series of 25 microscopic fields chosen at random. In each field, the tissue element immediately underneath each of the 16 points of reference is termed a *hit*; thus, 16 hits are examined per field. A total of 400 hits are examined in each section. The hits are categorized as (1) normal parenchymal hepatocyte, (2) degenerated parenchymal hepatocyte, (3) necrotic parenchymal hepatocyte, and (4) other cellular structure [201]. Hits on each of the first three categories are recorded and expressed as a percentage of the total number of hepatocytes examined in that section. Accumulation of the data from three to five animals in each treatment group provides a base of sufficient size for statistical analysis. This type of quantitative histological analysis was useful for determining the relative ability of various solvents to potentiate $CHCl_3$-induced hepatotoxicity (Table 29.2). In this study [201], rats were pretreated with an equimolar dose of *n*-hexane (H), acetone (A), 2,5-hexanedione (2,5-HD), or methyl *n*-butyl ketone (MBK); 18 h later the rats received a small challenging dose of $CHCl_3$, calculated to produce minimal signs of liver injury. Hepatic damage was assessed 24 h after $CHCl_3$ administration. Although each of the solvents was capable of potentiating the hepatotoxic effects of $CHCl_3$, it is clear (Table 29.2) that a marked difference exists in the severity of the potentiation produced. Normal hepatocytes accounted for approximately 88%, 75%, 52%, and 45% of the total of $CHCl_3$-challenged rats pretreated with H, A, 2,5-HD, or MBK, respectively. Degenerated hepatocytes accounted for approximately 6% of the total in rats receiving the combination of H and $CHCl_3$ and rose to approximately 18% in rats treated with MBK + $CHCl_3$. The percentage of necrotic hepatocytes was greatest in rats treated with MBK + $CHCl_3$ (37%) and decreased in the following order: 2,5-HD + $CHCl_3$ (35%), A + $CHCl_3$ (15%), and H + $CHCl_3$ (6%). These results indicate that this method for quantifying histological alterations provides an index of toxicity sensitive enough to discern the varying potentiating capacities of the four solvents tested. It is also noteworthy that the use of histological criteria to rank these solvents in order of increasing potentiating ability (H < A < 2,5-HD ≈ MBK) provided results similar to those obtained via determination of serum ALT (Table 29.2) and serum OCT activity [201]. Furthermore, the quantitative histological analysis provided a greater degree of discrimination between the solvents tested than did determination of total plasma bilirubin content (Table 29.2). In general, this procedure for quantifying histological abnormalities correlates well with other indices of liver injury [381]. An example of correlations between histopathological alterations and changes in functional indices of liver injury is given in Table 29.3. The severity of the hepatic lesion, expressed as the percentage of degenerated hepatocytes, the percentage of necrotic hepatocytes, or the percentage of abnormal hepatocytes (necrotic plus degenerated) is compared to alterations in ALT and OCT or to the plasma content of bilirubin. Regardless of the parameters assessed, a linear correlation was observed between the extent of the lesion as quantified by light microscopy and the severity of the biochemical alteration. Marked differences, however, were observed in the strength of correlation between the different combinations of parameters examined. In general, elevations in the serum activity of ALT were most strongly correlated with the histopathological alterations. The correlations between the severity of the lesion and alterations in relative liver weight, however, were not strong.

Minor modifications of this method have been used. Mitchell et al. [320] used an eyepiece containing eight points of reference and examined 50 random microscopic fields to collect 400 hits. It appears that the arrangement and number of reference points examined per field are of little consequence so long as a sufficient number of hits are collected

TABLE 29.2
Histologic Evaluation of the Effects of Pretreatment with Various Agents on $CHCl_3$-Induced Hepatotoxicity in Male Rats[a]

Treatment	Normal Hepatocytes (%)	Degenerated Hepatocytes (%)	Necrotic Hepatocytes (%)	ALT Activity (units/mL)	Total Bilirubin (mg/dL)
$CHCl_3$	99.7 ± 0.1	0.3 ± 0.1	0	37 ± 3	0.18 ± 0.01
n-Hexane + $CHCl_3$	97.9 ± 2.3	5.8 ± 0.9	6.3 ± 2.2	347 ± 100	0.24 ± 0.01
Acetone + $CHCl_3$	76.2 ± 4.7	9.2 ± 2.1	14.6 ± 3.0	1177 ± 534	0.26 ± 0.02
2,5-Hexanedione + $CHCl_3$	51.9 ± 5.0	13.5 ± 2.6	34.7 ± 3.1	2228 ± 477	0.82 ± 0.32
Methyl *n*-butyl ketone + $CHCl_3$	45.2 ± 2.0	17.5 ± 2.0	27.2 ± 1.8	4910 ± 631	1.35 ± 0.17

Source: Data obtained from Hewitt, W.R. et al., *Toxicol. Appl. Pharmacol.*, 53, 230, 1980.

[a] $CHCl_3$ (0.5 mL/kg i.p.) was administered 18 h after oral dose (15 mmol/kg) of vehicle, *n*-hexane, acetone, 2,5-hexanedione, or methyl *n*-butyl ketone. The animals were killed 24 h later. Values represent the mean ± SE determined in 4–15 rats.

TABLE 29.3
Linear Regression Analysis of the Relation between Histologic Evaluation of Severity of Liver Injury and Alterations in Various Parameters of Hepatic Damage

Correlation Coefficient (r)	x-Axis (% Hepatocytes)	y-Axis (Parameter)	Regression Line [$y = m(x) + (b)$]	Points/Line
0.959	Abnormal	Log (ALT activity)	$y = 0.0397(x) + (1.5538)$	49
0.950	Necrotic	Log (ALT activity)	$y = 0.0566(x) + (1.5881)$	49
0.926	Degenerated	Log (ALT activity)	$y = 0.1186(x) + (1.5386)$	49
0.922	Necrotic	ALT activity	$y = 99(x) + (–74)$	49
0.903	Abnormal	ALT activity	$y = 67(x) + (–106)$	49
0.885	Abnormal	Log (OCT activity + 1)	$y = 0.0550(x) + (0.5728)$	47
0.879	Degenerated	Log (OCT activity + 1)	$y = 0.1691\ (x) + (0.5281)$	47
0.865	Necrotic	Log (OCT activity + 1)	$y = 0.0773(x) + (0.6331)$	47
0.830	Necrotic	Bilirubin concentration	$y = 0.0025(x) + (0.15)$	49
0.820	Abnormal	Bilirubin concentration	$y = 0.017(x) + (0.14)$	49
0.816	Degenerated	OCT activity	$y = 116(x) + (–64)$	47
0.813	Degenerated	ALT activity	$y = 188(x) + (–69)$	49
0.799	Abnormal	OCT activity	$y = 37(x) + (19)$	47
0.770	Necrotic	OCT activity	$y = 51\ (x) + (27)$	47
0.755	Degenerated	Bilirubin concentration	$y = 0.049(x) + (0.14)$	49
0.650	Abnormal	Relative liver weight[a]	$y = 0.016(x) + (4.22)$	49
0.645	Necrotic	Relative liver weight	$y = 0.023(x) + (4.23)$	49
0.628	Degenerated	Relative liver weight	$y = 0.049(x) + (4.21)$	49

Source: Data obtained from Plaa, G.L. and Hewitt, W.R., *Quantitative Evaluation of Indices of Hepatotoxicity*, G.L. Plaa and W.R. Hewitt, eds., Raven Press, New York, 1982, pp. 103–120.

[a] Relative liver weight = liver wt./body wt. × 100.

for analysis. In one study [200], 30 points of reference per field and 50 fields were examined for a total of 1500 hits/section. However, H. Miyajima (unpublished observations) found no statistical difference between the results obtained by examining 1500 versus 400 hits per section. Thus, for routine usage, it appears that collection of 400 hits per section is satisfactory.

The quantitative method described above does not allow one to visualize the lobular distribution relative to the zonal configuration of the hepatic lobule. Iijima et al. [216] devised a semiquantitative morphologic method that permits such visualization. To evaluate the morphologic patterns, 10 hexagonal lobules are chosen randomly for each liver section. The distance of the injured area from the hepatocytes adjacent the terminal hepatic venule (THV; central vein) to the portal area is measured in one fixed direction per lobule using a micrometer ocular disc (5 mm divided into 100 parts). This distance is measured at a magnification of 100×. The sections are then examined at a magnification of 400× to classify the cellular changes observed. The damage is classified using six categories: (1) necrosis, (2) ballooning of hepatocytes, (3) swelling of hepatocytes, (4) inflammatory cell infiltration, (5) presence of lipid droplets, and (6) normal hepatocytes. The results are expressed in absolute mean distances (micrometers) from the THV. The mean distance for each category observed in a treatment group is calculated for four to six animals (total of 40–60 hexagonal lobules). The graphic representation of such an analysis is depicted in Figure 29.3, in which the lesions produced by two dosages of CCl_4 (0.1 and 1.0 mL/kg, i.p.) were monitored over a 120 h period [72]. It is evident that the severity of the lesion and recovery were dose dependent. The major advantage of this semiquantitative morphologic procedure is that it permits the investigator to prepare a graphic representation of what is visualized after examination of many microscopic sections. It can be particularly useful for the preparation of toxicological reports.

Electron microscopy is also of value in toxicological studies, as it permits a correlation between the ultrastructural and functional changes induced by foreign chemicals. Grice [173] delineated several of the advantages and disadvantages encountered in the application of electron microscopic techniques to the study of chemically induced liver injury. In general, electron microscopy provides a much earlier demonstration of hepatocyte injury and is of value for detecting minimal and often reversible pathologic changes that may be evident before they are detectable by light microscopy. The ability to detect subtle ultrastructural defects early in the course of poisoning often permits identification of the initial site of the lesion and thus can provide clues to possible biochemical mechanisms involved in the pathogenesis of liver injury. In addition, the power of these techniques can be enhanced through a quantitative, morphometric analysis of chemical effects [47,106]. Serious restrictions involving proper fixation techniques, sampling procedures, and the

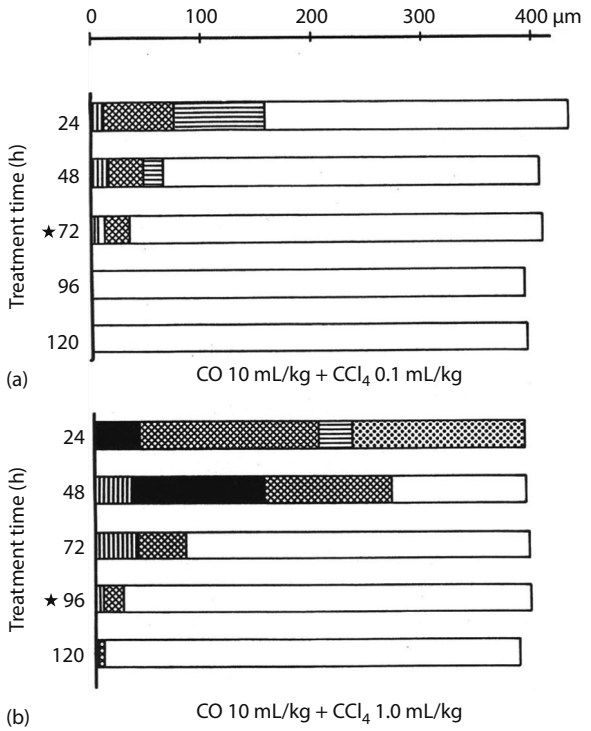

FIGURE 29.3 Lobular morphologic patterns of 0.1 mL/kg (a) or 1.0 mL/kg (b) of CCl_4-induced liver injury for a 120 h posttreatment period, according to the technique of Iijima et al. [216]. The left side (0 μm) of the figure corresponds to the centrilobular region, whereas the right side (400 μm) corresponds to the periportal space of the hepatic lobule. (From Charbonneau, M. et al., *Toxicology*, 35, 95, 1985. With permission.)

complexity of sample preparation, however, argue against the routine use of electron microscopy in the initial evaluation of the hepatotoxic potential of a new chemical. Rather, this technique is probably of greatest use for confirming a suspected alteration or defining a pathologic event [173].

Hepatic Excretory Function

Chemicals entering the systemic circulation may be excreted by the liver unchanged or after modification within the hepatocyte. Compounds that undergo biliary excretion have been divided arbitrarily into three classes [395] based on the bile/plasma concentration ratios obtained during their excretion [251]. Examples of class A substances include sodium, potassium, and chloride ions as well as glucose; these compounds have a bile/plasma ratio of about 1.0. Class B substances, for example, bile salts, bilirubin, BSP, and many xenobiotics, achieve a bile/plasma ratio of more than 1.0, usually between 10 and 1000. Among class C substances, which have a bile/plasma ratio of less than 1.0, are macromolecules such as inulin, phospholipids, mucoproteins, and albumin.

Transport systems in the sinusoidal and canalicular membranes of hepatocytes have been characterized [228,250,367,452]: sodium-taurocholate cotransporting polypeptide (NTCP), organic anion transporting polypeptides (OATPs), organic cation transporters, organic anion transporters (OATs), the ATP-dependent transporters multidrug resistance P-glycoproteins (MRP1, MRP2, MRP3, and MRP6), bile salt export pump (BSEP), and multidrug resistance–associated proteins (MDR1, MDR2, and MDR3). NTCP is associated with the sinusoidal sodium-dependent uptake of conjugated bile salts, while OATP is a multispecific carrier for sodium-independent uptake of bile salts, organic anions, and other amphipathic organic solutes. Several MRP efflux pumps are present in the basolateral membrane. They are said to function as reverse transporters [228] and may reduce intracellular concentrations (monovalent glucuronides and glutathione S-conjugates) when secretion via the canalicular route is impaired. Canalicular transport systems are important for the biliary excretion of bile salts (BSEP), bilirubin glucuronide (MRP2), nonbile salt organic anions (MRP2), glutathione (MRP2), and phospholipids (MDR2 and MDR3). Microsomal enzyme inducers can increase bile flow and biliary excretion of glutathione-derived sulfhydryls by affecting Mrp2 in rats [231]; they also induce the Mrp family in mice [302] via distinct transcription factors. Hereditary defects of intrahepatic transporters have been described (progressive familial intrahepatic cholestasis, Dubin–Johnson syndrome, Wilson's disease) [299,367]. Perturbations associated with extrahepatic or chemical-induced cholestasis [91,92,153,228,452,471] are known, although the mechanisms involved and causality are still unclear. Mrp2 and Bsep can be affected by taurolithocholate-, troglitazone-, or estradiol glucuronide-induced acute decreases in bile flow in rats [91,92,146,153,445], while Mdr2 does not appear to be involved in the bile flow deficiency following manganese–bilirubin combined treatment in mice [409]. Differential expression of hepatic transporter genes has been demonstrated in mice [503] following treatment with the hepatotoxicants acetaminophen and CCl_4.

Sulfobromophthalein Retention

The most common class B chemical used for the detection of liver injury is BSP. In 1925, this anionic phthalein dye was used in clinical medicine by S. Rosenthal and E. White after preliminary tests with other phthalein dyes proved less satisfactory [420]. Since its introduction, this substance has been used extensively for the assessment of liver function in humans and laboratory animals. After intravenous injection, BSP is present in the cardiovascular compartment. Its disappearance from the circulatory system depends on its uptake by the liver. The use of BSP to assess liver function is based on the observation that dye removal from blood is delayed by hepatic dysfunction. Commonly, BSP concentration in plasma is determined at a specific time after a standard dose of dye (per unit of body weight) is administered intravenously. Selection of the optimal dose of BSP is essential for correct interpretation of functional impairment.

The removal of BSP from the plasma is dependent on the simultaneous operation of a number of hepatic processes, for example, active transport across the plasma membrane into a storage compartment, metabolic transformation, and ATP-dependent transport across the canalicular membrane [521].

The most critical step in this process is thought to be the transfer of BSP from liver to bile. Most important in terms of selecting an optimal BSP dose is that its biliary excretion can be saturated and a transport maximum (Tm) exists; the clearance of BSP by laboratory animals is dose dependent [247]. Usually one observes that small doses are rapidly removed from the circulation; this rate of removal continues as one increases the dose, until a dosage level of BSP is reached where the rate of disappearance becomes longer. For example, with the isolated perfused rat liver [382], 5, 10, and 20 mg of BSP are cleared at the same exponential rate; the capacity of the liver to extract BSP from the perfusate, however, becomes saturated when 30 or 40 mg is injected. With the latter dose, the rate of disappearance becomes zero order, which indicates that the maximal capacity of the transport system is reached. This same type of phenomenon occurs in mice in vivo [118].

A marked species difference exists in the ability of the rat, rabbit, and dog to remove BSP from the plasma; this difference can be readily discerned by administering varying BSP doses to these laboratory animals (Figure 29.4). Both the rat and the rabbit have a remarkable ability to clear BSP from the plasma, whereas the dog has a relatively poor capacity [247]. If the overall BSP Tm for biliary excretion is measured, large differences are observed [247]. The significance of these findings is that the optimal dose of BSP for measuring liver function depends on the species employed. The dose should be one that is relatively close to the one that indicates BSP clearance capacity is being exceeded.

Determination of the BSP Tm has been used as an index of hepatic function in humans. Wheeler et al. [506] devised a procedure that can be employed in conscious dogs. With this technique, one infuses BSP at three different rates and measures the serum concentration at varying times. From the data, one can calculate the Tm and the relative storage capacity (S) for BSP. In addition, methods have been devised for making similar measurements in rabbit and rat [246]. The use of these techniques has not been widespread in laboratory animals. They are useful for assessing excretory capacity, however, and are employed in mechanistic studies to determine specific functional lesions involved in the reduction of BSP clearance. For example, defects in (1) transfer of BSP from plasma to liver, (2) storage of BSP within the hepatocyte, (3) conjugation of BSP with glutathione, or (4) transfer of BSP from the liver cell into the bile could participate in the CCl_4-induced depression of BSP clearance.

When these possibilities were evaluated, it appeared that the major effect of CCl_4 was to decrease the transfer of BSP from the hepatocyte to the bile [248,394]. Klaassen and Plaa [248] found that 24 h after a single intraperitoneal dose of CCl_4 both the BSP Tm and hepatic BSP conjugating activity were depressed; no change in hepatic BSP storage was detected. Because CCl_4 reduced plasma disappearance and the Tm of phenol-3,6-dibromophthalein disulfonate (DBSP), a nonmetabolized analog of BSP, and depressed excretion of both BSP and DBSP under submaximal conditions, it was concluded that the excretory parameter was probably the prime event altered by CCl_4. Subsequently, Priestly and Plaa [394] demonstrated that impaired BSP excretion, bile flow rate, relative hepatic storage, and BSP retention were observed as early as 3 h after CCl_4 administration. Impaired BSP conjugation, however, was not unequivocally demonstrated until 12 h after CCl_4. Thus, although impairment of both conjugation and excretion contributes to BSP retention, the effect on excretion appears to be more important.

Although BSP was introduced in 1925, it was not until 1950 that it was realized that this dye is excreted in a conjugated form into the bile [51,52]. Up to that time, it was assumed that this material did not undergo biotransformation prior to its excretion. BSP is conjugated with glutathione in humans, rats, and dogs. A number of other conjugates, including BSP-cysteinylglycine and BSP-cysteine, are also formed, presumably by cleavage of glutamic acid and glycine from the glutathione moiety. BSP conjugation, catalyzed by a glutathione S-transferase, is a cytoplasmic process. Under certain conditions, the impairment of

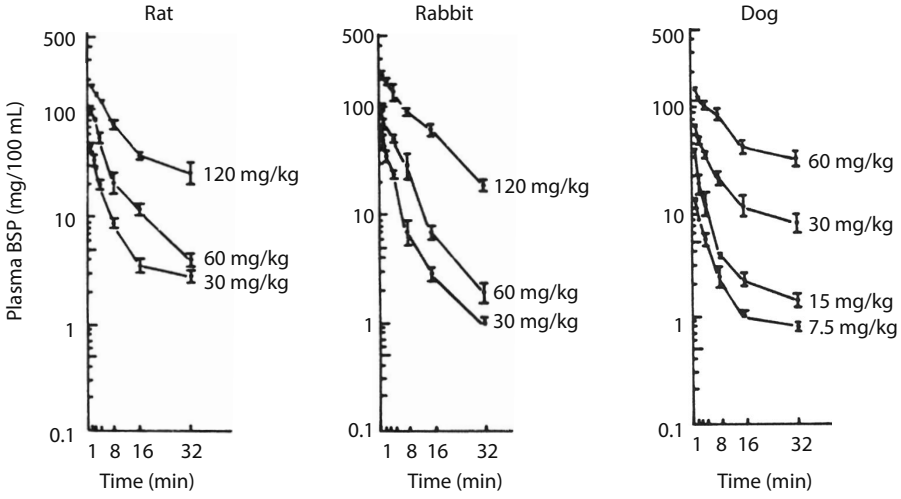

FIGURE 29.4 Plasma disappearance curves for BSP administered in varying doses in the rat, rabbit, and dog. (From Klaassen, C.D. and Plaa, G.L., *Am. J. Physiol.*, 213, 1322, 1967. With permission.)

BSP conjugation with hepatic glutathione can lead to depression of BSP excretion in the bile without impairment of general excretory function [50,84,392,393].

Although BSP is a useful and sensitive test of liver function, a variety of events can cause BSP retention. Diffuse and severe hepatocellular damages are associated with an increase in dye retention [189]. Liver injury of the cholestatic type, however, usually decreases biliary excretion to a greater extent than does parenchymal cell injury [515]. For example, using ANIT, Becker and Plaa [29] showed that the amount of BSP retained following such treatment is much greater than that observed after the necrotic effects of CCl_4. In this instance, ANIT affects BSP retention by decreasing the biliary excretion of BSP [380]. In rabbits, treatment with anabolic steroids can result in BSP retention owing to a decrease in excretory capacity [285]. In contrast, bunamiodyl [sodium 3-(3-butyramide-2,4,6-triiodophenyl)-2-ethylacrylate] seems to diminish uptake of BSP by the hepatocyte [39]. Finally, decreased hepatic blood flow can also cause BSP retention [118].

Indocyanine Green Retention

Several other compounds have been introduced into clinical medicine for the purpose of measuring liver function by the dye-clearance principle. The rose bengal test appeared in 1931 [108], and a third useful dye, indocyanine green (ICG), was introduced in 1959. ICG was originally used to measure cardiac output by the indicator-dilution technique. It was subsequently found in the dog, however, that 97% of the administered dose was eventually recovered from the bile in an unaltered form [505]. No dye was found in the urine. ICG has about the same spectrum of sensitivity and specificity as BSP, but it has a number of properties that make it more desirable to employ under certain circumstances. Cherrick et al. [76] found the following: (1) ICG is rapidly and completely bound to plasma protein, of which albumin is the principal carrier; (2) the dye is excreted in bile in an unconjugated form; (3) there seems to be no extrahepatic mechanism for removing the material; (4) ICG is nonirritating when inadvertently introduced subcutaneously, and it produces no untoward reactions upon single or repeated intravenous injections; and (5) the plasma disappearance of ICG is similar to that of BSP.

In laboratory animals, ICG is usually employed to supplement BSP tests. In the dog, Hunton et al. [214] found that (1) the plasma disappearance rate of ICG is exponential for at least 15 min and usually for 30–60 min; (2) the amount removed per minute seems to be inversely related to the dose administered; (3) the maximal rate of excretion of ICG into the bile is about 0.4 mg/min/kg; and (4) substances such as bilirubin, rose bengal, and BSP interfere with ICG excretion. Klaassen and Plaa [250] found that over a 32 min period, the rate of disappearance of ICG was exponential in the rat, rabbit, and dog (Table 29.4). The rabbit exhibited a greater capacity to remove ICG from plasma than did the rat, and the dog had the lowest capacity. It appears that the optimal dosage for ICG clearance in the dog is about 1.5–2.0 mg/kg; in the rat, approximately 16 mg/kg; and in the rabbit, 25–30 mg/kg.

TABLE 29.4
ICG Plasma Disappearance Rates in Rats, Rabbits, and Dogs

ICG Dose (mg/kg)	$T_{1/2}$ (min)	K (% Removed/min)
Rat		
4	2.5	28
8	4.0	17
16	6.5	11
32	8.5	8
64	18.0	4
Rabbit		
8	1.5	46
16	3.5	20
32	7.0	10
Dog		
1	7.0	10
2	17.0	4
4	30.0	2

Biliary excretory maximum and hepatic storage values for ICG could not be determined [250], as infusion rates sufficient to produce a biliary excretory Tm produced a marked decrease in bile flow. Decreased bile flow was observed in all three species but was most pronounced in the rat and least in the dog. Rapid administration of ICG, as used in plasma clearance experiments, does not produce marked alterations in bile flow. Other investigators [193] reported maximal biliary excretion rates for rats given ICG that were comparable to the peak excretion rates obtained by Klaassen and Plaa [250]. In Mdr2-deficient mice, the biliary excretion of ICG was reduced by 90%, while the excretion of total glutathione was decreased by 65%, relative to wild mice [213].

The major advantage of ICG in the detection and evaluation of hepatic function is that the material is not biotransformed prior to excretion. In addition, ICG is directly determined in plasma, without chemical treatment. In practice, it simply involves diluting an aliquot of plasma (0.1–1.0 mL) with water and determining the absorbance at 805 nm, the wavelength for peak ICG absorption. ICG, however, is unstable in aqueous solutions, which can be prevented by mixing ICG directly with serum or with an albumin solution. The dye is unstable when mixed with heparin solutions containing bisulfite [82], indicating that preservatives of the same type may also have an effect on ICG.

Other Anionic Chemicals

Although rose bengal was introduced before ICG, it has not been extensively employed in laboratory animals; in humans, the dye is used to diagnose hepatic disorder, especially in children. Rose bengal, like ICG, has the advantage that it is apparently not biotransformed before excretion into the bile [245,272]. It is available commercially in a radioactive form, and, therefore, its concentration can be quantified in small blood samples. Its uptake in isolated hepatocytes is similar

to that of BSP [521]. Klaassen [245] examined the pharmacokinetics of rose bengal in four species (rat, rabbit, dog, guinea pig) and found that, with the exception of biotransformation, the dye appears to be handled by the liver in a manner similar to BSP; it is a class B anion that is actively excreted into the bile. A marked species variation in the rate of biliary excretion of rose bengal exists [245]. The rat and rabbit excrete rose bengal into the bile at comparable rates, whereas the guinea pig is much more efficient and the dog much less efficient. Like BSP, the removal of rose bengal from the blood is altered by changes in hepatic excretory function [245,317,462], and Klaassen [245] indicated that the dye could be used as a measure of hepatic excretory function in laboratory animals. Because it is not biotransformed, alterations in its clearance would reflect primarily changes in its uptake into the liver or its excretion into the bile.

Other agents exist for monitoring hepatic excretory function. Mehendale and coworkers [314,316] used phenolphthalein glucuronide (PG), imipramine (IMP), and the polar metabolites of imipramine (PMIMP) as model compounds to characterize the hepatobiliary dysfunction produced by mirex. This pesticide did not suppress hepatic uptake and metabolism of IMP but inhibited the movement of PMIMP from the hepatocyte to bile; it also inhibited the biliary excretion of PG, a model anionic substrate that does not undergo biotransformation. These model substrates allowed the investigators to localize the site of mirex-induced dysfunction. In another report, they suggested that PG may be a more sensitive model compound than BSP for detection of hepatobiliary dysfunction. CCl_4 at 100/μL/kg depressed biliary excretion of PG in rats [95]; hepatobiliary dysfunction was undetectable with BSP at this dosage of CCl_4 [52,248]. The plasma disappearance and biliary excretion kinetics of PG in the rat have been described [314]. The biliary excretion of phenolphthalein sulfate is reported to be markedly delayed in Mrp2-deficient rats, suggesting that this substance is handled by Mrp2 [461].

The effects of some known potential cholestatic agents were investigated in vitro using taurocholate in regular and collagen-sandwich-cultured human hepatocytes [267]. Cyclosporin A, bosentan, glyburide, erythromycin estolate, and troleandomycin all inhibited bile acid efflux. Glyburide administered to rats resulted in elevated serum total bile acids in rats. These preliminary findings are quite encouraging and warrant more extensive studies.

Endogenous Cholephiles

At least one endogenous substance, bilirubin, has been used to evaluate chemically induced hepatic injury. Normally, bilirubin is excreted into the bile. Elevation of serum bilirubin concentration accompanies sufficiently severe parenchymal injury, but it is a relatively insensitive measure of chemically induced hepatic injury. The degree of change in serum bilirubin that one obtains with experimental hepatotoxicity in mice is summarized in Table 29.1. CCl_4, although producing sufficient parenchymal injury to cause a large increase in serum ALT activity, does not affect bilirubin concentrations greatly. On the other hand, bile duct occlusion does elevate serum bilirubin considerably. ANIT also elevates serum bilirubin but not to the extent that biliary occlusion does. The BSP retention values indicate that those experimental procedures that markedly impair biliary excretion also affect BSP retention, whereas CCl_4 causes a lesser degree of retention. However, if one assumes that BSP retention and bilirubin concentrations measure relatively the same type of liver function, it is evident that the changes occurring with BSP are considerably greater than those occurring with bilirubin. A likely explanation is that the measurement of endogenous concentrations of bilirubin may not assess the total capacity of the liver to clear bilirubin, as does a load of BSP selected to be a near-capacity dose for the particular species of animal employed. Indeed, if one does administer exogenous amounts of bilirubin and follows its plasma disappearance, as with BSP, the sensitivity of the bilirubin clearance procedure can be increased. Nevertheless, BSP clearance is simpler and more sensitive than bilirubin and is therefore preferred for the measurement of hepatocellular injury.

Bile acids, a second group of endogenous chemicals that are normally excreted into bile, have been used to assess some hepatotoxicants. Unlike serum bilirubin retention, elevation of serum bile acid concentrations, presumably because of decreased biliary secretion, appears to be a highly responsive index of hepatobiliary dysfunction [18,137]. At least following $CHCl_3$ and CCl_4 treatments in rats, elevation of serum bile acids occurred at dosages that exerted no effect on serum enzyme activity or bilirubin concentration [18]. Furthermore, elevations of individual serum bile acids occurred at dosages of CCl_4 that produced no consistent histological change. Neghab and Stacey [340] recently demonstrated that toluene, a nonhalogenated aromatic hydrocarbon, also results in increases in serum bile acids in rats in a dose-dependent manner; the elevations occur in the absence of other abnormal liver enzyme findings. Xylene also results in toluene-like interference of hepatocellular uptake of bile acids in isolated hepatocytes [340]. While these findings are consistent with toluene and xylene actions on hepatocellular bile acid transport, they are not necessarily indicative of liver injury. The specificity of serum bile acid elevations and the role of such events in the evaluation of hepatotoxicity are yet to be determined.

Biliary Secretory Function

Techniques designed to assess bile secretory function are also available; these methods, however, lend themselves more to specific research problems than to overall toxicological assessment. Fujimoto [144] reviewed older methods that can be applied in vivo. The so-called bile acid-dependent and bile acid-independent fractions of total bile flow [24,48,251] have been studied extensively in several species; secretin-sensitive bile flow is thought to be small in the rat but more important in rabbit and dog.

Fujimoto [144] developed a number of new techniques, in which marker substances are injected retrogradely into the biliary tree to assess the permeability characteristics of the biliary system. ANIT, which produces intrahepatic cholestasis in rats, and bile duct ligation increase the distended

capacity of the biliary tree, whereas another cholestatic agent, taurolithocholate, decreases the distended capacity; CCl_4 seems to exert no effect [142,356,371].

The retrograde technique has been modified to become the *segmented retrograde intrabiliary injection* (SRII) procedure [355]. With the use of radioactive marker substances of varying molecular weights (D-glucose, mannitol, sucrose, inulin, or dextran), one can assess the *membrane* characteristics of the biliary tree (canalicular and tight-junction complexes). This procedure was used to study the hepatobiliary dysfunction produced by Amanita phalloides [142], taurolithocholate [144], colchicine [21], S,S,S-tributylphosphorotrithioate [21], manganese and manganese–bilirubin combinations [15], and sequential treatments with ketones and chloroform [199]. These studies have been useful for discerning the site and possible mechanisms of action involved in their hepatobiliary effects.

Determination of Biliary Function

Sulfobromophthalein Clearance The BSP test dose is first injected intravenously. After 30 min, a suitable amount of blood is withdrawn and plasma is prepared by centrifugation. Aliquots of plasma are placed into tubes containing alkalinized or acidified saline. The BSP plasma content is determined by the difference between the absorbance in alkalinized and acidified saline. The BSP dose administered depends upon the species of animal being employed. It should be one that normal animals can readily clear in a 30 min collection period. Thus, the amount of BSP retained at 30 min should be about 1% of the dose administered. For the dog, rat, rabbit, and mouse, these dosages are about 15, 75, 50, and 100 mg/kg, respectively [247,376].

Biliary Sulfobromophthalein Transport Maximum The procedure can be performed in anesthetized rats or rabbits [246]. With the rat, the bile duct is cannulated as well as the femoral vein; the rectal temperature is maintained at 37°C to prevent hypothermic alterations in Tm [413]. The BSP solution is infused (2.5 mg BSP/kg/min) at a rate of 0.03 mL/min for 60 min, and bile is collected at 15 min intervals. The amount of BSP excreted (min^{-1} kg^{-1}) is calculated; the maximum value attained is the Tm. In the Sprague-Dawley rat, the BSP Tm is about 1.0 mg/kg/min.

SRII Method The procedure can be performed in anesthetized rats [144], and the rectal temperature should be maintained at 37°C to prevent hypothermic alterations in bile flow [413]. The bile duct is cannulated with PE-20 tubing just distal to the bifurcation of the biliary tree; this tubing is attached to a longer length of PE-20 tubing capable of containing a calibrated (40 mL) amount of solution. An exact amount of radioactive marker substance (D-glucose, sucrose, mannitol, inulin, or dextran) is infused into the bile duct and washed through with saline. Bile flow is then reestablished and bile drops collected serially. The bile flow rate is calculated by determining the time required to form each drop; the content of each drop is determined by liquid scintillation spectrometry, and the recovery expressed as a percentage of total marker recovered. The volume of the distended biliary tree can also be evaluated by the SRII method [144,371].

Mitochondrial Function

Mitochondrial function was one of the first biochemical processes investigated in chemical-induced hepatotoxicity, and many studies were performed to establish its role in various experimental models [528]. With carbon tetrachloride, unfortunately, it fell in disrepute as a possible initiating event, because the temporal appearance of the biochemical lesions in this organelle seemed to follow, rather than precede, histological changes [379]. More recent studies with acetaminophen [237] and 1,1-dichloroethylene (DCE) [307] have demonstrated the importance of mitochondrial dysfunction in liver injury. With DCE, dose-dependent and time-dependent mitochondrial parameters (oxygen consumption, state 3/state 4 oxygen consumption ratio, ADP phosphorylation per oxygen consumed) in isolated liver fractions obtained from treated mice showed that state 3 (ADP-stimulated) respiration rates for glutamate (complex I)- and succinate (complex II)-supported respiration were decreased 20 and 90 min, respectively, after treatment, whereas state 4 (resting) respiration was unaffected. Serum ALT was significantly elevated at 2 h, and centrilobular necrosis was observed 24 h after treatment. Thus, with DCE, mitochondrial dysfunction was one of the early events observed in the progression of the lesion.

Mitochondria play a vital cellular role in fat oxidation and energy production [370]; the β-oxidation and tricarboxylic acid cycles are key elements; cytochrome *c* oxidase and ATP synthase are critical enzymes. Microvesicular steatosis results from acute impairment of fatty acid oxidation. This lesion has been observed in humans and in laboratory animals following exposure to valproic acid, pirprofen, tetracyclines, and amiodarone [237,528]. Several dideoxynucleosides and the experimental agent, fialuridine, have resulted in inhibition of mtDNA replication in humans with mitochondrial cytopathy [370]. The treatment of rats with the cholestatic agent ANIT for 16 weeks resulted in impaired mitochondrial bioenergetics [361].

Mitochondria are the main source of reactive oxygen species (ROS), which, in turn, can lead to oxidative damage of mtDNA and cell death. ROS are important cytotoxic and signaling mediators in inflammatory liver diseases [225]. Ethanol exposure can promote oxidative stress by increasing ROS formation and decreasing cellular defense mechanisms [206]. Oxidative stress also appears involved in acetaminophen hepatotoxicity [226]. The mitochondrial permeability transition (MPT) pore, located in the inner membrane, allows cell survival when closed, but leads to cell death when open [370]. If all mitochondria have open pores, ATP synthesis is decreased; ATP depletion and necrosis occur. If only some pores are open, unaffected organelles still synthesize ATP, whereas the open ones cease to form ATP, release cytochrome *c*, thus activating caspases, which leads to apoptosis. Overall, the importance of mitochondria in the development of hepatic dysfunction seems well established. Pessayre et al. have even

suggested [370] that the possible action of new pharmaceutical agents on mitochondria should be evaluated before marketing.

Lipid Peroxidation and Oxidative Stress

It is generally accepted that the toxicity of CCl_4 depends on the cleavage of a carbon–chlorine bond to generate a trichlormethyl free radical ($\bullet CCl_3$); this free radical reacts rapidly with oxygen to form a trichlormethyl peroxy radical ($\bullet CCl_3O_2$), which may contribute to the toxicity [499]. The work of a number of investigators [85,379,401,499] demonstrates that (1) the cleavage occurs in the endoplasmic reticulum and is mediated by the cytochrome P450 mixed function oxidase (MFO) system, (2) the product of the cleavage can bind irreversibly to hepatic proteins and lipids, and (3) the CCl_4-derived free radical(s) can initiate a process of autocatalytic lipid peroxidation by attacking the methylene bridges of unsaturated fatty acid side chains of microsomal lipids.

The peroxidative process initiated by the $\bullet CCl_3$ radical is thought to result in early morphologic alteration of the endoplasmic reticulum, loss of activity of the cytochrome P450 xenobiotic metabolizing system, loss of G6P activity, loss of protein synthesis, loss of the capacity of the liver to form and excrete very low density fraction of the lipoproteins (VLDL), and eventually, through as yet unidentified pathways, to cell death [85,379,401,499]. Alterations in these parameters have been used to monitor the course and extent of CCl_4-induced hepatic damage, and furthermore, have been applied to the evaluation of other hepatotoxicants.

Normal cellular metabolism can result in the production of reactive oxygen species (superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radical) and all cells contain defense systems to prevent or limit damage. Glutathione is the major component of this system, but μ-tocopherol and ascorbic acid play important roles [290]. The imbalance between prooxidants and antioxidants is known as oxidative stress [402].

Three groups of agents have been described to characterize the toxicants associated with the induction of lipid peroxidation or oxidative stress in liver cells [85,402]. One group consists of agents biotransformed to reactive free radicals that promote membrane lipid peroxidation directly; CCl_4 and $CBrCl_3$ are examples of this group. A second group consists of chemicals that are biotransformed to electrophilic intermediates, which then conjugate with glutathione and result in glutathione depletion; bromobenzene and acetaminophen are examples. The third group consists of substances that are converted to nonalkylating intermediates and generate reactive oxygen species by redox cycling; diquat and menadione serve as examples.

It is now established that nonparenchymal cells can be involved in oxidative stress responses leading to hepatotoxicity [279]. Reactive oxygen intermediates are generated by macrophages, endothelial cells, and stellate cells (Ito cells), but under physiologic conditions, cellular antioxidants normally present prevent the intermediates from producing cytotoxicity. Enhanced formation of oxygen intermediates was demonstrated with CCl_4, galactosamine, and 1,2-dichlorobenzene. With the latter agent, recent evidence indicates that Kupffer–cell–derived oxygen species are largely responsible for the lipid peroxidation [207], while with ANIT, neutrophils appear to be involved [261].

Several procedures for the detection and quantification of lipid peroxidation in tissue samples or whole animals have been developed [384,401]. The reaction of malonaldehyde (MDH), a degradation product of peroxidized lipids, with thiobarbituric acid (TBA) to produce a TBA–MDH chromophore has been taken as an index of lipid peroxidation and is the most widely used method for detecting lipid peroxidation in vitro. Because MDH is rapidly metabolized in whole animals [384] and in whole liver homogenates [400], the failure to detect TBA-reacting material is not an indication of the absence of lipid peroxidation.

The determination of conjugated dienes in lipid extracts of hepatic subcellular fractions is a second approach for detecting lipid peroxidation [401]. The ultraviolet difference spectra of peroxidized lipids show an absorption maximum at 233 nm with a secondary absorption maximum between 260 and 280 nm due to the presence of ketone dienes. The appearance of conjugated dienes after treatment in vivo with toxicants is an unmistakable indication that lipid peroxidation has taken place.

Several other methods for the measurement of lipid peroxidation have been described. For example, the iodometric procedure of Bunyan et al. [57] has been employed. A variety of molecules that occur commonly in tissue may react with MDH and yield characteristic fluorescent chromophores [79]. MDH undergoes decomposition, and the decomposition products may also lead to fluorescent products when they react with proteins [438]. Measurement of these fluorescent products seems to offer a workable way for detecting lipid peroxidation in biological systems and tissues [401]. A second approach is the measurement of hydrocarbon gases. These gases appear early in the course of autoxidation of edible fats [138,209]. Two gases, ethane and pentane, are useful for measuring the peroxidative process in vitro and in vivo. Ethane is the predominant gas produced during autoxidation of linolenic acid [289], and pentane is the major gaseous hydrocarbon arising during thermal decomposition [124,125] and iron-catalyzed decomposition of linoleic and arachidonic acid hydroperoxides [116]. Riely et al. [411] initiated the use of ethane analysis in biological systems. They observed that ethane production was a characteristic of spontaneously peroxidizing mouse tissue (liver and brain) and was found in mice injected with CCl_4. In addition, they found that CCl_4-induced ethane evolution in vivo was potentiated by prior administration of phenobarbital and diminished by α-tocopherol, an antioxidant. Several other groups of investigators used ethane production to monitor the course of lipid peroxidation in vivo [58,187,266,292]. Dillard et al. [111] suggested that pentane expiration was a more sensitive index of lipid peroxidation than ethane in rats fed a vitamin E–deficient diet containing a high content of linoleic acid. Pentane production has also achieved considerable use as an index of the lipoperoxidative process [293,423,424]. A method has been devised for monitoring lipid peroxidation in humans by quantifying the excretion

of ethane and pentane in exhaled breath during a 2 h period [495]. Although the measurement of hydrocarbon gas production is an alternate procedure for the determination of lipid peroxidation, this technique cannot identify the tissue or subcellular organelle from which these substances arise. In addition, precautions must be taken to prevent or estimate the evolution of hydrocarbon gases by microorganisms in the gastrointestinal tract when in vivo studies are undertaken.

Prostaglandin F2–like compounds (F2-isoprostanes) are produced by peroxidation of arachidonic acid in phospholipids and released into the circulation [85,436]. The presence of lipid peroxidation in rats exposed to halothane has been demonstrated by the quantification of F2-isoprostane in plasma and liver [13]. The measurement of F2-isoprostanes in vivo is said to represent a promising method for detection of lipid peroxidation because of its reliability and sensitivity [14,85], but its utility in various forms of chemical-induced lipid peroxidation remains to be investigated.

Formation and Binding of Reactive Metabolites

Although the concept of lipid peroxidation is one of the truly important concepts of experimental pathology and toxicology, it does not appear to serve as a universal mechanism of liver injury. A number of drugs and chemicals, for example, acetaminophen [156,321], furosemide [173,321], 1,1-dichloroethylene [224,310,311], trichloroethylene [3], bromobenzene [157,410], and dimethylnitrosamine [157], produce hepatic damage but do not appear to promote lipid peroxidation. Rather, these agents are converted to highly reactive, electrophilic metabolites by the hepatic MFO system. Following formation, the metabolites, which are considered to be the ultimate toxicants, can interact with hepatic constituents (e.g., protein, lipid, RNA, DNA) to form alkylated or arylated derivatives. Even with CCl_4, the effects on lipids and lipoprotein metabolism (leading to steatosis) appear likely due to covalent binding [499]. Various investigators postulate that the binding of reactive metabolites to hepatic macromolecules can initiate cellular damage through processes as yet unidentified. This can result in intrinsic or idiosyncratic liver injury.

A detailed discussion of the formation and detoxification of reactive metabolites and their interaction with hepatic constituents is beyond the scope of this chapter, and the reader is referred to several excellent reviews [157,158,319,321,443]. However, the experimental work carried out to unravel the mechanisms by which several toxicants produce liver injury has led to some important observations that need to be discussed. One is that hepatotoxicity need not be correlated with the pharmacokinetics of the parent substance or even its major metabolites, but may be correlated with the formation of quantitatively minor, highly reactive intermediates. Assuming that a relation exists between the severity of the lesion and the amount of covalently bound metabolite, the covalent binding of the metabolite can be used as an index of its formation. Indeed, this parameter might well be the most reliable estimate of the availability of the metabolite for production of damage at the target site, as most of the metabolite may undergo decomposition or further metabolism before it can be isolated in body fluids or urine [319]. Thus, one widely used maneuver to assess the contribution of formation and binding of metabolites in chemically induced hepatotoxicity is to determine if radiolabeled chemicals administered to animals over a wide dosage range are covalently bound to macromolecular constituents in tissues that subsequently become necrotic [319].

A second concept is that a threshold tissue concentration of the metabolite must be attained before liver injury is elicited; if it is not attained, injury does not occur. Endogenous substances, such as glutathione, play an essential role in protecting hepatocytes from injury by chemically reactive metabolites. The mechanism that establishes a dose threshold, however, may vary from compound to compound. Finally, other enzymic pathways, for example, glutathione-*S*-transferase and epoxide hydrolase, also play a role in protecting the hepatocyte by catalyzing the further degradation of the toxic reactive intermediates.

The studies mentioned earlier have provided relatively straightforward biochemical strategies for uncovering the possible existence of potentially toxic chemically reactive metabolites in new compounds [319]. In general, a dose–response study employing the radiolabeled compound over a wide dosage range is perhaps the single most important facet of the overall study in that it can provide information relating to a dose threshold for toxicity, possible mechanisms for the threshold response (e.g., glutathione depletion), and the degree of covalent binding of metabolites to target organs or constituents with the target organ. This latter information, in conjunction with dose–response studies documenting the dosages required to produce necrosis (or other endpoint), provides strong presumptive evidence favoring toxicity mediated by a reactive metabolite. Subsequent efforts should include the use of inducers (e.g., phenobarbital, 3-methylcholanthrene) and inhibitors (e.g., SKF-525A, $CoCl_2$, piperonyl butoxide) of the MFO system. Enhancement of in vivo or in vitro covalent binding of the radiolabeled compound, as well as toxicity by an inducing agent, can provide support for the contention that a reactive metabolite mediates toxicity. A similar conclusion can be drawn if an inhibitor of the MFO system depresses covalent binding and toxicity. However, the observation that inhibitors increase the response or that inducers decrease the response does not preclude the possibility that the toxicant exerts its effect through the formation of chemically reactive metabolite [319]. For example, an inducing agent may stimulate the activity of a detoxifying pathway to a greater extent that a toxifying pathway. In addition, manipulation of the concentration of hepatoprotective substances, such as glutathione (e.g., depression of hepatic GSH concentration by diethylmaleate administration), can alter covalent binding or toxicity of a compound and support the likelihood that the toxic effects are mediated by a metabolite. Correlation of the data from several of these studies with a pharmacokinetic analysis can delineate the participation of a chemically reactive metabolite in the production of toxicity. For a more detailed discussion of these concepts, the reader is referred to the reviews cited earlier.

LIVER STEATOSIS

In addition to producing elevations in serum enzyme activities and altering hepatocyte transport processes, chemical hepatotoxicants can produce changes in structural and functional hepatic constituents that have been found useful for detecting and quantifying the degree of liver injury produced, as well as elucidating the mechanism(s) involved in producing the lesions. A number of agents that produce liver injury also cause the accumulation of abnormal amounts of fat, predominantly triglycerides, in the parenchymal cells. In general, triglyceride accumulation can be thought of as resulting from an imbalance between the rate of synthesis and the rate of release of triglyceride by the parenchymal cells into the system circulation. Nonesterified fatty acids (NEFAs) removed from the circulation or synthesized endogenously are processed through two major pathways in the liver: (1) mitochondrial β-oxidation for production of metabolic energy; and (2) incorporation into complex lipids, especially triglycerides, phospholipids, cholesteryl esters, and glycolipids. Once synthesized, the complex lipids may be used for production of cellular membranes (structural lipids) or be continuously secreted from the liver into the blood. The latter pathway appears to be of greatest interest in the triglyceride accumulation observed in steatosis.

Blockage of the secretion of hepatic triglyceride into the plasma is the basic mechanism underlying the fatty liver induced in the rat by CCl_4, ethionine, phosphorus, puromycin, or tetracycline, by feeding a choline-deficient diet, or by feeding orotic acid [210,296]. When hepatic triglyceride is released into the plasma, it is not released as such but as a lipoprotein. The VLDL is the major transport vehicle for endogenously synthesized triglyceride; there is some evidence indicating that CCl_4 and ethionine cause a fall in the level of circulating lipoprotein, principally VLDL. The composition of VLDL by weight is 8%–10% protein and 90%–92% lipid. Of the lipids, triglyceride is the most abundant component (56%); the average content of phospholipid is 19%–21% and cholesterol 17% [223].

Elevated triglyceride could result because of an increase in the rate of synthesis of this substance. There is evidence that the rate of synthesis is directly proportional to the concentration of the substrates present (NEFAs and glycerophosphate), and therefore it is theoretically possible that increased hepatic triglyceride synthesis could occur because of increased NEFAs or increased glycerophosphate. Increased NEFAs could result from decreased oxidation, increased synthesis, or increased mobilization from peripheral stores. In the case of ethanol-induced fatty liver, impaired mitochondrial oxidation of NEFAs appears to be the primary abnormality seen in humans [210] due to a shift in redox potential (increased NADH/NAD ratio). It may be accompanied, however, by other abnormalities [221]. There is little evidence to support the idea that fatty acid synthesis is involved in the development of fatty liver.

In humans, two types of steatosis, macrovacuolar and microvacuolar, have been characterized as hepatic lesions. With macrovacuolar steatosis, the hepatocyte contains a single, large, vacuole of fat and the nucleus is displaced to the periphery of the cell. In microvacuolar steatosis, the lipid exists in the hepatocyte as numerous small lipid vesicles and the nucleus remains in the center of the cell. Impaired mitochondrial ß-oxidation of NEFAs has received more attention as a possible explanation for the presence of microvacuolar steatosis [141]. Among the drugs reported to be associated with such a lesion, one finds salicylates, hypoglycin, valproic acid, amineptine, 2-arylpropionic acids, tetracyclines, zidovudine, and fialuridine.

Determination of hepatic fat content remains a reliable technique for demonstrating alterations by agents that produce steatosis with little or no necrosis (ethionine, phosphorus, tetracycline) and that are poorly reflected by serum enzyme measurement [528]. Alterations in hepatic triglyceride content have also been used as one of a battery of tests to determine the relative hepatotoxic potential of various halogenated hydrocarbons in rats [249] and to determine the ability of various alcohols to potentiate the hepatotoxic actions of CCl_4 [470] (Table 29.5).

TABLE 29.5
Effect of Alcohol Pretreatment on CCl_4-Induced Hepatotoxicity in Rats[a]

Treatment	ALT Activity (units/mL)	Triglycerides (mg/g Liver)	G6Pase activity (mg Pi/g Liver/20 min)
Ethanol (5.0 mL/kg)	50	8	6.7
Isopropanol (2.5 mL/kg)	50	6	6.2
CCl_4 (0.1 mL/kg)	100	9	6.0
CCl_4 (1.0 mL/kg)	500	17	3.8
Isopropanol + CCl_4 (0.1 mL/kg)	2250[b]	22[b]	2.8[b]
Ethanol + CCl_4 (0.1 mL/kg)	500[b]	13[b]	4.8[b]

Source: Data obtained from Traiger, G.J. and Plaa, G.L., *Toxicol. Appl. Pharmacol.*, 20, 105, 1971.

[a] The alcohol was given orally 18 h before intraperitoneal CCl_4 administration. Liver function was assessed 24 h after CCl_4 in 10 rats.

[b] Significantly different from the group given alcohol alone; $p < 0.05$.

LIVER FIBROSIS

Fibrosis, the accumulation of collagen, represents a key phenomenon in chronic liver disease [416]. Septal fibrosis is the principal feature of experimentally induced liver cirrhosis. CCl_4 and ethanol have been the toxicants most frequently used to induce liver fibrosis and cirrhosis. In the rat, twice weekly administrations of CCl_4 for 7–12 weeks have been shown to produce cirrhosis [63,73,197]. Ito cells (also called lipocytes, fat-storing cells, or stellate cells) acquire characteristics of myofibroblasts [415] and play an important role in the formation of collagen in liver fibrosis [459]. Ito cells isolated from fibrotic livers have significant increases in mRNA levels of type I, III, and IV procollagen compared to normal cells [501]. Serum concentration of the aminoterminal propeptide of procolagen type III (PIIIP) can be used as a fibrogenic marker for the period progressing to cirrhosis, but its use in cirrhosis seems to be limited by factors other than liver fibrogenesis [197].

High concentrations of the amino acid 4-hydroxy-L-proline are found only in collagen. Determination of hepatic hydroxyproline content represents a valuable marker to evaluate total collagen content and thus fibrosis in liver tissue [416]. The hepatic levels of hydroxyproline are well correlated with the degree of liver fibrosis measured histologically [73,197,239,353]. Figure 29.5 illustrates the effect of repeated CCl_4 gavage on hepatic hydroxyproline concentrations in corn oil– and acetone-pretreated rats; after 10 weeks of treatment, acetone + CCl_4-treated animals showed a fully developed cirrhosis, whereas a much less severe lesion was observed in corn oil + CCl_4-treated rats (37% ± 2% and 16% ± 3% of the liver occupied by fibrous connective tissue, respectively). Finally, the serum activity of immunoreactive prolyl hydroxylase, the enzyme responsible for proline hydroxylation, is elevated in rats with fibrotic livers, but Okuno et al. [353] suggested that these elevations should be carefully evaluated when being used as a parameter to estimate the activity of fibrogenesis in the liver.

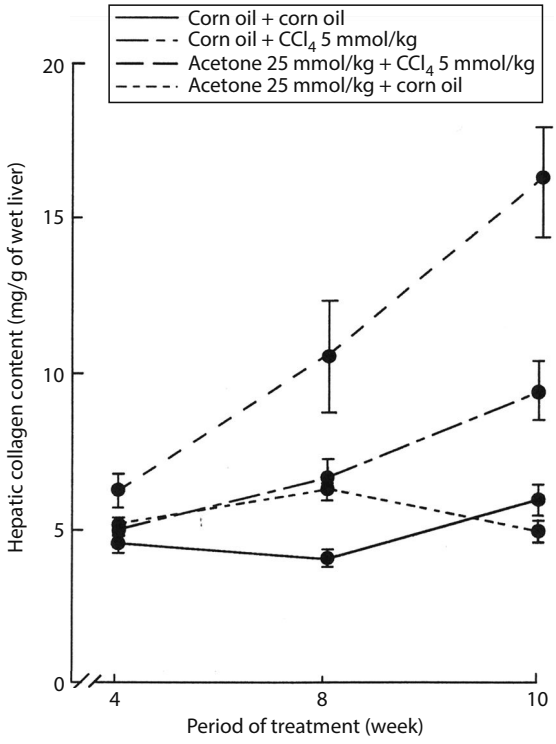

FIGURE 29.5 Temporal progression of hepatic collagen content in rats treated with corn oil or acetone twice weekly (Tuesday and Thursday) and challenged with corn oil or CCl_4 18 h later. Values represent the mean ± SE determined in eight rats. (Data obtained from Plante, I. et al., *Carcinogenesis*, 23(7), 1243, 2002.)

Analytical Determination of Hepatic Triglyceride, Hepatic Malonaldehyde, and Hepatic Collagen Contents

Hepatic Triglycerides

The hepatic triglyceride assay described by Butler et al. [61] is an adaptation of the method of Van Handel and Zilversmit [485], originally developed for the direct determination of serum triglycerides. The procedure consists of five steps: (1) homogenization of liver; (2) adsorption of phospholipids onto zeolite, followed by extraction of triglycerides into chloroform; (3) hydrolysis of triglycerides to fatty acids and glycerol; (4) oxidation of glycerol with $NaIO_4$ to formic acid and formaldehyde; and (5) formation of a colored complex of formaldehyde and chromotropic acid [61]. The procedure is relatively simple and can be used to analyze a large number of tissue samples in a single day.

Hepatic Malonaldehyde

The analysis is based on the reaction of MDA with TBA to produce a fluorescent complex. The method described by Buege and Aust [56] employs fluorescent measurements as put forth by Yagi [516] with the exception that the excitation wavelength is 532 nm. A calibration curve (0.15–2.5 mM) is prepared using 1,1,3,3-tetraethoxypropane (TEP), a chemical releasing MDA under acidic conditions [445]. The sample (1 mL) for analysis (e.g., mitochondrial fraction, cytosol, or homogenate) is prepared to yield a protein concentration of 4–6 mg/mL in phosphate buffer, is mixed with 2 mL of TBA solution, and is incubated at 100°C. After adding butanol and centrifuging, one analyses the organic phase for fluorescent content (532 and 553 nm for excitation and emission, respectively). The MDS content is expressed as pmol MDA/mg protein.

Hepatic Collagen

Liver content of hydroxyproline is measured by the colorimetric method of Edwards and O'Brien [120]. Hydroxyproline is liberated from collagen by acid hydrolysis, oxidized to pyrrole with chloramine-T, then transformed into a red chromogen using a p-dimethyl-aminobenzaldehyde solution, commonly called the Ehrlich reagent. The hydroxyproline is calculated from the absorbance at 500 nm; assuming 12.5% of collagen is constituted of hydroxyproline residues, the total collagen content is derived.

INTERCELLULAR JUNCTIONS IN HEPATOCARCINOGENESIS

The liver is constituted of parenchymal and various types of nonparenchymal cells, which all have to cooperate to perform a plethora of specialized functions. The establishment of cell–cell interactions mediated, in large part, by intercellular junctions is required to coordinate these functions. Cells junctions are specialized regions on the borders of cells that can be classified in three functional groups: occluding junctions (tight junctions), anchoring junctions (adherens junctions and desmosomes), and communicating junctions (gap junctions) (Figure 29.5). Many studies have shown that junctional proteins from the three types of junctions interact, colocalize, and coimmunoprecipitate and that they can regulate the expression or localization of each other in various tissues [109,198,318,500,514]. In hepatocytes, assembly of adherens junctions is needed for the formation of gap junctions [145]. Similarly, gap junctions expression is necessary for the formation and function of tight junctions, and upregulate the expression of tight junctions components [255,258]. Zonula occludens-1 (ZO-1), a tight junction protein, is thought to (1) coordinate the cellular dynamics of various associated junctions and (2) maintain the structural functionality of this multijunctional network [109,155,283]. Cell–cell interactions are required for the regulation of homeostasis and diverse cellular and tissue functions related to cell specialization, growth, and differentiation. In the liver, albumin secretion, ammonia detoxification, glycogenolysis, and bile secretion depend on intercellular junctions [45,192,337,341,343,440–442,454,463,522]. Thus, changes in junctional regulation by environmental contaminants can result in loss of tissue function and have been linked to hepatocarcinogenesis.

HCC, the most common type of liver cancer, arises from hepatocytes [473]. HCC is a progressive disease, divided in three distinct phases: molecular, preclinical, and clinical [473]. During this progression, hepatocytes gradually lost their architecture, polarity, and interactions, adopting a fibroblast-like phenotype, a process referred to as epithelial-to-mesenchymal transition (EMT). During EMT, disassembly of junctional structures is also observed. As a result, many junctional proteins have been associated with HCC progression and are considered as tumor suppressor.

Occluding Junctions and Hepatocarcinogenesis

Tight junctions are complex structures composed of integral membrane and cytoplasmic proteins (Figure 29.6). Integral membrane proteins include occludin, claudins, coxsackieadenovirus receptor (CAR), junctional adhesion molecules (JAMs), and tricellulin [305], each of them forming homodimers with transmembrane protein from adjacent cells. Cytoplasmic components of tight junctions are subdivided into two classes according to either the presence of a postsynaptic density protein-95/disc large protein/zonula occludens-1 (PDZ) domain or their functional properties [309,493]. The latest system distinguishes adaptors or scaffold proteins (e.g., ZO-1, -2, and -3), membrane-associated guanylate kinase–inverted protein (MAGI-1, -2, and -3), regulatory proteins (e.g., Rab13, aPKC, PTEN), and (post)transcriptional regulators (e.g., Symplekin, ZONAB). Tight junctions form highly polarized paracellular barriers that are selectively permeable to ions and small hydrophilic molecules, an essential function for homeostatic maintenance. Claudins and occludin are critical components of tight junction strands as their types and concentrations are thought to determine the ions and size selectivity of the barrier.

Tight junctions are found in two localizations in the liver: between adjacent hepatocytes and between bile duct epithelial cells. They form the blood–biliary barrier that keeps the bile in the bile canaliculi, apart from blood circulation, and allow the maintenance of hepatocytes polarity [260,282]. In human and rodent, claudin-1, -2, -3, -5, -7, -8, -14, occludin, JAM-1, -2, -3, -4, CAR, tricellulin, ZO-1, -2, -3, MAGI1, MUPP1, Cingulin, Symplekin, and 7H6 antigen are detected, with species variations in expression and localization [218,257,493].

HCC generally results from chronic liver diseases, in large part associated with viral hepatitis B and C, or cirrhotic livers [473]. Tight junctions are considered as the *door* for entry of hepatitis viruses, as hepatitis C virus can bind to claudin-1 and occludin, which act as coreceptors to facilitate entry of the virus into hepatocytes [167,282,369,525]. Tight junctions molecules also act as coreceptors for other viruses, such as adenoviruses and coxsackievirus [185]. Thus, on one side, tight junctions can promote liver hepatocarcinogenesis by facilitating viral infections, which render the liver more susceptible to HCC. On the other side, loss of cell interactions

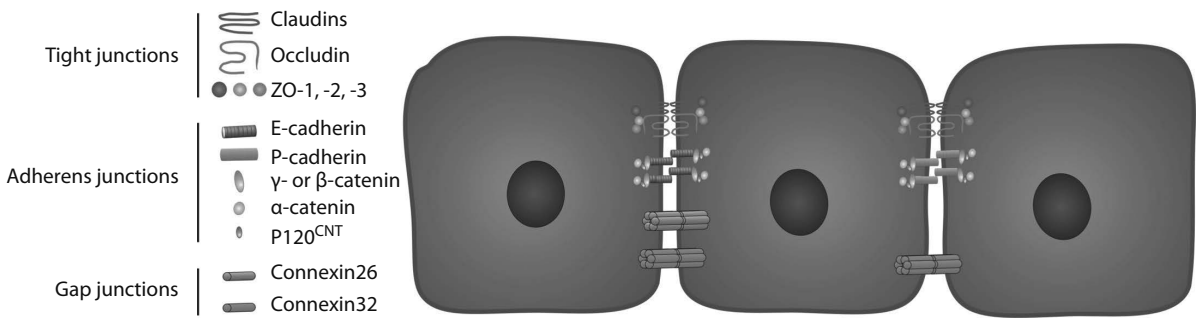

FIGURE 29.6 Schematic representation of tight, adherens, and gap junctions in hepatocytes.

is a hallmark of cancer and EMT, and many tight junctional components are considered as HCC biomarkers. Claudin-1, occludin, and CAR expressions are reduced in human HCC as compared to normal liver tumors [263,358], and downregulation of claudin-1 has been associated with poor survival in patient [203]. Recently, a decreased expression of symplekin in premalignant and malignant liver lesions was observed, suggesting that reduction of tight junction proteins may be an early step in the transformation of a normal hepatocyte [64].

On the other hand, overexpression of claudin-10 is observed in HCC and is thought to be associated with angiogenesis and invasiveness [212]. Introduction of claudin-10 in cultured Hep3B HCC cells, which lack claudin-10 expression, promotes cancer cell survival, motility, and invasiveness [220]. Silencing of the same protein in HLE, an invasive cell line with high level of claudin-10 expression, abolished invasiveness [220]. As a result, it has been suggested that claudin-10 protein could be a useful marker to predict poor prognosis of HCC patients after hepatectomy [212].

Anchoring Junctions

Anchoring junctions include two major groups, adherens junctions and desmosomes. Both are formed by transmembrane proteins, the cadherins, and cytosolic proteins (catenins for adherens junction and Armadillo proteins and plakins for desmosomes) [117,171,488]. Cytosolic components link adherens junctions and desmosomes to the cytoskeletal microfilaments and intermediate filaments, respectively. The role of anchoring junctions is to attach adjacent cells together by dimerization of their cadherins, which is dependent on the presence of Ca^{2+} ions (Figure 29.6).

In human and rodent livers, only stellate cells and hepatocytes form adherens junctions; Kupffer cells express β-catenin without forming junctions. Hepatocyte adherens junctions are formed by E-cadherin and N-cadherin, binding to α-, β-, γ-catenin, and $p120^{CTN}$, while adherens junctions in stellate cells involve N-cadherin and α-, β-catenin, and $p120^{CTN}$ [202,215,327]. As E- and N-cadherin are expressed in hepatocytes, four different cadherin/catenin complexes are present in the liver: E-cadherin/β-catenin/α-catenin/$p120^{CTN}$, E-cadherin/γ-catenin/α-catenin/$p120^{CTN}$, N-cadherin/β-catenin/α-catenin/$p120^{CTN}$, and N-cadherin/γ-catenin/α-catenin/$p120^{CTN}$ [268]. Many desmosomal components were also detected in liver extract, including desmoplakin 1 and 2, desmoglein 1 and 2, and desmocollin 2, which are expressed in hepatocytes [64,196,217,252,359,428,468].

While only few studies focused on desmosomal components in cancer [117], the role played by adherens junctions has been well studied [488]. In many tissues, including liver, E-cadherin is considered as a tumor suppressor [70,229,368]. Moreover, alterations in the expression of adherens junctions α-, β-, and γ-catenin were observed in hepatic tumors [105,215,219,268,324]. Interestingly, Kozyraki and collaborators [268] found a loss of E-cadherin, but not in N-cadherin, in HCC, suggesting that both complexes can have distinct function or stability in the liver. Desmosomes and adherens junctions have been studied in normal, premalignant liver lesions and HCC in a recent study [64]. Expression and localization of anchoring junctions in benign and premalignant lesions were uniform in hepatocytes and did not differ from normal tissue. In contrast, expression of components of desmosomes and adherens junctions was heterogeneous in HCC, ranging from none to very high per cell, and differs also between samples. Similarly, localization of junctional proteins in HCC was either at the plasma membrane, as in normal liver, or cytoplasmic and/or nuclear. Finally, the authors showed that the expression levels of the adherens junctions and desmosomes correlated with the grades of HCC cell differentiation by both immunohistochemical and Western blotting analysis [64].

Communicating Junctions

Communicating junctions, or gap junctions, are formed by a family of transmembrane protein named connexins (Cxs). The family is composed of 20 members in the mouse and 21 members in humans, named according to their predicted molecular weight, with different temporal and spatial expression in different cell and tissue [274]. Within cells, connexins form hexameric hemi-channels, called connexons, which are delivered to the plasma membrane. Once inserted in the plasma membrane, connexons freely diffuse within the cytoplasmic membrane bilayer and are clustered at the edges of specific area, named gap junction plaques [148,280,467]. While the mechanisms are not totally understood, guidance of connexons toward gap junction plaque seems to implicate N- and E-cadherin [232,318,500]. Connexons can then dock with connexons from adjacent cells to form gap-junction channels. Gap junctions allow direct passage of small molecules, such as cAMP, $Ins P_3$, adenosine, ADP and ATP, and second messengers, between cells, a process known as gap junctional intercellular communication (GJIC). Gap junctions are removed from the cell surface by internalization in specialized vesicle named annular junctions or connexosomes [148,375].

In rodent and human livers, Cx43 is expressed in endothelial, Kupffer, and stellate cells, as well as in the Glisson's capsule, Cx40 and Cx37 are expressed in vascular cells, while transcripts of Cx37, Cx31.9, and Cx30.2 were detected in whole liver [33,41,172,347,422,439,510]. Hepatocytes, on the other hand, are loaded with Cx32 and Cx26, as gap junction channels occupy about 3% of their membrane surface [330,450] (Figure 29.6). Cx32 is predominant, representing about 90% of the total hepatic connexins content, and uniformly distributed, while small amount of Cx26 is expressed mainly in the periportal acinar area [65,344,450].

Similar to E-cadherin, connexins are considered as tumor suppressor genes in many tissues [338]; inactivation of connexins genes in normal cells results in increased cancer-like phenotype, and cancer cells adopt a more normal phenotype upon transfection with connexins [97]. As Cx32 is the major hepatic connexin, Cx32-knockout mice are more susceptible

to diethylnitrosamine (DEN)-, radiation-, and chemical-induced liver hepatocarcinogenesis [126,242,243]. Similarly, transgenic rats carrying a dominant-negative mutant of Cx32 under the control of the albumin promoter (Cx32DTg) are more susceptible to DEN-induced hepatocarcinogenesis [208]. Interestingly, hepatic GJIC function decreases with aging, which can also render aged rats more susceptible to hepatocarcinogenesis [334]. Interestingly, Cx43, which is expressed in fetal but not in adult hepatocytes, is re-expressed in damaged hepatocytes [333], in primary culture and in many cell lines derived from normal liver [256,456,509]. However, Cx43 is usually localized in the cytoplasm or in the nucleus, and its reappearance could be considered as part of a dedifferentiation process, whereby hepatocytes gradually adopt a more fetal-like pattern [509]. Consistently, Cx43 is detectable in HCC [360].

Cell Interactions in Toxicological Studies

The liver is the primary site of detoxification, which involves cytochrome P450–mediated xenobiotic biotransformation. Many studies have suggested that cytochrome P450 expression is linked to the presence of Cx32 and E-cadherin; however, the mechanism is not clear [192,343,440–442]. Yet, in all previous studies, exposure to xenobiotic (β-naphthoflavone, phenobarbital, or clofibrate) resulted in a decrease in Cx32 expression and a concomitant induction of cytochrome P450 isoenzymes. Similarly, alcohol-, carbon-, tetrachloride-, and thioacetamide-induced acute hepatic injuries, and chemically induced chronic liver diseases are associated with downregulation of junctional components [43,80,259,322,335,336,426,518].

As cell junctions are critical for proper hepatic functions and in functions related to cell differentiation and proliferation, it has been suggested that they could be used as biomarkers in toxicological screening of chemical and epigenetic carcinogens [493]. In particular, inhibition of GJIC has been reported following exposure to numerous epigenetic carcinogens, whereas genotoxic compounds usually do not affect gap junctions [304,419,474–477,482,519,520]. Although not clear yet, some mechanisms and pathways have been associated with chemically induced downregulation of junctional components, focusing mainly on Cx32 in the liver.

Mechanisms Implicated in Connexin32 Downregulation

GJIC is regulated by both short-term and long-term mechanisms. Long-term regulation is mainly achieved by biosynthesis, trafficking, and turnover of connexins [274] and is more likely to be involved in hepatocarcinogenesis. Connexins half-life is short for transmembrane proteins: less than 2 h in cultured cardiomyocytes and 5 h in hepatocytes for Cx43 and Cx32, respectively [127,276]. As a result, downregulation of connexins transcription shortly results in decreased GJIC, which in the long term can promote carcinogenesis. Epigenetic mechanisms such as histone acetylation, DNA methylation, and micro-RNA-related control have been implicated in connexins regulation [490,492]. Using two cell lines of liver cells, one expressing Cx32 but not Cx43, the other expressing Cx43 but not Cx32, Piechocki and collaborators showed that connexins expressions correlate with their respective promoter methylation status, hypermethylation of the promoter region leading to silencing [373]. In rat HCC induced by a choline-deficient L-amino acid–defined (CDAA) diet, Cx26, and E-cadherin promoters were hypermethylated, resulting in gene silencing, as compared to normal liver tissue [478]. Interestingly, changes in methylation seem to be an early phenomenon even in hepatocarcinogenesis [437], and have been observed in tumors from other tissues [74,75,205,460].

Albeit its promoter has been identified, little is known about transcriptional regulation of Cx32 [19,20,131,342]. The human Cx32 gene is driven by two tissue-specific promoters containing binding sites for transcription factors, including hepatocyte nuclear factor-1 (HNF-1), Sp1, YY1, and NF-1, which role in carcinogenesis remains to be elucidated [254,329,374]. Pathways implicated in the regulation of these transcription factors remain, for the most part, to be identified. Snail, a tumorigenic transcription factor, mediates inhibition of E-cadherin gene expression in HCC [323,457]. In a study looking at mechanisms implicated in female rats susceptibility to hepatic tumor promotion by hexachlorobenzene (HCB) [269,278], Plante and collaborators demonstrated that both Cx26 and Cx32 were decreased in HCB-treated female rats, but not in males, resulting in downregulation of GJIC [385]. They demonstrated that downregulation of Cx32 was induced by the female-specific activation of the integrin-linked-kinase [508] pathway and resulted from decreased binding of transcription factors on the Cx32 promoter [386–388]. The same studies also showed that the activation of ILK pathway concomitantly reduced E-cadherin expression, but through different effectors, identifying for the first time a pathway implicated in the regulation of junctional proteins from two different types of junctions [386,387]. From this study, two important points were made: (1) exposure to environmental pollutants can promote tumorigenesis by inhibiting intercellular junctions, and (2) members of the junctional complexes can concomitantly be regulated by signaling pathways. Together, these data support the premise that cell–cell junctions are important early biomarkers of chemically induced hepatic cancer.

Methods to Evaluate Hepatic Connexins and GJIC

Changes in hepatic connexins expression in cancer or following exposure to chemicals are normally assessed by Western blot analysis, as good antibodies are available for Cx26, Cx32, and Cx43 [385,386,389]. Cellular localization is also critical when evaluating gap junctions since connexins need to be at the cytoplasmic membrane to be able to achieve their function [274,275]. Typically, gap junctions present as punctate structures at the junction of two adjoining cells [155]. Consistently, trafficking defects were associated with diseases and intracellular localization of connexins has been observed in tumors from diverse tissues [235,509]. While plasma membrane localization of connexins is required for

gap junction functions, it is not sufficient as channels can be closed by different mechanisms [301]. Some disease-linked connexin mutants showed proper localization but are not functional [274,432].

Because gap junctions allow the direct passage of ions and small molecules from one cell to the other, methods developed to evaluate GJIC usually involve tagged small molecular size molecules [1]. Transfer of endogenous and biologically relevant compounds can be monitored from loaded donor cells to unlabeled recipient cells using radiolabeled precursors, such as uridine. In this type of assay, GJIC is evaluated by autoradiographic labeling of the cytoplasm of recipient cells [162–164]. Increase in intracellular calcium, which propagate from cell to cell after stimulation, a process known as intercellular calcium waves (ICW), is linked to the presence of functional gap junctions and can be monitored using calcium-sensitive fluorescent dyes as another indicator of functional GJIC [427]. Gap junctional electrical conductance can also be monitored using dual voltage patch clamp technique, which is a very sensitive method, allowing the recording of a single gap junction channel [191,345].

The most commonly used methods, especially in carcinogenesis and toxicological studies, involve small fluorescent dyes transferring from adjacent cells through gap junction channels [1]. Dyes can be introduced into a confluent cell culture in one single cell, or in a restricted number of cells, by micro injection [236], scraping [122,357], or electroporation [398], and GJIC evaluated either by counting the number of receiving cells (or layers of cells) or as incidence of coupling (*yes–no* evaluation). Usually, a combination of two dyes is used: the first one, which is able to pass through gap junctions (i.e., Lucifer Yellow [LY]), is used to evaluate GJIC, and the second one, which cannot pass through gap junctions (i.e., Rhodamine-dextran [RH]), is used to evaluate transfer due to cell damage or other nonjunctional mechanisms. Alternatively, cells can be loaded with lipophilic cell plasma membrane permeable dyes, such as calcein acetoxymethyl ester, which once hydrolized by cytoplasmic esterases becomes fluorescent and membrane impermeable. Fluorescence is then irreversibly photobleached in a cell, or in a small number of cells, and fluorescence recovery after photobleaching (FRAP), coming from the transfer of fluorescent dye from the adjacent cells, is monitored [496]. Cells can also be loaded with a gap junction–permeable dye, such as calcein, and a gap junction–impermeable dye, such as DiI, and either cocultured with unloaded cells (preloading assay [161]) and allowed to form a monolayer, or spread on top of a monolayer of unloaded cells and allowed to adhere to them (parachute assay [527]). GJIC between donor preloaded cells and receiving unloaded cells is measured by fluorescent microscopy. More recently, Dakin and collaborators [100] used tracers that are nonfluorescent (or weakly fluorescent) when key functional groups of fluorophores are masked by photolabile protecting groups (cages), and become fluorescent upon removing of the protecting group by photoactivation (uncaging). The gap junction–permeable tracers can be activated (uncaged) by illumination in a single, or a small group of, preloaded cells (Local Activation of Molecular Fluorescent Probe [LAMP]) and fluorescence followed. This latest technique can also be applied in three-dimensional systems (infrared-LAMP assay [99,523].

These methods have been successfully used to study toxicological effects of compounds on GJIC in hepatocytes in vitro [1,491]. Ex vivo, GJIC can be determined in freshly removed precision-cut liver slices by injection of fluorescent dyes [222,270,418]. A modified version of the scrape loading has also been successfully developed in order to evaluate the effect of carcinogen in vivo [425]. Sai et al. [425] demonstrated a dose-dependent decrease in GJIC in the liver of mice treated with pentachlorophenol, which correlates with the dose dependency of tumor-promoting activity previously observed by the same team [481]. Similarly, Plante and collaborators showed that exposure to HCB, an epigenetic carcinogen, decreases GJIC in female rat liver (Figure 29.7) [385]. Likewise, exposure to cadmium, perfluorooctanoate (PFOA), perfluorooctane sulfonic acid (PFOS), phenobarbital, and HCB all decrease GJIC in vivo [211,230,385,483,498].

Role for Gap Junction Intercellular Communication in Spreading Liver Damage and Immune Responses: The Bystander Effects

In the last few years, increased connexins and GJIC have been shown to be beneficial in cancer treatments through *bystander effects*. Bystander effects refer to biological effects in cells that are not directly targeted by a treatment, such as transmission of damage signals from irradiated to nonirradiated cells that kill an increased number of tumors cells [190]. A promising approach combining the bystander effect and GJIC involves the herpes simplex virus-thymidine kinase (HSV-TK). In this system, cells expressing the TK gene can phosphorylate an antiviral drug, the ganciclovir (GVC), to GVC triphosphate, a nucleotide analog that causes DNA chain termination and apoptosis. This toxic metabolite can then be transferred to cells that do not express the TK gene, presumably through gap junction, and thereby induce apoptosis in TK-negative cells [190,346].

Paradoxically, in recent years, the GJIC-related bystander effects have also been associated with spreading of liver damage and immune response induced by drugs or chemicals. When developing new drugs, hepatotoxicity is a major concern as toxic metabolites can lead to oxidative stress and DNA damage in hepatocytes [339]. It has been suggested that GJIC should decrease to avoid transfer of these toxic metabolites to neighboring cells and thereby reduce liver toxicity [333,364]. Using rats carrying a dominant-negative mutation in the gene coding for Cx32, Naiki-Ito et al. [333] demonstrated that the loss of Cx32 is protective against acetaminophen-induced hepatotoxicity, a drug involved in many cases of drug-induced liver injury. More recently, Patel et al. [364] treated Cx32-knockout mice with acetaminophen or thioacetamide (TAA), a classic hepatotoxin known to cause fulminant hepatic failure, and showed that they had less liver damage and a reduced inflammatory response as compared

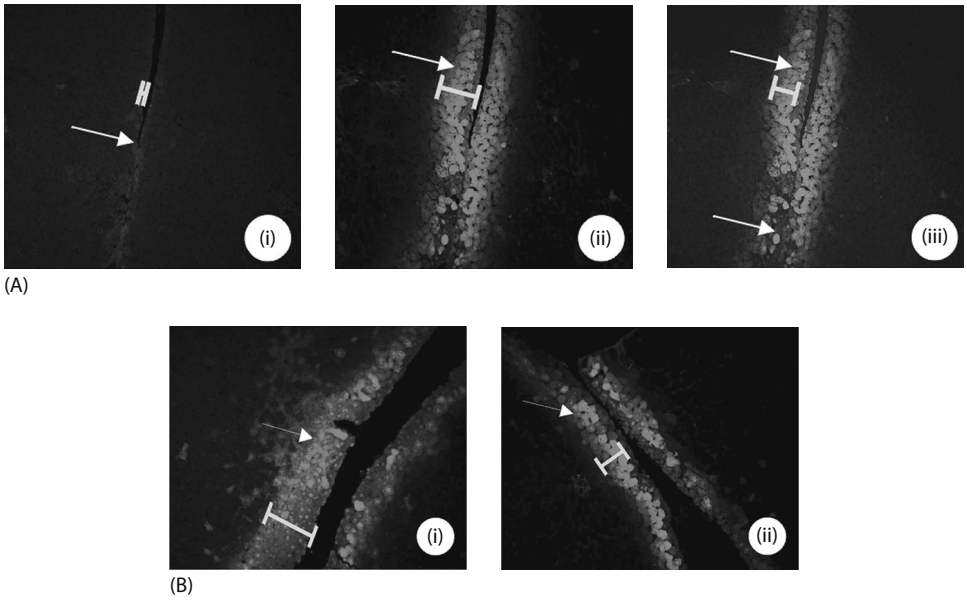

FIGURE 29.7 Effect of hexachlorobenzene (HCB) on gap junctional intercellular communication in female rat liver. Rats were administrated HCB (100 mg/kg) by gavage for 5 consecutive days and samples on day 100 of the experiment. Liver were excised and subjected to incision-loading/dye-transfer analysis. (A) Rhodamine-dextran (RhD) (panels i and iii) was used as a control for damaged cells, while Lucifer-Yellow (LY) was used to measure cell–cell communication (panels ii and iii). Distance of LY migration is indicated by a white lane (A and B). (B) LY migration in a control (panel i) and a HCB-treated female liver (panel ii). (Data obtained from Plante, I. et al., *Carcinogenesis*, 23(7), 1243, 2002.)

to wild-type mice. Moreover, TAA-induced mortality was extensively reduced after exposure to a lethal dose of the drugs. Oxidative stress is a well-known effect of hepatotoxicans; decreased Cx32 and GJIC in knockout mice would prevent transmission of an oxidative stress signal throughout the liver. Interestingly, pretreatment, coadministration, or posttreatment with a Cx32 inhibitor had similar effects in TAA- or acetaminophen-exposed wild-type mice, suggesting that targeting gap junction proteins could be a broad strategy for preventing drug-induced liver injury at all of these stages.

While GJIC-mediated bystander effects have received a lot of attention regarding drug-induced liver injury in the last few years, little is known about such hepatic effects induced by chemicals. Asamoto and collaborators demonstrated that exposure to D-galactosamine or carbon tetrachloride results in milder liver damage in transgenic rats expressing a dominant-negative Cx32 than in their wild-type counterparts [11]. Whether or not other toxicological damages could be amplified by GJIC remains to be determined.

LIVER REPAIR AND RECOVERY

The liver is a remarkable organ in its ability to regulate its growth and gain or loss of optimal mass. The biology of proliferative properties of the organ, the mechanisms initiating regeneration, the relationship between hepatocyte proliferation and apoptosis, and the involvement of nonparenchymal cells (Kupffer cells and stellate cells) have been well described [129,500]. Liver regeneration is a multistep process involving priming and progression. Although various forms of chemical-induced liver injury have been studied extensively in terms of biochemical events for over 50 years [379], interest generally has focused on the early initiating effects leading to the appearance of hepatocellular dysfunction, rather than on the later recovery phase of the lesion. In the rat, organ recovery after acute hepatotoxic insult is largely dose dependent; recovery time is longer when the lesion is more extensive. As the lesion progresses, hepatocellular regeneration appears; it appears within 6 h after administration of a low dose of CCl_4 in the rat, even though the centrilobular necrosis is just becoming evident [294,295]. When the hepatic lesion is enhanced by the introduction of another agent, however, recovery time can be an important consideration for assessing possible mechanisms of action involved in the potentiations [377]. With the combination of CCl_4 and several potentiating agents (*n*-hexane, 2-hexanone, 2,5-hexanedione, isopropanol, and acetone), this relationship was assessed using both biochemical indices (serum ALT and OCT activities) and morphological patterns (quantitative histology) of liver injury; appropriate dose–response curves were established from the percentage of animals affected [72]. Time of recovery was shown to be due to the maximal severity of the lesion, regardless of the potentiation. Although pretreatment with the potentiator resulted in an enhanced hepatotoxic response from a low dose of CCl_4, the dose–response curve for the enhanced response was no different than that produced by a higher, but equitoxic dose of CCl_4 administered alone. These data were interpreted as an indication that the five potentiators did not alter the temporal progression of CCl_4-induced liver injury.

Mehendale and his collaborators have performed an extensive series of experiments to assess the role of tissue repair in potentiated liver injury. The studies originated from the observation that chlordecone-potentiated CCl_4 hepatotoxicity in rats was quantitatively quite remarkable and resulted in enhanced lethality. Two tissue repair responses were observed after exposure to a low dose of CCl_4 [62]; the early phase regeneration (EPR) response (arrested G2 hepatocytes activated to proceed through mitosis) occurs quickly (peaks at about 6 h) and is followed (at about 24 h) by the secondary phase regeneration (SPR) response (hepatocytes mobilized from G0/G1 to proceed through mitosis). During chlordecone potentiation of CCl_4 hepatotoxicity, the EPR phase appears to be eliminated and the SPR phase decreased; thus, the progression of the severe injury is facilitated and leads to lethality. There is evidence that induction of EPR may accelerate SPR. Interestingly enough, large doses of CCl_4 given alone also result in regeneration responses similar to those obtained with chlordecone and a small dose of CCl_4. Experiments performed with colchicine, partial hepatectomy, CCl_4 autoprotection, nutritional factors, and different animal species have provided data consistent with the purported roles attributed to EPR, SPR, and liver injury [62,449].

The role of tissue repair has been assessed with other hepatotoxicants [315,449]. Dose–response studies indicate that thioacetamide when given alone affects hepatic tissue regeneration (measured with ^{3}H-thymidine incorporation into DNA; proliferating nuclear cell antigen) in a manner not unlike that observed with CCl_4. Comparable observations were obtained with o-dichlorobenzene and trichloroethylene, although the dose-dependent relationships were not as evident with these agents. Increased lethality was not observed with isopropanol-potentiated CCl_4 liver injury. Expression of stimulators of promitogenic signaling interleukin-6, inducible nitric oxide synthase, hepatocyte growth factor, transforming growth factor-α, and epidermal growth factor receptor were studied after thioacetamide hepatotoxicity in rats; they were modified by moderate caloric restriction [315]. With a binary mixture of chloroform and allyl alcohol, tissue repair was shown [6] to exert a key role in the final outcome of the liver injury in rats. Mehendale and his collaborators have proposed [291,449] a two-stage model for chemical-induced hepatotoxicity. Stage one would involve initiation and infliction of injury; stage two would lead to recovery or progression to massive injury depending on the effects of the toxicant on cellular regeneration (enhancement would lead to recovery; inhibition would lead to massive injury). They hypothesized that progression of the liver injury might be mediated by cytoplasmic and lysosomal degradative enzymes leaking from dying cells [291]. The experimental results carried out following CCl_4- and acetaminophen-induced hepatotoxicity suggest that calpains may be involved. Although various aspects of the repair–recovery process are still hypothetical or speculative, the concept as such is thought provoking and certainly an important contribution to the understanding of chemical-induced liver injury. It will be interesting to see how it evolves.

APOPTOSIS

Previous sections highlighted the fact that necrosis of hepatocytes is a common type of cell death occurring after chemical exposure. There exists, however, another type of cell death, called apoptosis (from the Greek "apo" meaning "away from" and "ptosis" meaning falling: cells "falling away from" a tissue) [86] that is recognized as an important process in chemically induced effects in the liver and liver disease [421]. In a landmark article, Kerr et al. [240] defined a form of cell death morphologically distinct from necrosis, which they called apoptosis. Apoptotic cells exhibited nuclear and cytoplasmic condensation followed by dissociation of the cell into membrane-bound fragments similarly to the events observed in a phenomenon previously described by embryologists as programmed cell death. For this reason, apoptosis has been inappropriately used as a synonym for programmed cell death. Apoptosis is defined as an active mode of cell death, since it requires RNA and protein syntheses, is controlled by pro- and antiapoptotic genes, and is induced by physiological stimuli in addition to the typical pathological stimuli associated with necrosis [83]. On the other hand, necrosis is considered to be a form of *passive* cell death. Recently, however, necrosis has also emerged as an alternate form of programmed cell death, whose activation might have biological consequences, including the induction of an inflammatory response [119].

Apoptosis is morphologically defined by a progressive condensation of the chromatin to the inner face of the nuclear membrane (DNA hyperchromicity and cresenteric caps), convoluted cell shape (*blebbing/budding*), dilatation of the endoplasmic reticulum, cell shrinkage with consequent loss of membrane contact with neighboring cells, and fragmentation of the cell with formation of membrane-bound acidophilic globules (apoptotic bodies) often containing nuclear material. The latter are frequently found within the cytoplasm of intact cells, indicating they are phagocytized by adjacent cells [101]. In addition, apoptosis is not commonly associated with the inflammatory response that accompanies necrosis [240]. Other morphological criteria specific for necrotic cells are cellular and nuclear swellings, patchy chromatin condensation, swelling of mitochondria, vacuolization in cytoplasm, plasma membrane rupture (*ghost-like* cells), and dissolution of DNA (karyolysis). Histological criteria of cell death, such as pyknosis and karyorrhexis, can be applied to both apoptosis and necrosis at certain stages and hence cannot distinguish between these two modes of cell death [366]. Finally, from the above it is apparent that apoptosis involves only scattered single cells, whereas necrosis affects large areas of liver lobules. In the normal liver, apoptosis is predominant in acinar zone 3 and is thought to subserve the elimination of senescent cells [34].

Apoptosis and mitosis play complementary contrasting roles in tissue homeostasis as the former leads to cell removal and tissue hypoplasia, whereas the latter causes tissue hyperplasia. For example, the induction of altered hepatic foci appears to be related to compensatory cell proliferation

in PCB-77-treated rats, whereas the inhibition of apoptosis appears to be important in PCB-153-treated rats [464]. Because apoptosis plays a critical role in deleting cells from tissues, it is not surprising that failure of apoptosis leads to imbalanced cell proliferation and is now recognized as a phenomenon associated with carcinogenesis. Bursch et al. [59], however, recently suggested that in contrast to rat liver, inhibition of apoptosis in mice appears to be a minor determinant of tumor promotion. Tumor promoters and nongenotoxic carcinogens inhibit active cell death, thereby increasing the accumulation of (pre)neoplastic cells and accelerating the development of cancer [430]. Sustained activation of the aryl hydrocarbon receptor (AhR) is postulated to generate a strong selective pressure in liver treated with 2,3,7,8-tetrachlorodibenzodioxin (TCDD), leading to selection and expansion of clones evading growth arrest and apoptosis [44]. Suppression of apoptosis with TCDD coincided with a marked hyperphosphorylation of p53 mediated by the AhR [429]. Using the inhibition of UV-induced apoptosis in rat hepatocytes in primary culture as an in vitro model for mechanistic studies on the inhibition of apoptosis, Bohnenberger et al. [46] showed that *non-dioxin-like* PCBs are likely to promote liver carcinogenesis via the suppression of apoptosis. Heptachlor strongly inhibited TGF-induced apoptosis and cytochrome *c* release into the cytosol in rat hepatocytes [352]. Peroxisome proliferators, a large and chemically diverse family of nongenotoxic rodent hepatocarcinogens that activate the peroxisome proliferator-activated receptor alpha (PPARα), have been shown to suppress apoptosis induced by both spontaneous and transforming growth factor ß1, the physiological negative regulator of liver growth [412]. Experimental evidence supports a mechanism involving diminished apoptosis in the age-related difference in sensitivity, where PPAR agonists produce a five- to seven-fold higher yield of grossly visible hepatic tumors in old-relative-to-young animals [524].

Examples of Chemically Induced Liver Apoptosis

In addition to being present in various viral, immunological, malignant, or drug-induced human liver diseases, hepatocyte apoptosis in animals can be triggered either in vivo or in vitro by many toxic agents [130]. Histopathologic features of apoptosis are frequently observed in chronic cholestatic disorders as a result of accumulation of toxic bile salts within hepatocytes [366]. Bile salt–induced apoptosis occurs in a concentration-dependent manner at concentrations that are far smaller than those critical for micelle formation and do not cause cell necrosis [365]. Hydrophobic bile salts, such as glycodeoxycholate, glycochenodeoxycholate, or taurochenodeoxycholate, cause apoptosis in isolated hepatocytes [78,366]. The hydrophilic bile acid tauroursodeoxycholic acid significantly reduced glycochenodeoxycholate-induced hepatocyte apoptosis [36]. Death receptor activation is an important endpoint in bile acid–induced apoptosis. Higuchi et al. [204] reported that deoxycholate is more potent than glycodeoxycholate, chenodeoxycholate, and glycochenodeoxycholate to upregulate death receptor 5 (DR5)/tumor necrosis factor–related apoptosis inducing ligand (TRAIL) receptor 2 sensitizing hepatocytes to TRAIL-mediated apoptosis. Glycochenodeoxycholate-induced hepatocyte apoptosis is modulated by caspase cascade activation [497]. Hepatobiliary diseases are characterized by elevated caspase activation and apoptosis, which can be specifically detected in situ by a cleavage site-specific antibody against cytokeratin-18 [26].

Increases in the number as well as changes in the distribution of apoptotic bodies within the liver were observed in ethanol-fed rats and mice [35,165]. When compared with normal livers, the increased apoptosis was more pronounced as the duration of ethanol exposure increased and was reversed by ethanol withdrawal. All the putative mechanisms for alcohol-induced hepatocellular damage (i.e., oxidative stress, toxic acetaldehyde adduct formation, hypoxia, or immunologically mediated destruction) can induce apoptosis, such that it may represent a final common mechanism mediating hepatocellular damage by ethanol [366]. Alcohol increases liver apoptosis predominantly through an intrinsic signaling pathway [107].

Metals cause changes in liver apoptosis. Mice injected with 5–60 μmol/kg i.p. of cadmium showed both a time- and dose-dependent increases in liver apoptosis, which peaked at 9–14 h after cadmium administration, then decreased [186]. The time-course of apoptotic DNA fragmentation index, monitored by quantification of oligonucleosomal DNA fragments, correlated with the results obtained by histopathological analysis and a commercial in situ apoptotic DNA detection kit. An interesting conclusion of this work is that apoptosis is a major mode of elimination of critically damaged cells in acute cadmium hepatotoxicity in the mouse, and it precedes necrosis. Cadmium also causes a biphasic elevation in parenchymal cell apoptosis in the rat [480]; apoptosis of nonparenchymal cells is the basis of the pathogenesis of peliosis hepatitis. An organic metal, tributyltin, which is a highly toxic water contaminant, also induces apoptosis in rainbow trout hepatocytes through a step involving Ca^{2+} efflux from the endoplasmic reticulum or other intracellular pools and by mechanisms involving cysteine proteases, such as calpains, as well as the phosphorylation of apoptotic proteins, such as Bcl-2 homologues [399]. Denizeau et al. [234] reported the involvement of initiator and effector caspases, cleavage of their intracellular substrates and activation of both death receptor and mitochondrial pathways in TBT-induced apoptosis in rat hepatocytes. Long-Evans Cinnamon (LEC) rats, an animal model for inherited copper toxicosis, show liver apoptosis with increasing age [135]. Finally, chronic arsenic administration induces a specific pattern of apoptosis called postmitotic apoptosis [27].

Assessment of Apoptosis

Precision-cut liver slices provide a valuable in vitro system for studying drug-induced liver apoptosis [328]. Using a model of apoptosis-driven regression of rat liver hyperplasia induced by cyproterone acetate, the mean duration of the histological stages of apoptosis was found to be approximately 3 h

[60]. Since apoptosis occurs in isolated single cells and does not stimulate persisting tissue changes, such as inflammation and scarring that characterize necrosis, procedures based on biochemical endpoints, such as DNA cleavage patterns, have been developed as complementary tools for the analysis of cell morphology. Internucleosomal DNA cleavage caused by a Ca^{2+}/Mg^{2+}-dependent endonucleases is a prominent feature of apoptosis [8]. During apoptosis, DNA is cleaved in a non-random manner into 50–300 kilobases, then into 180 base-pair fragments. Several techniques are based on the analysis of DNA fragments. Separation of nuclear DNA from apoptotic cells on agarose gel yields a typical *ladder* appearance of fragments, whereas DNA from necrotic cells produces a smear on electrophoresis. The use of DNA electrophoresis as the sole criterion for apoptosis is not recommended, since DNA ladders can be observed in cells with necrotic-type morphology, and cells with ultrastructural characteristics of apoptosis can fail to produce DNA ladders.

Individual cell electrophoresis can be performed using the so-called *comets* assay [101]; the apoptotic cells appear as *comets* having characteristic *heads*, that represent the remains of the cells containing high molecular weight DNA and *tails*, which represent a fraction of the degraded DNA. Commonly used techniques involve in situ labeling of DNA strand breaks such as the TdT-mediated dUTP-digoxigenin Nick End Labeling (TUNEL) assay; deoxynucleotides can be either fluorochrome- or enzyme (phophatase or peroxidase)-tagged. For a description of this procedure used in the context of liver toxicity assessment, see Habeebu et al. [186]. Wheeldon et al. [504] suggested that further validation is required before in situ end labeling can be used confidently alone, since they observed variation in apoptotic body indices after cyproterone acetate withdrawal between in situ end labeling and hematoxylin and eosin staining techniques. Similarly, Grasl-Kraupp et al. [170] concluded that DNA fragmentation is common to different kinds of rat liver cell death and that its detection in situ should not be considered a specific marker of apoptosis.

Several techniques for measuring apoptosis rely on flow or laser scanning cytometry (for a review, see pp. 49–61 in [101]). Dive et al. [112] proposed a rapid multiparameter flow cytometric assay that discriminates and quantifies viable, apoptotic, and necrotic cells via measurement of forward- and side-light scatter (proportional to cell diameter and internal granularity, respectively) and the DNA-binding fluorophores Hoechst 33342 and propidium. For a comparison of approaches for quantifying hepatocyte apoptosis, see Goldsworthy et al. [166], and for a discussion on the selection of methods and the inappropriate uses of methodology, see pp. 61–69 in Ref. [101]. Annexin V is a 36-kDa protein that binds with high affinity to phosphatidylserine lipids in the cell membrane, a component that goes from the inner membrane leaflet to the outer cell surface in apoptotic cells. Therefore, Annexin V has been shown useful for detecting the earliest stages of apoptosis. Recent advanced positron emission tomography (PET) techniques using either ^{18}F-annexin V [517] or ^{124}I-annexin V [238] have opened the way for in vivo measurement of chemically induced apoptosis in rats.

Modulation of molecular endpoints based on proteins involved in signaling cascades controlling apoptosis can be used to demonstrate chemically induced apoptosis. For example, the caspase (cysteine–aspartyl–proteases) enzymes play a central role in the cell death machinery as indicated in chemically induced apoptosis discussed earlier. They are subdivided into initiator (caspase-2, -8, -9, and -10) and effector (caspase-3, -6, and -7) caspases. Two main pathways are well established in apoptosis, that is, the death receptor pathway and the mitochondrial pathway. The first pathway requires membrane receptors such as Fas, which is involved in a signaling complex activating caspase-8, ensuring activation of caspase-3. The second pathway involves the release of a small apoptogenic molecule, cytochrome *c*, leading to formation of apoptosome, which also leads to caspase-3 activation. The two pathways can be linked through Bid, a pro-apoptotic member of the Bcl-2 family of proteins [234]. At least 15 different Bcl-2 proteins have been identified and can be subdivided into pro- and anti-apoptotic members. Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl-1, Brag-1, and A1 inhibit apoptosis, whereas Bax, Bak, Bcl-xS, Bag, Bid, Bik, Hrk, and Bad promote apoptosis in mammalians [421].

A simple cytological method has been used for evaluating the apoptotic rate of human neutrophils, since these cells are known to spontaneously undergo apoptosis without any stimulation and are recognized as an excellent model for studying this biological process [150]. The assay can be applied to tissue slices, primary cells, cell lines, freshly isolated cells, and can be performed with cells of virtually any origin. Furthermore, observations made using this technique correlate well with the results obtained by conventional Hoechst 3342 and propidium iodide cytofluorimetric analyses [159]. This simple and low-cost histologically based procedure is performed at room temperature. Isolated cells (suspension of approximately 1.5×10^6 cells/mL) are loaded into a cytospin chamber and gently spun with a cytocentrifuge to adhere the cells onto a microscope slide, whereas adhering cells can be directly grown on glass coverslips and tissues slices used. A rapid coloration procedure is performed using the Diff-Quick staining kit (Baxter, Miami, FL) according to the manufacturer's instructions. The slides are rinsed and sealed with warm parafilm or nail varnish; the sealed slides can be kept for several days. Cells are observed with a light microscope at a magnification of 400× or 1000×; nuclei appear as dark purple structures. Apoptotic cells are easily distinguished from normal cells by examining morphological changes: cell shrinkage, nuclear collapse (crescent shape or small dot profiles), dense white inner cell vacuoles, and apoptotic bodies (plasma membrane blebs/buds or granules). It is recommended that a minimum of 100 cells from different fields be counted in two replicate slides. Results are expressed as the percentage of cells in apoptosis (100 × the number of apoptotic cells/the total number of cells). For new applications

using isolated or cultured cells, it is strongly recommended that cell viability be verified by trypan blue exclusion before performing the assay; unlike necrotic cells, apoptotic cells exclude trypan blue.

CONCLUDING REMARKS

It can be seen that although techniques for determination of chemically induced liver injury in laboratory animals are readily available, no single technique is satisfactory for the detection and quantification of all forms of injury. Rather, a battery of procedures consisting of one or more of the biochemical/functional techniques coupled with a histological analysis of the liver is essential for the correct evaluation of the hepatotoxic potential of a chemical agent.

DEDICATION

This chapter is dedicated to the memory of two internationally recognized toxicologists, Gabriel L. Plaa (May 15, 1930 – November 11, 2009) and Michel Charbonneau (April 10, 1959–June 27, 2013).

Gabbie was a hepatotoxicologist who set up the basis for many assays and mechanisms that are still in use these days. Among others, he served as president of the STC, the SOT, as well as the Pharmacological Society of Canada.

Michel's main interests were to understand the risks associated with pollutants exposure on human health and cancer. His scientific achievements were recognized by numerous awards, and by his implication in SOT, as president of STC and director of Réseau de Recherche en Santé Environnementale. Above all, Michel was my first mentor, recently my colleague, and forever my friend and "scientific father." This wonderful person left us too early and will be missed by many.

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30 Principles and Methods for Renal Toxicology

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CONTENTS

INTRODUCTION

This chapter focuses on guiding and underlying principles and methods used to study kidney function and toxicity on levels ranging from the whole animal or humans, to the organ, cellular, subcellular, and all the way down to the level of individual molecules. This broad range reflects the need to have noninvasive means of detecting renal function and toxicity in the clinic as well as to develop more effective therapeutic approaches to treat renal disease and renal injury resulting from exposures to toxic chemicals. The latter requires an understanding of the molecular changes that accompany exposures to toxic chemicals and in various disease states that affect the kidneys. An important theme that underlies much of the chapter is that of biomarkers. Although the concept and use of biomarkers (also called biological markers) to assess renal function or exposure to nephrotoxicants is not new,[1] recent advances in methodology and in our understanding of renal function have identified new biomarkers that can be very useful for identifying chemical exposures or indicating changes in renal function.

To be most effective and useful in the clinic, the biomarker must be something that is readily measured in a noninvasive manner, such as in a blood or urine sample. One can divide biomarkers into three types[1]: biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility. Biomarkers of exposure (to a drug or chemical contaminant) are typically either the chemical itself, a key stable metabolite, or a product derived from interaction between the chemical and a component in the target cell. An example of the latter might be a protein with a specific adduct or a chemically modified residue. Utility of such markers for therapeutics is questionable because so many drugs have common metabolites or produce similar molecular changes in target cells. Biomarkers of effect are by far the most common and include some measurable cellular, physiologic, or biochemical change. For these to be most useful, they must be tissue- and chemical-specific and highly sensitive. Finally, biomarkers of susceptibility are those that can identify a genetic factor or preexisting disease that alters response to a chemical exposure. The study of pharmacogenetics or pharmacogenomics has greatly increased our understanding of potential variations in the response of the human population. By identifying genetic polymorphisms in processes such as drug metabolism or transport or ligand–receptor binding, for example, one can identify those individuals who are more likely than the majority of the population to experience adverse effects from chemical exposure and at lower doses of the chemical. From this has developed the concept of personalized medicine.

The text is divided into four main sections. The first section reviews some key properties of renal structure and function as they relate to the kidneys as a target organ for chemically induced toxicity. Although much more detailed information on renal structure and function can be found in several texts, books, and book chapters,[2,3] it is important to provide some foundation to address the critical issue of what makes the kidneys particularly susceptible to many forms of injury. The second section considers the various experimental models that can be used to study renal function and toxicity. Key considerations in the use of these models are presented, focusing on advantages and limitations to their use in renal toxicology. The third section considers several selected examples of assays used to quantify renal cellular function. These range from parameters that can be measured in blood or urine, and are thus potentially applicable to clinical use to cellular and subcellular parameters that can be used to help understand mechanism and develop novel therapeutic approaches to treating renal injury. These assays are presented to illustrate examples of both some classic assays as well as newer approaches that can provide insight into cellular metabolism and molecular regulation. The fourth section discusses the topic of human disease and risk assessment, which integrates discussion of experimental models of renal disease and chemically induced nephrotoxicity and that of how animal models are used to make predictions for humans.

It is important to note that this chapter is meant to provide a summary and overview of factors that determine or contribute to nephrotoxicity and the experimental models and assay methods used in studies of renal function and toxicity. Presentation of methods and assays is not in the form of detailed, step-by-step procedures; rather, presentation of the highlights, advantages, and limitations are the major emphases.

RENAL PHYSIOLOGY, ANATOMY, AND BIOCHEMISTRY

The kidneys are uniquely sensitive to many toxicants because they receive and filter a large quantity of blood relative to its weight. On average, the two kidneys receive 25% of cardiac output while comprising only about 1% of body weight. Additionally, plasma membranes of renal epithelial cells, especially those of the proximal tubule, possess a myriad of transport proteins. Thus, the combination of renal blood flow and membrane transporters can often lead to a high degree of accumulation and high concentration of chemicals relative to that in plasma within the tubular epithelial cells. The presence of drug metabolism enzymes, which can result in bioactivation of inert or nontoxic chemicals, further contributes to the potential susceptibility of the kidneys to chemically induced injury.

The major function of the kidneys is to excrete waste products while maintaining total body salt, water, and acid–base balance. The kidneys are thus the primary organs responsible for maintaining the constancy of the internal environment. This is accomplished by three mechanisms: (1) glomerular filtration, (2) tubular reabsorption, and (3) tubular secretion. Each mechanism, primarily with a focus on its contribution to renal susceptibility and injury, are discussed later in this chapter. The following subsection will briefly discuss some of the key structural and functional features of the mammalian kidney. A critical point is that the kidneys exemplify

form following function. It is also important how these functions, in a toxicological or pathological context rather than under normal physiological conditions, make the kidneys a unique target organ.

Overall Structural Organization of the Kidneys

The major tissue types in the kidneys are vascular tissue and tubular epithelia. Kidney structure can be considered at multiple levels of organization. At the highest level, the kidney is subdivided into cortex, outer stripe of the outer medulla, inner stripe of the outer medulla, and inner medulla or papilla. Humans and larger mammals have multipapillate kidneys whereas small mammals, such as rat and rabbit, are unipapillate. Each papilla descends into a renal fornix. In multipapillate kidneys, fornices merge to form the renal pelvis, which is an expanded upward extension of the ureter. There is a close association, both physically and functionally, between the renal vasculature and epithelial tissue. The renal artery enters the kidney alongside the ureter, branching to become progressively the interlobular arterioles that lead to the glomerular capillary network. The venous system has subdivisions with similar designations, terminating in the renal vein, which also flows beside the ureter.

At the closed end, the nephron is extended to form the cup-shaped Bowman's capsule. The lumen of the capsule is continuous with the narrow lumen that extends through the renal tubule. Associated with the capsule is the tuft of capillaries that forms the glomerulus inside Bowman's capsule. This structure is responsible for the initial step in urine formation. A filtrate of plasma passes through the single-cell layer of capillary walls, through the basement membrane, and finally through another single-cell layer of epithelium that forms the wall of Bowman's capsule. The filtrate then passes into the lumen of the tubules to begin its passage through the various segments of the nephron, eventually passing into the collecting duct and the renal pelvis. The wall of the renal tubule is one cell layer thick and this epithelium functions to separate the plasma and urinary filtrate in the lumen from the interstitial fluid.

The nephron is considered the functional unit of the mammalian kidney, and consists of a glomerular capillary network surrounded by Bowman's capsule, a proximal tubule (convoluted and pars recta or straight segment), a loop of Henle (thin descending limb, thick ascending limb), a distal tubule (convoluted tubule and collecting tubule), and a collecting duct (Figure 30.1). The nephron is an epithelial tubule that is closed on one end and opens into the renal pelvis and collecting ducts at the other end. Nephrons are divided into three classes, based on the location of their glomerulus: superficial, midcortical, and juxtamedullary. Glomeruli located near the surface of the kidney give rise to superficial nephrons, which generally only descend into the outer medulla. These nephrons are also called short-looped nephrons and lack thin ascending limbs. Glomeruli located deep within the cortex, near the corticomedullary border, give rise to juxtamedullary nephrons. These nephrons are called long-looped nephrons and enter the inner medulla and have thin descending limbs. In general, mammals that produce highly concentrated urine have longer papillae and a higher proportion of long-looped versus short-looped nephrons. Glomeruli located between the superficial and juxtamedullary glomeruli give rise to

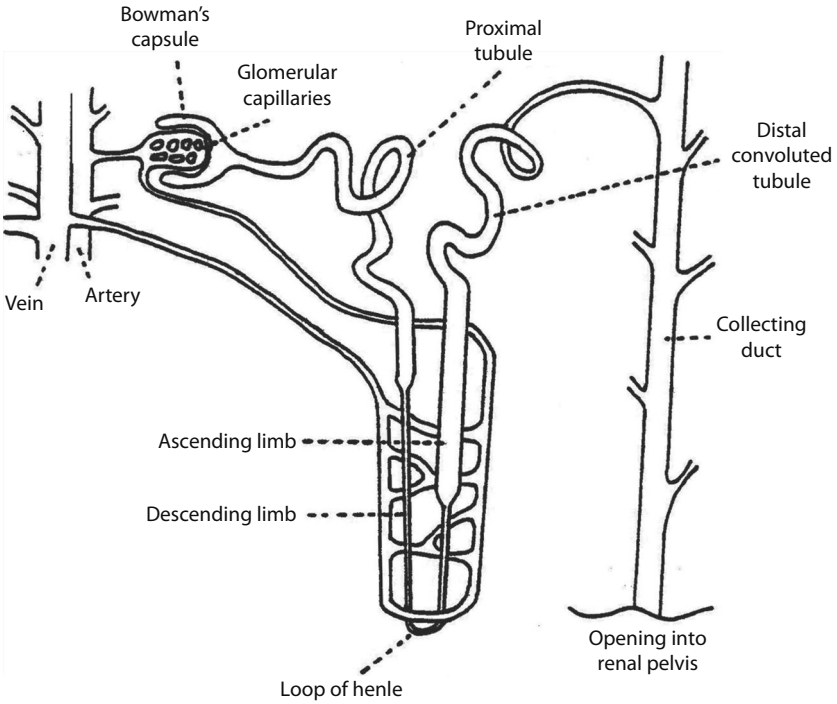

FIGURE 30.1 Diagram of basic nephron structure and the associated renal vasculature.

midcortical nephrons. These may be either long-looped or short-looped, although the deeper glomeruli are more likely to be associated with long-looped nephrons.

Basic nephron structure can also be considered on the basis of localization relative to the vascular or urinary poles. The urinary pole starts at the proximal convoluted tubule in the cortex. The tubule enters the medulla where it forms the loop of Henle. The distal tubule returns to the glomerulus of origin and then enters the cortex where it is somewhat convoluted. The distal tubule then leads into the collecting duct, which goes into the medullary pyramid and then into the papillae. The nephron ends at the junction of the distal tubule and the collecting duct. Several nephrons feed into a single collecting duct. The vascular pole is the juxtaglomerular region, which contains mesangial cells and the returning distal portion of the distal tubule. This region has many closely packed nuclei and is called the macula densa. The afferent arteriole breaks up into 8–10 capillary loops in the glomerulus. On the opposite side of the glomerulus is the urinary pole where the proximal tubule begins.

An important region of the nephron that is critically involved in regulation of renal blood flow and urine formation is the juxtaglomerular apparatus (JGA), which consists of specialized epithelial cells, the macula densa cells, and specialized secretory or granular cells at the vascular pole where the afferent and efferent arterioles enter and leave the glomerulus. The JGA is thus a combination of specialized tubular and vascular (i.e., smooth muscle) cells. A key function of the JGA is secretion of renin, which is involved in the formation of angiotensin and ultimately, in secretion of the mineralocorticoid aldosterone. This process is part of the feedback system that regulates glomerular filtration and renal blood flow.

Glomerular Filtration and Urine Formation

Each kidney receives its blood supply from a single renal artery and renal vein. The renal arteries originate from the aorta and the renal veins drain into the interior vena cava. The urine formed in each kidney is drained via a single ureter into the bladder. The initial stage of urine formation is filtration of plasma and accumulation of the ultrafiltrate in the lumen of Bowman's capsule. The glomerular filtrate contains nearly all the constituents of the blood except for the blood cells and large proteins. Approximately 15%–25% of the water and solutes are removed from the plasma during a single pass through the renal circulation by filtration alone. In humans, the glomerular filtrate is typically produced at a rate of 125 mL/min or about 200 L/day. Consideration of normal fluid intake implies that most of the glomerular filtrate must be reabsorbed into the bloodstream to maintain water balance and prevent dehydration. The critical importance of the kidneys in this function is illustrated in a bar graph comparing major sources of fluid input and output under normal conditions (Figure 30.2). Input of fluid comes from metabolism and the gastrointestinal (GI) tract. Both of these are highly adaptable, although water production from metabolism typically is geared toward energy production rather than volume regulation. Input of water from the GI tract can be modulated by activation of the thirst mechanism in dehydration. The prominence of the kidneys is clearly seen in a comparison of the primary sources of water output: Although the lungs, GI tract, and skin are significant sources, the kidneys provide 68% of the output under basal conditions.

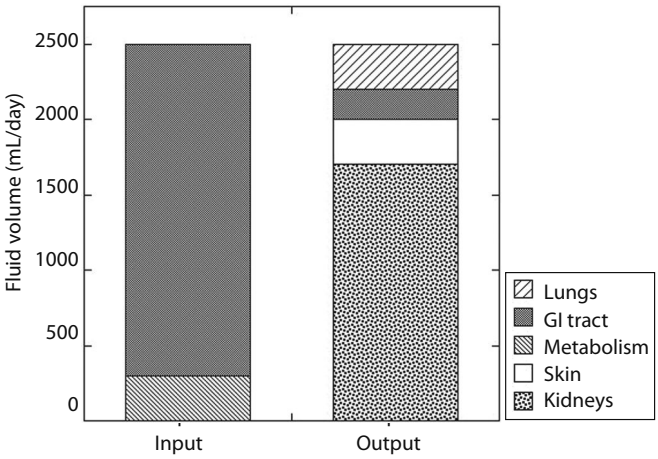

FIGURE 30.2 Summary histogram showing the function of different organ systems in fluid volume homeostasis.

Glomerular filtration refers to the movement of water and solutes across the glomerular membrane to form an ultrafiltrate of plasma in the tubular lumen, and is the first step in urine formation. It depends on two factors: (1) a pressure gradient from the lumen of the capillary to the lumen of Bowman's capsule and (2) the sieve-like properties of the three-layered tissue separating the two compartments. Water and nonprotein solutes (e.g., inorganic anions and cations, intermediary metabolites such as glucose and urea) are found in the filtrate at approximately the same concentrations at which they occur in plasma. The exclusion of most proteins above a threshold molecular weight from the glomerular filtrate is critical for maintenance of the osmotic pressure difference across the glomerular membrane. As described in the following text, appearance of significant amounts of high-molecular-weight proteins in the urine is an obvious indicator of glomerular damage. Thus, filtration occurs because forces moving fluid out of the capillary (i.e., hydrostatic pressure) exceed forces preventing both movement out of the capillary (i.e., oncotic pressure due to plasma proteins) and into Bowman's space (i.e., hydraulic pressure within Bowman's capsule).

The glomerular filtration rate (GFR) is affected by changes in blood pressure, plasma protein concentration, and the ultrafiltration coefficient (K_f). GFR varies directly with blood pressure and inversely with protein concentration in the afferent arteriole. Although GFR is highly dependent on blood flow, renal blood flow is normally independent of systemic blood pressure and is autoregulated (i.e., to be approximately constant) as long as blood pressure is above about 80 mmHg. K_f is not a constant, but varies in response to agents such as angiotensin II, vasopressin, and norepinephrine.

Contraction of the mesangium reduces K_f. Alterations of this coefficient are thought to be important in the progression of glomerular damage but not tubular damage.

The two other processes, besides glomerular filtration, that account for the ability of the kidneys to regulate electrolyte, organic metabolite, and water balance are tubular reabsorption and tubular secretion. Tubular reabsorption refers to the movement of substances from the tubular fluid, through the epithelial cell, back into plasma. In this manner, substances that would otherwise have been excreted in urine are reabsorbed. The functional significance of tubular reabsorption is evident from an evaluation of the renal handling of Na^+ ions and glucose. The kidneys actively reabsorb >99% of the Na^+ ions and glucose that emerge in the tubular fluid after glomerular filtration. Although reabsorption of the filtered NaCl load occurs along all segments of the nephron, the proximal tubules account for approximately 60% of the total reabsorption under normal conditions. The remainder is reabsorbed in the thick ascending limb (~30%), distal convoluted tubule (~7%), and collecting duct (~2%–3%). Depending on the filtered NaCl load, however, these proportions can be greatly modified as Na^+ ion transport in the more distal portions of the nephron and the collecting duct is under hormonal control. Tubular secretion, in contrast, refers to the movement of substances from plasma, through the epithelial cell, into the tubular fluid and ultimately the urine. Tubular secretion is a critical component of how the body handles many drugs and xenobiotics.

The processes described earlier lead to a glomerular filtrate in the proximal tubular lumen that is isosmotic with plasma (i.e., ~300 mOsM). As the filtrate progresses along the nephron, however, there is a countercurrent mechanism that generates an extremely high osmolarity in the medullary interstitium. Thus, the medullary interstitium at the base of the loop of Henle in long-looped nephrons can reach as high as 1200 mOsM. As fluid progresses through the thick ascending limb to the distal and cortical collecting tubules, osmolarity decreases to ~100 mOsM. This behavior is dependent primarily on three factors: (1) active chloride or sodium reabsorption in the ascending limb, (2) the anatomical relationship between the descending and ascending limbs (i.e., both limbs are close together and flow in each is in the opposite direction), and (3) the parallel vascular structure to the nephron structure that forms a countercurrent exchange of blood and prevents solutes from coming out of the descending limb and being washed away.

Nephron Heterogeneity: Physiological, Biochemical, and Toxicological Implications

The mammalian kidney is a complex organ whose basic structural unit, the nephron, is composed of several cell populations, each exhibiting diverse morphological, biochemical, and physiological properties.[4,5] As briefly described earlier, specific properties of each segment are critical to overall kidney function in reabsorption and secretion. As presented in the following text, each segment is both morphologically and biochemically designed to perform specific functions in urine formation and maintenance of electrolyte, fluid, and metabolite homeostasis. Additionally, these features also give each nephron segment a characteristic susceptibility to either chemical toxicants or to pathological states, such as hypoxia or ischemia/reperfusion. As will be briefly described in the following subsections, these susceptibility differences are due in part to the distinctive composition of membrane transporters and enzymes in each segment but also to differences in cellular energetics and oxygenation.

An important feature of all nephron cell types, and is also characteristic of transporting epithelia in general, is that they are polarized cells, meaning that the plasma membrane is physically separated by tight junctions into distinct regions (luminal or brush-border membrane and basolateral membrane). A critical feature of this separation is that each region comprises distinct enzymes, carriers, channels, and other proteins. This separation is essential for functions such as vectorial transport as part of tubular reabsorption and tubular secretion; disturbances in the processing of key membrane proteins have been identified as a response to nephrotoxicants and disease states.

Nephron Cell Types: Structure and Function

The subsections that follow will summarize some key morphological and functional features of several major nephron segments. Additional details and discussion of other nephron segments can be found in any number of texts or book chapters.[2,3] An overview of the major nephron segments, highlighting some of their key morphological features and physiological roles, is presented in Table 30.1.

Proximal Tubules

The first nephron segment to which the tubular filtrate comes in contact after glomerular filtration is the proximal tubule. The proximal tubule is subdivided into three segments, S1, S2, and S3. The first segment (S1) is located within the pars convoluta or proximal convoluted tubule, and is found in the cortex. The S2 segment is localized at the end of the convoluted and beginning of the straight section (pars recta or proximal straight tubule), and the S3 segment is localized entirely within the proximal straight tubule, which is in the outer stripe of the outer medulla.

The epithelial cells of the S1 segment are rather leaky, allowing movement of solutes between cells by a paracellular route. Approximately 60%–70% of the initial tubular filtrate is reabsorbed in the S1 segment. Reabsorption occurs via an array of active and passive transport processes, with water flowing passively down the osmotic gradient. Hence, the tubular fluid remains isosmotic with respect to the plasma in spite of the large reduction in fluid volume to approximately 25% of its original volume. Cells of the S1 segment are tall and have a prominent brush border, extensive infoldings of the basal membrane, and interdigitations of the lateral borders of adjacent cells. The extensive brush border, or microvilli, and the infoldings of the basal membrane illustrate the correlation between form and function, as these structures

TABLE 30.1
Summary of Morphology and Physiology of Selected, Key Nephron Segments from Mammalian Kidney

Nephron Cell Type	Morphology	Physiology
Proximal tubule	Tall and prominent microvilli on luminal membrane (especially S1)	75% of filtered Na^+ removed by active transport
		Passive water and Cl^- reabsorption
	Cuboidal shape	Organic anion secretion (S2, S3)
	Extensive basolateral infoldings	Most glucose, amino acid reabsorption
	Leaky epithelium	Tubular fluid remains isosmotic to plasma
	High density of mitochondria	
Thin descending limb	Squamous epithelium	Extremely high osmotic water permeability
	Little cellular interdigitation (type I)	Aquaporin 1 on luminal and basolateral membranes
	Better developed lateral interdigitations (types II, III)	Low permeability to Na^+
	Deep tight junctions (type I)	Variable permeability to urea (low to moderate)
	Shallow tight junctions (types II, III)	Tubular urine concentration
	Low density of mostly spherical mitochondria	
Thin ascending limb	Flat, moderately interdigitated cells	Water impermeable; no aquaporin
	Shallow tight junctions	Little, if any, active Na^+ transport
	Low density of mostly spherical mitochondria	Highly permeable to NaCl
		Vasopressin-sensitive C1C-K1 Cl^- channel
		Variable permeability to urea (low to moderate)
Thick ascending limb	Extensive interdigitation	CYP-dependent arachidonic acid metabolism
	Shallow tight junctions	Water impermeable; no aquaporin
	Large number of elongated, rod-shaped mitochondria	Na^+–K^+–$2Cl^-$ cotransport
	Cortical cells are thinner and have less mitochondria than medullary cells	Active Na^+ transport
		Active Ca^{2+} and Mg^{2+} transport
		Dilution of hyperosmotic tubular urine
Distal tubule (distal convoluted and connecting tubule)	Distal convoluted tubule (DCT)—appears bright under microscope short deep tight junctions; numerous long mitochondria	High rates of Na^+ reabsorption
		Thiazide-inhibitable Na^+–Cl^- cotransporter
	Cortical collecting tubule (CCT)—appears granular under light microscope wider than DCT	K^+–Cl^- cotransporter
		Ca^{2+} reabsorption
		DCT—water impermeable
		CCT—vasopressin-regulated aquaporin 2 water channel
Cortical collecting duct	Two cell types: principal cells (PC), intercalated cells (IC) (2:1)	IC—abundant carbonic anhydrase
		PC—no carbonic anhydrase
	PC—few cellular organelles including mitochondria sparse, short microprojections on apical membrane	Vasopressin-dependent water permeability
		Low urea permeability
	IC—abundant cytoplasmic vesicles higher mitochondrial content densely packed microvilli on apical membrane	Active and passive transport of Cl^- and K^+ ions
		Acid–base regulation

provide a large surface area for reabsorption. The cells possess a high content of mitochondria near the basal pole, a characteristic feature of Na^+-transporting epithelia. The vast majority of filtered glucose and amino acids are reabsorbed in this segment as well.

Cells of the S2 segment are structurally less complex than those of the S1 segment; S2 cells have a shorter, less dense brush border, less basolateral interdigitation, and a lower content of mitochondria. While the rate of volume reabsorption is thought to decrease in vivo going from S1 to S2 segment, in vitro studies show a similar capacity for the two segments. The results in vivo are, therefore, likely due to the decreased concentration of electrolytes in the S2 tubular fluid due to the large reabsorption that occurred in the S1 segment. Certain carriers, however, are present at higher activities in the S2 segment, such as many of those for organic anions.

The transition between S2 and S3 segments is relatively subtle, and species-dependent differences exist in S3 ultrastructure. For example, S3 cells are shorter and less complex than S2 cells in rabbits whereas S3 cells in rats have a well-developed brush border that is longer than that in other proximal segments. Although glucose reabsorptive capacity is much lower in S3 segments than in either S1 or S2 segment, organic anion and organic cation transport is high in S3. Consistent with this distribution, several enzymes involved in drug metabolism are present at the highest levels among the three proximal tubular segments in the S3 segment.

Thin Descending and Thin Ascending Limbs

Thin descending limbs are present in both short-looped and long-looped nephrons. They are subdivided into three subtypes in most mammalian species, types I, II, and III.

Short-looped nephrons contain only type I cells, which form a squamous epithelium with little cellular interdigitation and deep tight junctions. Long-looped nephrons of rat and hamster, in contrast, contain type II cells in the more proximal portion of the thin limb and type III cells as the thin limb enters the inner medulla. Type II cells contain well-developed lateral interdigitation and shallow tight junctions. A key physiological feature of type I, II, and III thin descending limb cells is their extremely high osmotic water permeability, which is mediated by aquaporin 1 water channels in both luminal and basolateral plasma membranes. Urea and sodium permeability exhibits a good deal of heterogeneity among both the different cell types of thin descending limbs and across species that have been studied, ranging from very high to low.

Thin ascending limbs are only present in long-looped nephrons, and begin slightly before the bend of the loop of Henle in most species. There is an abrupt conversion of cell type to the medullary thick ascending limb as the tubule progresses past the inner–outer medullary border. The thin ascending limb contains flat, moderately interdigitated cells that are connected by shallow tight junctions. Unlike the thin descending limb, cells of the thin ascending limb are water impermeable in all species studied and do not express any aquaporin water channels. Thin ascending limb cells have very low Na^+ pump activity, suggesting little if any active Na^+ transport. In contrast, there is a high passive NaCl permeability, which is thought to involve paracellular flux. Vasopressin-sensitive, transcellular Cl^- transport occurs and is mediated by the ClC-K1 Cl^- channel that is present on both the luminal and basolateral plasma membranes. Cells from both thin descending and thin ascending limbs contain a low density of mostly spherical mitochondria, which is consistent with the low activities of active transport processes.

Thick Ascending Limb

The thick ascending limb is divided into two segments, the medullary thick ascending limb, which is located in the outer medulla, and the cortical thick ascending limb, which is located in the medullary rays within the cortex. The medullary thick ascending limb originates at the inner–outer medullary border, either from the thin ascending limb of long-looped nephrons or from the thin descending limb of short-looped nephrons. The cell type becomes the cortical thick ascending limb at the outer medulla–cortex junction, although the transition between medullary and cortical cell types is not abrupt.

Medullary thick ascending limbs are longer in long-looped as compared with short-looped nephrons. There are extensive interdigitation and shallow tight junctions between adjacent medullary thick ascending limb cells. They contain a large number of elongated, rod-shaped mitochondria and one or two cilia on their luminal surface. Medullary thick ascending limbs are subdivided into two types, based on the smoothness or roughness of their luminal surfaces. Although the transition is fairly subtle from medullary to cortical thick ascending limb, the cortical thick ascending limbs are thinner than those of the medullary thick ascending limb and cells from the cortical region contain a lower density of mitochondria. The cortical thick ascending limb extends past the macula densa, varying in length among species.

Although most of the renal cytochrome P450 (CYP) activity is found in the proximal tubules (predominantly the S3 segment), both medullary and cortical thick ascending limbs contain CYP activity that metabolizes arachidonic acid to several biologically active products, indicating the importance of these nephron segments in regulation of renal hemodynamics. Like the thin ascending limb, both medullary and cortical thick ascending limbs are water impermeable and do not express aquaporin water channels. Unlike the thin ascending limb, medullary and cortical thick ascending limbs have active ion transport processes. Active transporters include the bumetanide-sensitive Na^+–K^+–$2Cl^-$ cotransporter (NKCC2, BSC1) in the luminal plasma membrane, the (Na^++K^+)-stimulated adenosine triphosphatase (ATPase) on the basolateral plasma membrane, and the membrane potential-dependent, divalent cation transporter for Ca^{2+} and Mg^{2+}. These active solute reabsorption processes provide the primary mechanism for dilution of the luminal fluid as it moves up the nephron through the thick ascending limbs.

Distal Tubule

The term "distal tubule" is generally used to refer to the portion of the nephron that lies between the macula densa and the first junction of two tubules, although it is actually composed of several structurally and functionally distinct cell types. The most proximal portion begins just beyond the macula densa and contains cortical thick ascending limb cells. These cells abruptly transition to distal convoluted tubules. The distal convoluted tubules appear bright under light microscopy and are short. The cells contain deep tight junctions, short blunt apical microvilli, deep basolateral invaginations, and numerous long mitochondria oriented perpendicular to the basolateral membrane. The third portion of the distal tubule, the connecting segment or connecting tubule, appears granular under light microscopy and is wider than the distal convoluted tubule. This segment is also sometimes referred to as the granular distal tubule.

Functionally, both the distal convoluted tubule and connecting tubule exhibit high rates of Na^+ reabsorption that are mediated by the thiazide-inhibitable Na^+–Cl^- cotransporter (NCC1, TSC). Potassium is transported by the K^+–Cl^- cotransporter (KCC1) on the luminal plasma membrane and a conductive K^+ channel is expressed in rabbit distal convoluted tubules. Calcium ion reabsorption is also a prominent function of the distal convoluted tubule but occurs at a lower rate than in the cortical thick ascending limb. Finally, the distal convoluted tubule is water impermeable, but the connecting tubule in rat possesses a vasopressin-regulated water channel, aquaporin-2, indicating this segment can be water permeable.

Cortical Collecting Duct

The cortical collecting duct originates at the convergence of two collecting tubules and extends down to the corticomedullary

TABLE 30.2
Substrate Preferences and Predominant Pathways for Energy Metabolism among Nephron Cell Types

Nephron Cell Type	Substrate Preference	Mitochondrial Density	Pathway Preference for ATP Generation
Proximal tubule	Fatty acids, ketone bodies, lactate, glutamine, glutamate, pyruvate, citrate, acetate	High	Oxidative phosphorylation, citric acid cycle, gluconeogenesis
Thick ascending limb (medullary and cortical)	Lactate, glucose, ketone bodies, fatty acids, acetate	Moderate to high	Oxidative phosphorylation and glycolysis
Distal convoluted tubule	Glucose, lactate, β–hydroxybutyrate	High	Glycolysis
Cortical collecting duct	Glucose, lactate, β-hydroxybutyrate, fatty acids	Low	Glycolysis

border. This segment contains two cell types, the principal (or light) cells and the intercalated (or dark) cells, at a ratio of approximately 2:1. In comparison with the intercalated cells, principal cells have fewer cellular organelles, including mitochondria, a less electron-dense cytoplasm, and sparse, short microprojections on their apical membranes in contrast with the abundant cytoplasmic vesicles and more densely packed microvilli on apical membranes of intercalated cells. Another important difference between the two subtypes of cortical collecting duct cells is that carbonic anhydrase is abundant in intercalated cells but is either absent or present at relatively low levels, depending on species, in principal cells. This has functional implications for pH regulation of the urine.

Physiologically, the collecting duct regulates the final composition of the urine. Although the collecting duct has a low capacity for reabsorption, it is important in regulating the reabsorption of water, solutes, electrolytes, bicarbonate, and protons. Water permeability of the cortical collecting duct is almost completely dependent on vasopressin, which stimulates baseline osmotic water permeability by a factor of 10–100 in rats and rabbits. The cortical collecting duct has a low permeability for urea that is unaffected by vasopressin. In the presence of vasopressin, therefore, this property enables the kidneys to increase the concentration of urea within the lumen of the cortical collecting duct. This nephron segment also contains both active and passive transport processes for Cl^- and K^+ ions and plays an important role in acid–base regulation.

Cellular Energetics

Patterns of cellular energy metabolism differ widely among the cell types of the nephron. These patterns are based on a number of factors, including cellular energy (i.e., adinosine triphosphate [ATP]) requirements, mitochondrial density, and oxygenation.[6] The first factor, cellular energy requirements, is largely dependent on active transport capacity. As described earlier, each nephron cell population exhibits markedly different levels of various active transport pathways. Thus, while the proximal tubules contain high activities of active Na^+, organic anion, glucose, and amino acid transport pathways, and have correspondingly high needs for ATP and high densities of mitochondria, thin descending and ascending limb cells have low activities of active transport pathways and correspondingly low needs for ATP and low densities of mitochondria. Thick ascending limb cells, particularly those in the medullary region, have high activities of active Na^+ and divalent cation transport and high densities of mitochondria. Distal tubular cells also have high rates of active Na^+ transport and high amounts of mitochondria.

In terms of substrate utilization, each segment has a characteristic preference for energy needs and exhibits distinct patterns of activity for pathways of carbohydrate utilization and synthesis (Table 30.2). For example, although many segments contain a high density of mitochondria, ATP generation primarily comes from glycolysis in the more distal segments of the nephron. Although fatty acids and ketone bodies can be used by several nephron segments, they are the preferred source of reducing equivalents for ATP generation in the proximal tubules. Because many toxicants target mitochondria as an early step in their mechanism of action, cells such as those in the proximal tubule are particularly susceptible to injury, provided that the chemical is transported into the cell.

An example of two nephron cell populations exhibiting a markedly different susceptibility to inhibition of mitochondrial function and ATP depletion, is illustrated in our studies of the susceptibility of suspensions of freshly isolated proximal tubular and distal tubular cells from the rat to hypoxia or chemically induced ATP depletion.[7,8] In the hypoxia study,[7] cells were incubated under either hyperoxic (95% O_2/5% CO_2), normoxic (21% O_2/74% N_2/5% CO_2), or hypoxic (95% N_2/5% CO_2) conditions to determine the extent of cell death over time. Distal tubular cells were markedly more sensitive to cytotoxicity from O_2 deprivation than those from the proximal tubule; moreover, this difference was not correlated with the extent of ATP depletion, as proximal tubular cells exhibited ~75% ATP depletion yet little or no cytotoxicity. Essentially, similar results were obtained with the chemical model of cellular ATP depletion[8]: Proximal tubular cells exposed to iodoacetate + KCN exhibit little cytotoxicity, but significant ATP depletion, whereas distal tubular cells exhibited a similar degree of ATP depletion but markedly higher release of lactate dehydrogenase (LDH). This suggests that nephron cell populations have differing abilities to withstand marked (>80%) ATP depletion.

Another factor that relates to differences in cellular energetics is in vivo oxygenation. As described earlier and in our hypoxia study,[7] proximal and distal tubular cells respond differently in vitro to O_2 deprivation. In the intact animal or in humans, however, the situation is more complex. On the one

hand, each nephron cell population has different ATP consumption rates and differing mitochondrial densities. Beyond that, however, it is known that an O_2 gradient exists in the kidneys as one goes from cortex to medulla such that the inner medulla has been described as being on the brink of anoxia. Accordingly, the pars recta (S3) segment of the proximal tubule and the medullary thick ascending limb in isolated, perfused rat kidneys are particularly susceptible to injury from hypoxia.[9–13] An additional series of studies from Epstein and colleagues[14–17] correlated the degree of cellular injury from hypoxia or ischemia with either ATP consumption (i.e., cellular work) or the inverse of mitochondrial activity.

Organic Anion and Cation Transport

As indicated earlier, an important component of susceptibility of a specific cell type to chemically induced injury is whether that cell type possesses the capacity to transport and accumulate the chemical. Although there are several classes of transporters involved in renal physiology that are localized in various nephron segments, perhaps the most important for toxicology and experimental therapeutics relating to the kidneys are those for transport of organic anions and cations because most therapeutically used drugs belong to these two classes of molecules. Although these carriers are also distributed in multiple segments of the nephron, they are expressed at the highest levels in the proximal tubules. As shown in Figures 30.3 and 30.4, transport of organic anions and cations, respectively, occurs by multiple carriers and mechanisms. While each carrier certainly has a discrete substrate specificity pattern, there is also considerable overlap between carriers, providing functional redundancy. Most of these carriers are polyspecific, that is, they transport multiple substrates. Several recent reviews have been published on the properties and regulation of these carriers and their importance in renal function.[18–27]

Organic anions, including many Phase II metabolites of xenobiotics such as glucuronide, glutathione (GSH), and sulfate conjugates, are transported across the basolateral and brush-border membranes of renal proximal tubular cells, which constitute the mechanism for their secretion into tubular urine. Uptake of organic anions across the basolateral plasma membranes of renal proximal tubules from the renal plasma and interstitial space occurs primarily by organic anion transporters 1 and 3 (Oat1/Slc22a6 in rodents and OAT1/SLC22A6 in humans; Oat3/Slc22a8 in rodents and OAT3/SLC22A8 in humans) (Figure 30.3). Oat1/OAT1 and Oat3/OAT3 are considered tertiary active transporters, because uptake of organic anions (OA^-) is dependent on the 2-oxoglutarate concentration gradient, which is in turn determined by counter transport with Na^+ ions as mediated by the sodium-dicarboxylate carrier (NaC3/SLC13A3). The Na^+ ion gradient used to drive 2-oxoglutarate transport is achieved by the (Na^++K^+)-stimulated ATPase, a primary active ion transporter. An additional member of the SLC22

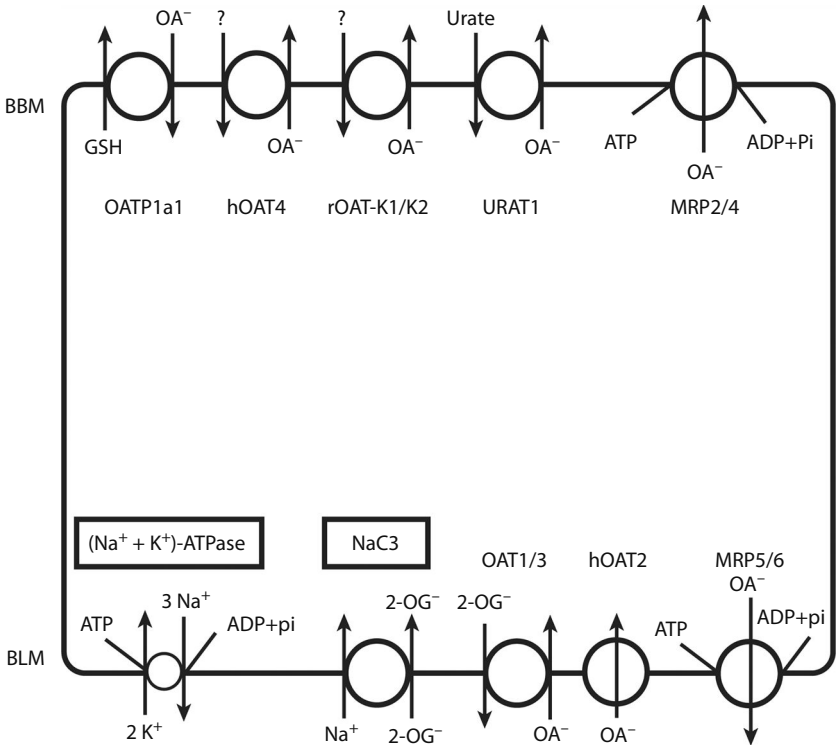

FIGURE 30.3 Scheme for organic anion (OA^-) transport in renal proximal tubule. BBM, brush-border membrane; BLM, basolateral membrane; MRP, multidrug resistance–associated protein; NaC3, sodium-dicarboxylate 3 cotransporter; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; 2-OG^-, 2-oxoglutarate. The "h" in front of some of the carriers indicates it has only been found in humans? = Unknown what, if anything, is coupled to organic anion efflux.

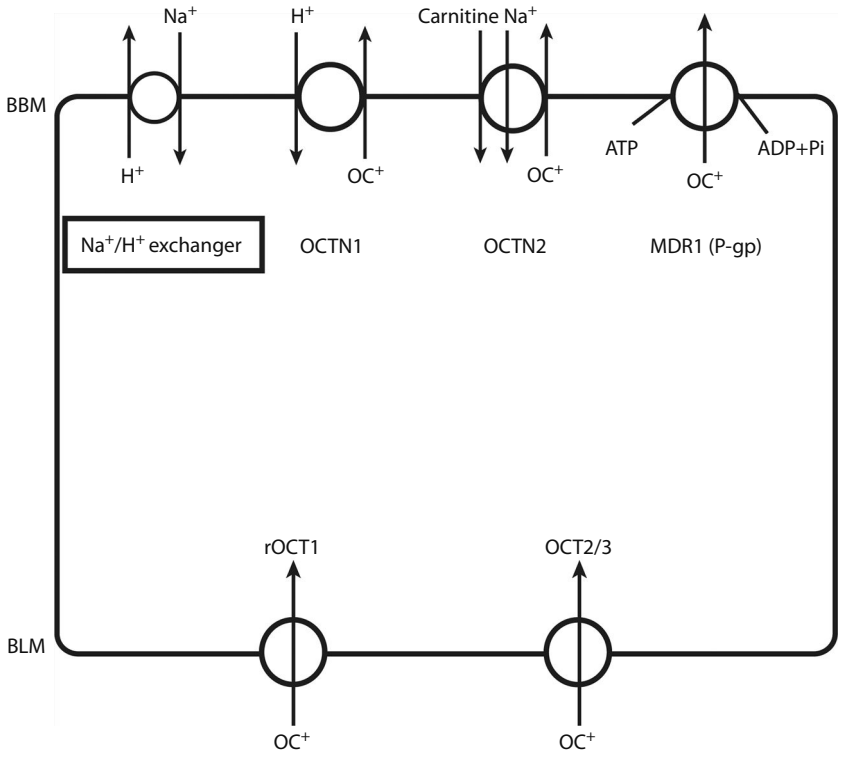

FIGURE 30.4 Scheme for organic cation (OC^+) transport in renal proximal tubule. BBM, brush-border membrane; BLM, basolateral membrane; MRP, multidrug resistance–associated protein; OCT, organic cation transporter. The "r" in front of some of the carriers indicates it has only been found in rats.

transporter family, OAT2, is expressed in basolateral membranes of human kidney proximal tubules; in contrast, Oat2 is expressed primarily in liver and not in kidney of rodent kidney proximal tubules. OAT2 functions as an OA^- uniporter. Finally, OA^- uptake across the basolateral membrane, particularly for Phase II conjugates of xenobiotics, can occur by the multidrug resistance proteins 5 and 6 (Mrp5/6–Abcc5/6 in rodents; MRP5/6–ABCC5/6 in humans), which are primary active transporters using the free energy of ATP hydrolysis to drive uptake of OA^-.

In some respects, less is known about the mechanisms of the exit step by which OA^- are transported across the luminal or brush-border membrane of renal proximal tubules into the tubular lumen (Figure 30.3). The major carriers involved in the efflux step include the organic anion transporting polypeptide-1a1 (Oatp1a1/OATP1A1; Slco1a1 in rats and mice, SLCO1A2 in humans), the rat-specific Oat-k1 and Oat-k2 (Slco1a3), and two MRP isoforms (MRP2/4–ABCC2/4). Oatp1a1/OATP1A1 catalyzes uptake of OA^- in exchange for the tripeptide GSH, which may be the principal carrier responsible for the delivery of intrarenal GSH to the active site of γ-glutamyltransferase (GGT) for its turnover.[28] A member of the SLC22 family, URAT1 (SLC22A12), is expressed exclusively in renal proximal tubules and is responsible for the uptake of urate in exchange for OA^-, thereby maintaining blood levels of uric acid.

Many important drugs are organic cations or weak bases and are secreted into the tubular lumen by an array of organic cation transporters (OCTs) (Figure 30.4). These carriers, which are mostly members of the SLC22 carrier family, include two or three carriers, depending on species, on the basolateral plasma membrane, and three carriers on the brush-border plasma membrane of the renal proximal tubule. On the basolateral membrane, Oct1/OCT1 (Slc22a1/SLC22A1) is only expressed in the kidney in rodents and is mainly expressed in the liver in humans. Oct1/OCT1 transports a variety of organic cations (OC^+), some weak bases, noncharged compounds, and some anions. Oct2/OCT2 and Oct3/OCT3 (SLC22A2/3) exhibit a similar, broad substrate specificity. All three carriers are electrogenic, Na^+-independent, and reversible with respect to direction. Driving force is solely based on the electrochemical gradient of the transported substrate.

On the luminal membrane, two SLC22A family carriers, OCTN1 (SLC22A4) and OCTN2 (SLC22A5), are present and catalyze efflux of organic cations into the tubular lumen. OCTN1 may function as either an OC^+ uniporter or a H^+/OC^+ exchanger, with the H^+ gradient generated by the Na^+/H^+ exchanger. OCTN2, in contrast, may function as either an OC^+ uniporter or a Na^+/carnitine cotransporter. Finally, the multidrug resistance P-glycoprotein (MDR1; P-gp; ABCB1) catalyzes efflux of OC^+ into the lumen and is driven by the hydrolysis of ATP.

In addition to tissue- and species-dependent differences in expression of these various transporters, there exist both sex-dependent differences in expression due to regulation

by estrogens and androgens[29–31] and individual differences based on genetic polymorphisms.[32] OA^- transport in rats is higher in males than in females,[30] and this difference in activity correlates with Oat1 and Oat3 protein expression. Wright and colleagues[29,31] made the interesting finding that while the androgen-stimulatory and estrogen-inhibitory patterns exist in rodents (i.e., rats and mice), no sex-dependent differences in transport activity or Oat expression were observed in rabbits or rabbit proximal tubules. These observations highlight two key points: First, not all animal models are appropriate to reflect sex-dependent differences in OA^- or OC^+ transport that might occur in human kidney; second, the sex-dependent differences indicate that males and females will exhibit pharmacokinetic differences in the handling of many drugs that are organic anions or cations, which could lead to a greater susceptibility to potential drug overdose toxicity due to greater accumulation of drug. Several plasma membrane transporters from human proximal tubular cells have been documented to exhibit genetic polymorphisms,[32] which also indicates that some individuals will exhibit pharmacokinetic differences in drug handling, leading to altered accumulation and differences in susceptibility to potential drug overdose toxicity.

Drug Metabolism

Another key component of the intoxication process for most chemicals involves metabolism, as most chemicals are not toxic in their native form but must be metabolized to exert their effects. Thus, for polar or hydrophilic chemicals, once they are transported into the renal cell by one of the various carriers discussed earlier, they can then be bioactivated by a large array of Phase I and/or Phase II enzymes to yield potentially reactive metabolites. The traditional view of renal function is that the kidneys act as filters to remove toxic waste products from the blood via glomerular filtration or tubular secretion. Whereas the focus of most drug metabolism studies has been the liver, it became apparent that the kidneys have a significant capacity to carry out extensive oxidation, reduction, hydrolysis, and conjugation reactions.[33,34] Enzyme systems similar to those present in the liver and other extrarenal tissues are involved in renal drug metabolism. An important difference between the kidneys and other tissues is that many of the drug metabolism enzyme systems, such as CYPs, glutathione *S*-transferases (GSTs), and others, are differentially distributed among the different nephron cell populations.[4,5,35] Thus, for example, whereas CYPs are exclusively found in the cortex (primarily the proximal tubules), prostaglandin synthetase, which catalyzes co-oxidation of several drugs and xenobiotics, is localized in the medulla.

Knowledge of the nephron cell type localization of drug metabolism enzymes and drug accumulation patterns are critical to understanding nephrotoxicity. A clinically important example of this is for acetaminophen.[34] Whereas the acute nephrotoxicity of acetaminophen is characterized by proximal tubular necrosis and is associated with metabolism by CYP, chronic nephrotoxicity of acetaminophen is characterized by papillary necrosis and interstitial fibrosis and is dependent on oxidation by prostaglandin synthetase in the inner medulla. This difference is largely due to the accumulation of acetaminophen in the cortex under acute exposure conditions and in the inner medulla under chronic exposure conditions. The differential distribution of acetaminophen thereby provides access to different drug metabolism enzymes.

Nonpolar or hydrophobic chemicals may also be bioactivated within the kidneys. Because of their chemical nature, these chemicals generally enter renal cells by passive diffusion rather than by specific membrane carrier proteins. Two related examples of this process are for the halogenated solvents trichloroethylene[36] and perchloroethylene.[37] Each of these related chemicals may be metabolized by either CYPs or GSTs, although trichloroethylene is a far better substrate than perchloroethylene for CYPs. Both chemicals, besides sharing metabolism by multiple enzymatic pathways, also share having multiple target organs. For the renal-specific, toxic effects of tri- and perchloroethylene, metabolism by the GSTs is the relevant pathway. Both chemicals are converted, through a series of metabolic steps, involving both intracellular and plasma membrane-bound enzymes, to the corresponding cysteine *S*-conjugates. These metabolites are then substrates for either the cysteine conjugate β-lyase or the flavin-containing monooxygenase, which convert them to chemically reactive species that are directly associated with alkylation of DNA and protein, oxidative stress, and various signal transduction mechanisms that lead to altered cellular function.[36–38]

In the study of drug metabolism, human tissue is often unavailable and studies are conducted in animal models, most typically using rodents. One then extrapolates the animal data to humans by using physiological parameters of the animal model and humans to account for differences. Further, if information about relevant genetic polymorphisms in drug-metabolizing enzymes or preexisting diseases or health conditions is not available, uncertainty factors are included in such human health risk assessments based on the animal model data. For the kidneys, significant species differences exist between rodents and humans such that drug metabolism data from the former cannot be readily extrapolated to the latter. Examination of renal activities of CYPs provides a clear illustration of this. For example, whereas rodent kidneys, particularly in the proximal tubular segments of the nephron, contain a large array of CYP enzymes that is very similar to that found in the liver,[33,34,39,40] human kidney expresses a much smaller number of CYP enzymes.[41–43] Additionally, the pattern of inducibility of both CYPs and Phase II enzymes such as GSTs or UDP-glucuronosyltransferases in the kidney differs between rodents and humans.[44,45] Enzyme induction is the process by which prior or concurrent exposure to certain chemicals causes the increased expression of specific enzymes. For example, whereas CYP1A1/2, that metabolizes polycyclic aromatic hydrocarbons is expressed at low constitutive levels in rodent kidney, CYP1A1 is highly inducible; in contrast, this CYP enzyme is both not detectable and poorly inducible in human kidney. Another example is CYP4A2/3

in rodent kidney, that is found in the proximal tubules and is inducible by fibrates; the human ortholog CYP4A11 is also expressed in renal proximal tubules but is inducible by ethanol and dexamethasone. The toxicological implication of this is similar to that of genetic polymorphisms in humans, namely that different capacities to metabolize drugs will produce characteristic responses.

Regulation of Renal Function

Glomerular filtration is a primary function of the kidneys and is typically measured by either direct or indirect methods to gauge kidney function. GFR is primarily regulated by three factors: (1) the balance of pressures acting across the capillary wall (glomerular capillary hydraulic pressure and Bowman space oncotic pressure tend to favor filtration whereas glomerular capillary oncotic pressure and Bowman's space hydraulic pressure tend to retard filtration); (2) the rate of plasma flow through the glomeruli; and (3) the permeability and total surface area of the filtering capillaries. Each of these factors is, in turn, regulated by several physiologic factors, including local and systemic hormones. GFR is also regulated by a process called tubuloglomerular feedback, which involves transmission of a stimulus at the macula densa to the arterioles of the same nephron, based on the composition of the tubular fluid flowing past the macula densa. Tubuloglomerular feedback causes glomerular vasoconstriction when Na^+ delivery to the macula densa increases and glomerular vasodilatation when Na^+ delivery is decreased. The extent of the feedback response is also modulated by extracellular fluid volume. Thus, because Na^+ ions are the major solute available for renal excretion and Na^+ ions are the major cationic component of the extracellular fluid, changes in renal Na^+ excretion provide a primary driving force for these regulatory responses.

Renal effector mechanisms that modulate body fluid volume homeostasis include GFR, several peritubular and luminal factors (e.g., peritubular capillary Starling forces, luminal composition, medullary interstitial composition, and transtubular ion gradients), humoral mechanisms (e.g., the renin–angiotensin–aldosterone system, vasopressin, prostaglandins, atrial peptides, and endothelium-derived factors), and renal nerves. The reader is referred to textbooks of renal physiology[2] for more detailed information on these regulatory mechanisms.

Besides control of ionic composition and extracellular fluid volume, the kidneys play a central role in the handling of acids and bases. pH is controlled largely by the HCO_3^-/pCO_2 ratio. While the lungs regulate pCO_2, the kidneys control HCO_3^- concentration. Considering a plasma HCO_3^- concentration of 24 mEq/L and the normally high GFR, the kidneys typically must reabsorb 5000 mEq HCO_3^-/day. This occurs by two processes, the carbonic anhydrase reaction, which is found in most cells but is highest on the brush-border membranes and cytoplasm of proximal tubular cells, and transport across the luminal and basolateral plasma membranes. More than 95% of filtered HCO_3^- is reabsorbed in the proximal tubules, with the remainder being reabsorbed in the loop of Henle. By the time the filtrate reaches the distal tubule or collecting duct, essentially all the HCO_3^- has been reabsorbed.

The pH in plasma and extracellular fluids is thus determined by the following equation:

$$pH = 6.1 + \log\left\{\frac{[HCO_3^-]}{0.03\,pCO_2}\right\}$$

where

$[HCO_3^-]$ is regulated by the kidneys

pCO_2 is determined by the lungs

At the physiological extracellular pH of 7.40, the ratio $[HCO_3^-]/0.03\ pCO_2 = 20$. Normal arterial plasma concentration of HCO_3^- is 24 mM and normal arterial pCO_2 is 40 mmHg. Any primary change in either parameter causes the kidneys and lungs to respond to reattain the normal ratio.

Acid–base balance is also maintained by secretion of ammonia derived from amino acid catabolism. This occurs primarily in the distal nephron, where ammonia is secreted into the lumen and protonated. The protonated form of ammonia, NH_4^+, is impermeable to the luminal membrane and becomes trapped in the lumen and is excreted. During metabolic acidosis, for example, renal ammonia production is increased by induction of amino acid deamination reactions, thereby increasing excretion of H^+ ions. A summary scheme of mechanisms by which kidneys maintain acid–base balance is shown in Figure 30.5. Factors that regulate or influence renal acidification and alkalinization are summarized in Table 30.3. These factors include renal hemodynamics, filtered load of ions, systemic acid–base conditions, and hormones, among others.

As discussed earlier, organic anion and cation transport is an important step in the regulation of the volume, electrolyte, and acid–base homeostatic functions mediated by the kidneys, which influences susceptibility to toxicants. Modulation of organic anion secretion, which in turn influences Na^+ and fluid balance, is under hormonal control, with the subsequent receptor-mediated responses being signaled by protein kinases or other second messengers.[18,27,46] Protein kinase C activation reduces OA^- uptake mediated by OAT1 and OAT3, reduces OA^- efflux by MRP2, and reduces OA^- transport by OATP1A1. There are several potential biochemical and molecular mechanisms involved in this downregulation. For OAT1, for example, regulation has been shown to occur by the modulation of: (1) gene transcription, (2) mRNA stability, (3) mRNA translation, (4) phosphorylation of the carrier protein, (5) internalization of membrane transporters, (6) recruitment of preformed transporters into the membrane, or (7) by allosteric control by regulatory proteins. Additionally, the function of OA^- transport may be indirectly modulated by regulation of $(Na^+ + K^+)$-stimulated ATPase activity.

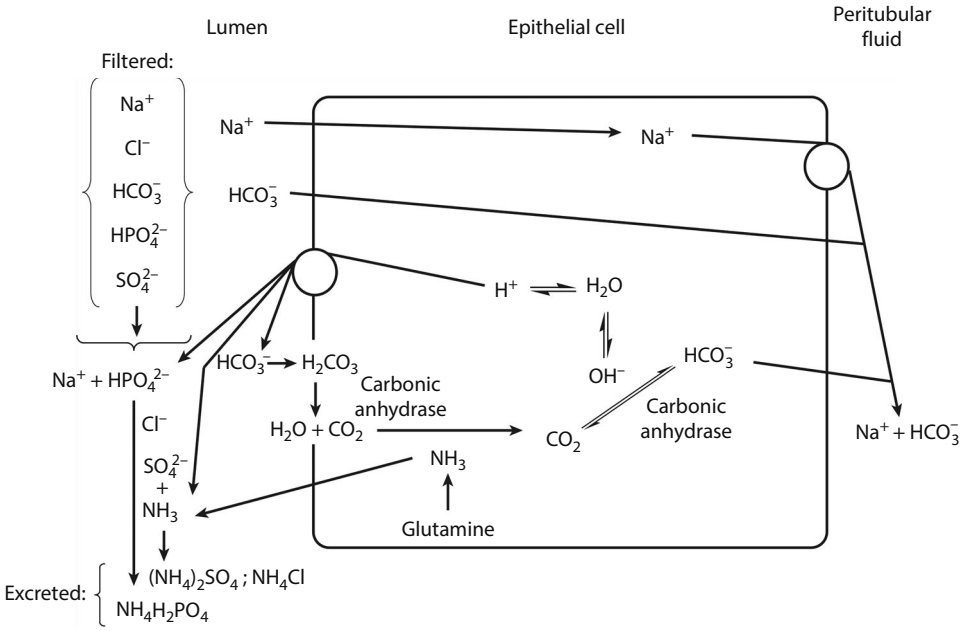

FIGURE 30.5 Summary scheme of how renal epithelium regulates acid–base balance.

TABLE 30.3
Factors Influencing or Regulating Renal Acid–Base Balance

Proximal Nephron	Distal Nephron
Filtered load: GFR, ultrafiltrate [HCO_3^-]	Aldosterone
Peritubular [HCO_3^-] and pH	Luminal buffering capacity
pCO_2	Systemic pH, pCO_2
Angiotensinaz II	Na^+ delivery and transport (cortical collecting tubule)
Extracellular volume	K^+ homeostasis
NaCl reabsorption	Anion transport
K^+ homeostasis	Parathyroid hormone, Ca^{2+} homeostasis
Parathyroid hormone, Ca^{2+} homeostasis	
Adrenergic nerve activity	

Besides protein kinase C, other protein kinases have been either shown or suggested to possibly regulate OA^- transport, including protein kinase A (inhibition or no effect), mitogen-activated protein kinase/extracellular regulated kinase (MEK; inhibition), tyrosine kinase (inhibition or no effect), phosphatidylinositol 3-kinase (PI3K; inhibition), and calcium/calmodulin-dependent protein kinase II (inhibition). Hormones or hormone-like compounds acting through these various kinases and other second messengers such as cAMP, either inhibit (e.g., phenylephrine, dopamine, parathyroid hormone, bradykinin, endothelin, estradiol) or stimulate (e.g., catecholamines, epidermal growth factor, thyroid hormone, testosterone, dexamethasone) OA^- transport and/or secretion. As discussed earlier, OA^- secretion is faster in males than in females, illustrating the influence of estrogen/androgen balance.

EXPERIMENTAL MODELS FOR ASSESSING RENAL FUNCTION AND TOXICITY

General Considerations for Choosing a Model

A large variety of experimental models can be used to assess renal function and toxicity. Each model has its advantages and limitations. The choice of experimental model is often dictated by the questions being asked and the extent of knowledge of the process under investigation. Factors to be taken into consideration include the potential involvement of extrarenal processes and hormones in the renal response, knowledge of the nephron cell type localization of any effects being studied, and the molecular mechanisms mediating the renal response. Some models are limited by an inability to distinguish nephron cell type localization or by the lack of functional expression of certain biochemical processes. The various experimental models that can be used to study renal physiology and toxicological responses are summarized in Table 30.4, and each is discussed in more detail in the following text. The reader is also referred to two reviews on this subject.[47,48]

To gain a full understanding of how a chemical may cause nephrotoxicity, more than one experimental model is usually necessary, both to enable different types of questions to be addressed and to validate certain approaches (e.g., in vivo model to validate an in vitro model). An example of a situation in which the use of only one approach and experimental model could lead to incorrect conclusions can be found in studies on the cytotoxicity of inorganic mercury salts.[49,50] In these studies, Lash and colleagues found that inorganic mercury was a more potent cytotoxicant in suspensions of freshly isolated rat renal distal tubular cells than in rat renal proximal tubular cells. In vivo, however, the proximal tubule

TABLE 30.4
Summary of Selected Experimental Models Used to Study Renal Physiology and Toxicity

Model System	Uses/Advantages	Limitations
Clinical measurements and whole animals	Assess integrated physiological responses and regulatory mechanisms Measure noninvasive markers of renal function Applicable to humans	Minimal control of experimental exposure conditions Limited ability to distinguish nephron cell type involved Limited ability to distinguish biochemical/molecular mechanisms
Isolated perfused kidney	Intact tissue structure Distinguish intrarenal from extrarenal effects	Short-term viability (≤2 h) Interanimal variability Incomplete definition of conditions Expensive
Micropuncture and microperfusion	Determine tubular site of action Quantitate transport processes in single nephrons	Sophisticated technology Subject to technical errors and misinterpretation of results
Renal slices	Intact tissue structure Ease of preparation Drug screening Measurement of drug metabolism	Short-term viability (≤2 h) Often limited access to luminal membrane Poor oxygenation Multiple nephron cell types present
Isolated perfused tubules	Determine tubular site of action Quantitate transport processes in specific nephron segments	Short-term viability (≤2 h) Sophisticated technology
Isolated tubules/tubular fragments	Intact tubular structure Precise definition of conditions Ease of preparation Several test samples with paired controls	Short-term viability (≤4 h) Often limited access to luminal membrane Some mixture of cell types
Freshly isolated renal cells	Bidirectional exposure Ease of preparation Several test samples with paired controls Cells from specific nephron segments Drug screening	Short-term viability (≤4 h) Loss of plasma membrane polarization
Primary renal cell cultures	Similar to in vivo kidney Longer-term viability Maintenance of polarity	Difficult to maintain Dedifferentiation Limited lifetime relative to cell lines
Renal cell culture lines	Precise definition of incubation conditions Immortalized Reproducible Easy to subculture Transfect with cDNAs	Dedifferentiation Often of ill-defined origin

is the primary nephron segment target for inorganic mercury and little cellular injury is seen in the distal nephron, except at very high doses.[51] The primary reason for the in vivo nephron-segment selectivity for mercury is that the proximal tubules are the first epithelial cells to be exposed to filtered or renal plasma mercury and possess sufficient membrane transporters and membrane and intracellular target molecules to bind and accumulate virtually all the mercury to which it is exposed. From the in vitro studies, therefore, one might erroneously conclude that it was the distal tubules and not the proximal tubules that were the primary target for mercury.

Whole Animal and Clinical Assessments: Markers of Renal Function and Toxicity in Blood and Urine

Studies of renal function in whole animals or in humans typically involve measurement of parameters in blood or urine.[52] These types of studies permit the collection of large amounts of data, but are often limited by the ability to define biochemical or molecular mechanisms of injury. In vivo studies are often necessary or are one necessary component to establish target organ specificity, involvement of extrarenal processes in nephrotoxicity, or when more invasive procedures are not feasible, such as with humans in the clinic. In experimental animals, however, in vivo studies have the advantage of allowing for the isolation of tissue for morphological analysis following chemical exposure or drug administration.

Urinalysis

Quantitative urine collection is used to more precisely determine treatment-related changes in renal excretory function and to define renal clearances (see next section). To enable quantitative and complete urine collection in animals, they are often housed in metabolism cages. A metabolism cage consists of an animal chamber mounted above an excrement collection system, which usually consists of a funnel and a

TABLE 30.5
Parameters Often Determined in a Typical Urinalysis

Parameter	Purpose or Use of Measurement
Urine osmolarity or specific gravity	Overall fluid homeostasis
Urinary pH	Acid–base balance
Urinary volume	Overall fluid homeostasis
Urinary electrolyte and solute concentrations: Na^+, K^+, Cl^-, urea	Extracellular fluid balance
Creatinine excretion	Indicator of glomerular filtration
Glucose excretion	Proximal tubule function
Aminoaciduria	Proximal tubule function
Proteinuria:	
Low-molecular-weight proteinuria (β_2-microglobulin, α_1-microglobin, retinol-binding protein), Kim-1	Proximal tubule function
Albuminuria	Glomerular damage
Enzymuria:	Specific enzymes derive from specific nephron segments
GSH *S*-transferase-alpha (GSTα/GSTA; proximal tubule)	
GSH *S*-transferase-pi (GSTπ/GSTP; distal tubule)	
N-Acetyl-β-glucosaminidase (NAG; papillary collecting duct, proximal tubule > distal tubule)	
γ-Glutamyltransferase (GGT; proximal tubule)	
Tamm-Horsfall glycoprotein (thick ascending limb)	
Lactate dehydrogenase (LDH; distal tubule > proximal tubule)	

urine/feces separator. Both urine and feces are collected for metabolism and metabolic balance studies. For assessment of renal function, urinalysis is performed. Major parameters determined in typical urinalysis are listed in Table 30.5.

The various parameters provide indications of the function of the kidneys in maintenance of fluid, pH, electrolyte, and solute balance. Additionally, the appearance of specific components in the urine at concentrations outside of the physiological range can provide information about the function of specific nephron segments. This application of urinalysis is particularly applicable in the clinical setting, where the search for biomarkers that can be early and sensitive indicators of exposure to toxic chemicals is a significant emphasis in research and was the subject of a National Research Council/National Academy of Sciences publication back in 1995.[1] Since that time, extensive work has been conducted to identify novel, more renal-specific, and sensitive biomarkers that can provide early indications of exposure to nephrotoxic chemicals prior to the onset of irreversible injury. Biomarkers can be divided into three categories: biomarkers of susceptibility, biomarkers of exposure, and biomarkers of effect. A marker of susceptibility is an indicator of an organism's inherent or acquired sensitivity to toxicity from exposure to a specific xenobiotic. Some general factors that serve as markers of susceptibility include age, gender, and environmental exposures. Genetic polymorphisms may also render an individual more susceptible to the nephrotoxic effects of a given chemical. In terms of a urinary biomarker, for example, one may administer a test chemical that is metabolized by a specific enzyme that is known to exhibit genetic polymorphisms and then quantify metabolite levels in urine. Because determinants of susceptibility are multifactorial, validation of a marker of susceptibility must be carefully performed.

A marker of exposure is a chemical or a metabolite of the chemical or a product of an interaction between the chemical and some target molecule. One typically measures markers of exposure as concentrations of chemicals or its metabolites in blood, urine, or other body fluid or tissue. Difficulties in the use of such markers include that many drugs and other chemicals have the same or similar metabolites, making identification of the causative agent uncertain. Exposures to large doses of toxic chemicals may alter their rate of metabolism and/or excretion, thereby making a correlation between measured levels of the chemical or metabolite and the actual exposure difficult to obtain. Moreover, without taking susceptibility into account, a marker of exposure in one individual may have markedly different functional implications than that in another individual.

As suggested by its name, a marker of effect should indicate the influence of a chemical exposure on some biological process. Markers of adverse effect can be indicators of altered biochemical processes in a target organ or cellular signals of tissue toxicity. The effects that occur may only be indirect in that they may only indicate a potential for toxicity but by themselves cannot be construed to directly produce toxicity. Examples of this type of marker are protein or DNA adducts. As discussed earlier in the section on whole animal and clinical assessments, parameters such as blood urea nitrogen (BUN) and serum creatinine (SCr) and urinary proteinuria can be indicators of renal injury. Their disadvantages, however, include a lack of sensitivity and an inability to indicate information about site or mechanism of action. As noted earlier, BUN and SCr do not become significantly elevated until renal toxicity occurs to a significant extent. Hence, an ideal biomarker of effect would be one that is highly sensitive, is measurable prior to a significant degree of renal toxicity has occurred, persists throughout the time course of the alterations in renal function, is specific to the kidneys, correlates in amount with the degree of injury, and is specific to a nephron cell type or specifically indicates a mechanism of action. Ideal markers should also be stable in urine over a long enough period of time and at a wide enough range of urinary pH values, and if they are enzymes, they should not be readily inactivated in the urine. Some of the more sensitive and specific markers are shown in the bottom row of Table 30.5; examples include the urinary secretion of enzymes that are derived from various nephron segments.

The ultimate goal of using biomarkers of effect for nephrotoxicity is to have an easily measured, sensitive, and stable indicator of renal injury at its very early stages so that measures to counter the nephrotoxicity can be instituted prior to a significant decrease in renal function or to the advent of irreversible renal cell death.

The traditional markers of renal function, BUN and SCr, although considered the gold standards for assessment of kidney injury, are not particularly sensitive.[53–56] Rather, a number of other urinary biomarkers have recently been validated in both humans and experimental animals with regard to both sensitivity and specificity, including kidney injury molecule-1 (Kim-1), cystatin C, clusterin, and lipocalin-2.[55,57]

Clearance Methods

Methods to assess renal clearance are fundamental to whole animal studies and have wide clinical applicability as well. These procedures involve the measurement of blood or plasma (P_x in mg/mL) and urine (U_x in mg/mL) concentrations of a substance. Urine flow rate (V in mL/min) is also taken into account. Depending on the test substance used, either glomerular or tubular function can be assessed. Combining these parameters, gives the classical clearance equation:

$$C_x = \frac{U_x V}{P_x}$$

The clearance, C, represents the quantity of blood or plasma cleared of the substance per unit time and has units of volume per unit time (usually mL/t).

The most common substances monitored for determination of renal clearance are inulin or creatinine. Inulin is the preferred indicator of GFR, as it is a fructose polysaccharide (M_r = 5200 Da) that is freely filtered and then is neither reabsorbed nor secreted. Hence, all of the inulin that appears in the urine arises from plasma via glomerular filtration. Inulin is considered the *gold standard* of exogenously administered markers for GFR. Its scarcity and high cost, however, have made its use less favored in the clinical setting. Inulin is readily measured in plasma or urine by a colorimetric assay, although glucose is also measured; separate measurement of glucose is needed for correction to obtain the inulin concentration.

Creatinine is also used to monitor GFR, and has the advantage of not requiring infusion of an exogenous substance. This makes creatinine useful for monitoring of GFR over a protracted period of time in the same individual. Because creatinine undergoes some reabsorption, however, measurement of its clearance tends to underestimate that of inulin by 10% or more, depending on species. Creatinine (M_r = 113) is a metabolic product of creatine and phosphocreatine, which are both found almost exclusively in muscle. Creatinine production is proportional to muscle mass and varies little from day to day, although it can be affected by diet. Thus, because of its convenience and low cost, creatinine is the most widely used indirect measure of GFR. Correct interpretation of data in a clinical setting, however, can be problematic.

One can also measure renal plasma flow if a test substance is used that undergoes both filtration and active tubular secretion. *p*-Aminohippurate (PAH) is often used for that purpose because it is freely filtered by glomeruli and undergoes active secretion by several OATs. PAH extraction by the kidneys can vary from about 70% to 90%, depending on species, due to heterogeneity of the distribution of OATs and blood flow, thereby causing incomplete extraction. Renal blood flow is converted from RPF by dividing RPF by the plasma fraction of whole blood, as estimated from the hematocrit (Hct), according to:

$$RBF = \frac{RPF}{(1 - Hct)}$$

Clearance ratio relative to a marker substance (usually inulin) can be calculated and used to determine whether a test substance is reabsorbed or secreted. Thus, if a substance has a C value less than that of inulin, this indicates reabsorption. Glucose is a good example of a highly reabsorbed substance whose C value is always less than that of inulin. C_{PAH}, in contrast, is usually greater than C_{inulin}, indicating secretion.

Another method for measurement of GFR is to determine plasma clearance of an intravenous bolus injection of an indicator radionuclide-labeled marker. Renal clearance is measured as the plasma clearance, or the amount of indicator injected divided by the integrated area under the plasma concentration curve; models to estimate plasma clearance assume that the volume of distribution and renal excretion are constant over time. Two indicators that have been used and validated are ^{125}I-iothalamate and ^{51}Cr-labeled ethylenediaminetetraacetic acid (EDTA). The basic procedure involves injection of radiolabeled marker into arterial blood and sampling of venous blood. After correcting for noninstantaneous equilibration between arterial and venous circulations, plasma levels are measured and clearance is calculated from the slope and intercept of a line plotted on a logarithmic scale, using the following formula:

$$C = V_o \frac{(\ln(2))}{t_{1/2}}$$

where

V_o is the volume of distribution

$t_{1/2}$ is the half-time for decay of marker levels in plasma

An additional parameter to indicate GFR that is popularly used in whole animal toxicity screening studies is measurement of BUN. As filtration slows, BUN rises and is generally paralleled by levels of SCr. Thus, in the absence of changes in protein intake or metabolism, BUN or SCr are useful indicators of GFR. An important point to realize is that although BUN may be the most commonly used measurement for

glomerular filtration, substantial loss of renal function (on the order of at least 50%) must occur before BUN rises above normal levels. BUN, like SCr, as discussed earlier, is thus not a very sensitive indicator of renal injury and thus does not have much preventive value.

Experimental Models

Isolated Perfused Kidney

As compared with in vivo studies in whole animals, the isolated perfused kidney shares the advantage of being a model that maintains intact renal structure. It also possesses the advantage of enabling separation of extrarenal from intrarenal factors that may be part of the mechanism of action. The methods used in animal preparation and kidney isolation and perfusion have been described in several reviews[58,59] and will only be highlighted briefly here.

Being at the highest level of structural organization for in vitro kidney models, it is not surprising that the isolated perfused kidney preparation has been in use for nearly 150 years. The basic isolated perfused kidney preparation consists of the whole kidney isolated from the systemic vasculature and usually removed from the animal, and perfused through the renal artery with a synthetic plasma- or blood-like medium. Oxygenated perfusate is delivered into the renal vasculature with an adjustable pump so that a constant, mean arterial pressure can be achieved and maintained during the course of experiments. Urine is collected through a cannula placed in the ureter. Several variations of the basic method have been implemented, depending on the purpose of the experiment. For example, various in-line monitoring devices have been included to measure perfusate temperature, pH, and oxygen content. The perfusate leaving the renal vein can either be recirculated or collected in a reservoir to achieve single-pass perfusion. The single-pass method has the advantage of ensuring a constant arterial perfusate composition, as it is not modified by renal metabolism or excretion, but has the disadvantage of being expensive because it consumes large amounts of perfusate, particularly if the perfusate contains physiologic colloids such as albumin. Although isolated perfused kidney preparations have been described for several species, including the dog, rabbit, guinea pig, pig, and toad, the most common species used in recent years has been the rat.

As summarized in Table 30.4, advantages of the isolated perfused kidney preparation are that it retains kidney structure, so is particularly well suited for studies requiring intact renal morphology. It is the only in vitro preparation in which glomerular filtration and tubular functions are both retained in manners similar to the in vivo kidney. Control over many of the aspects of the perfusate composition is possible, including concentrations of dissolved and gaseous metabolites and electrolytes, colloids, oxygen carriers, drugs and their metabolites, and other chemicals added to modify function. This type of control enables establishment of reproducible exposures to chemicals or drugs under investigation. Also, because the isolated tissue is being used, higher concentrations of test chemicals are possible to achieve than could be used or tolerated in vivo.

Some of the limitations with the isolated perfused kidney model are those that are inherent with any in vitro model. For example, once the tissue is removed from the animal, function (e.g., GFR and tubular function) decline progressively over the course of experimentation. Hence, the model can only be useful for a limited period of time, which is generally on the order of 2 h. Because of this time limitation, studies are naturally restricted to acute effects as responses that occur over longer periods of time, such as changes in gene expression or enzyme induction, require longer exposure and/or study times. As explained by Diamond,[59] the isolated perfused kidney exhibits several functional abnormalities that must be considered when evaluating results. These abnormalities include relatively high perfusion rates and low filtration fractions as compared with the in vivo kidney, loss of urinary concentrating ability, and glomerular proteinuria. Various structural abnormalities have also been observed in tubules of isolated perfused kidneys, including cellular necrosis of the medullary thick ascending limb and the S3 segments of the proximal tubule. The latter is particularly significant for drug metabolism and nephrotoxicity studies because the S3 segment of the proximal tubule is the site of a large proportion of the metabolism and toxic effects of exogenous compounds. Finally, other limitations exist in the ability of studies with the isolated perfused kidney to discriminate mechanisms of action and localization of processes in specific nephron cell types.

Micropuncture and Microperfusion Techniques

In vivo microperfusion and micropuncture are two techniques that are used to study the function of individual nephron segments in the intact kidney.[60] For example, these methods can be used to directly determine the tubular handling of drugs and nephrotoxicants, characterize the function of specific carrier proteins, or determine the effect of toxicant administration on tubular function. A particular application of micropuncture is to determine the in vivo concentration and profile of luminal fluid along the nephron. Microperfusion, on the other hand, is a valuable approach because a drug or other chemical of interest can be applied directly to the tubule in the luminal perfusate and the effect on tubular function determined.

Although micropuncture and microperfusion are performed in several mammalian species, the rat is the most common experimental animal that is used. Moreover, specific strains of rats are often used in preference to others for particular purposes; for example, the Munich–Wistar rat is often used in studies of glomerular function because glomeruli are visible and accessible at the surface of the kidneys. Other strains, such as the spontaneously hypertensive or Brattleboro rat, are used to study nephron function under specific pathophysiologic conditions. Rats of 200–250 g body weight are usually used for micropuncture of superficial cortical nephron segments whereas young rats weighing less

than 150 g are used for micropuncture of collecting ducts, loops of Henle, or vasa recta because they are more readily accessible at this stage of development. Micropuncture or microperfusion is most often performed on the left kidney because of its accessibility and longer vascular pedicle as compared with the right kidney.

Once the kidney is isolated and placed in a holder, superficial nephron segments can be visualized with a stereomicroscope. The majority of the tubules visible on the surface are proximal convoluted (S1) tubules. As noted earlier, superficial glomeruli are visible in kidneys of strains such as the Munich–Wistar rat. Peritubular capillaries are visible as thin red lines between and around the tubules. A few distal tubular segments are also visible on the surface of the rat kidney and can be distinguished from proximal tubules by their thinner epithelial walls and smaller lumina. Typically, approximately 60% of the length of proximal tubules from dog or rat is accessible to micropuncture. Besides visual identification, proximal and distal tubules can also be distinguished by use of dyes such as lissamine green. After intravenous infusion of a small bolus of the dye, a distinct time course for appearance in the tubules is used to enable the identification of specific tubular segments. Because of natriuretic effects and an increase in single nephron GFR caused by lissamine green, micropuncture or microperfusion experiments should begin only after the dye has completely disappeared from the kidney and the effects of the dye on renal function have reverted to normal, which typically takes approximately 30 min.

Micropuncture has been instrumental in demonstrating tubular reabsorption and secretion of a large variety of endogenous and exogenous chemicals. It has the advantage that the tubule is studied in the intact, functioning kidney, and can be studied as a physiologically stable preparation for several hours. One is limited, however, to using certain species. Because the method requires surgery, anesthesia is required, which necessitates consideration of potential effects of anesthesia on tubule function. This concern can be taken into account by the use of paired control and experimental conditions in the same anesthetized animal. Another concern is that because of internephron heterogeneity, sampling from a single nephron may not be representative of all nephrons. Microperfusion is useful in determining and discriminating between luminal and peritubular actions of a drug or nephrotoxicant in the in vivo kidney, with the chemical being added specifically to either the tubular perfusion fluid or the systemic circulation to study luminal or peritubular action, respectively. Another advantage of microperfusion is the ability to distinguish net reabsorption from secretion, although other models can accomplish that as well (see following text). Both micropuncture and microperfusion involve surgical procedures and careful placement of micropipets. Thus, significant skill is required to conduct such microprocedures.

Renal Slices

Renal slices have been a useful tool for many years to study renal physiology and biochemistry. Organic anion transport, for example, has been extensively studied with this technique. Most studies using renal slices have used slices from the cortex, in part because of the predominance of the proximal tubules in renal transport and toxic responses, and because of its ready accessibility. The simplest method to prepare renal slices is freehand, using a razor blade and a glass microscope slide as a slicing guide.[61] Slices need to be uniform in thickness and be taken only from the section of kidney desired. To address the issue of uniformity of slice thickness and the attainment of slices that are as thin as possible, several automated tissue slicers have been developed. The first slicer developed to prepare the so-called *precision-cut tissue slices* was the Krumdieck slicer.[62] The slicer is filled with an oxygenated, physiological buffer solution to preserve function and operates on the principle that a core of tissue is fed into an oscillating blade that is drawn across the core to make a slice. The slice is then swept away into a collection chamber. Slice thickness is adjustable, with optimum thickness considered to be around 250 μm.[63] The diameter of the core is preset and typically ranges from 4 to 8 mm. The Brendel–Vitron slicer was developed more recently[64] and differs from the Krumdieck slicer in having a simpler design that is possibly more rugged and *user friendly*.[63]

Once prepared, slices are typically incubated in a Dubnoff metabolic shaking bath, that permits control over temperature, shaking rate, and gas environment. Many assays are carried out in a bicarbonate-based buffer system, such as Krebs–Ringer, thus necessitating an atmosphere containing CO_2 (typically 95% O_2, 5% CO_2). There is some concern over oxygenation of the tissue slice and nephron heterogeneity. Improvement in procedures, however, has made poor tissue slice oxygenation less of a problem. Incubations are typically done as for short-term cell cultures, using a serum free, 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12. Medium is gassed with 95% O_2, 5% CO_2, and pH is adjusted to 7.4. Other media, such as Waymouth's culture medium or a Krebs-HEPES buffer, have also been used.

A variety of assays of renal function and toxicity have been used with renal slices. In most cases, parameters are normalized to slice weight rather than other parameters, such as cell number or protein, as this is the most convenient and appropriate factor to use. If the tissue slice becomes edematous or dehydrated, then tissue weight will be an inappropriate normalizing factor. Inasmuch as loss of protein may occur in nephrotoxicity, slice DNA content is recommended to use to normalize assays.[63] Tissue slice K^+ content is a good indicator of cell viability, as it reflects (Na^++K^+)-stimulated ATPase activity and membrane integrity. Losses in K^+ content are indicators of toxicity. As with other in vitro models, intracellular enzyme leakage (e.g., lactic dehydrogenase) is used as an indicator of membrane disruption and necrosis. Both cellular ATP content and respiration (O_2 consumption) are useful to assess energetic status of the tissue slice. Protein synthesis activity, as measured by incorporation of ^{3}H–L-leucine, is also used to assess functional competence. Because active transport of organic anions and cations is a

primary, energy-dependent function of proximal tubular cells, measurements of medium:slice ratios of transported substrates are often used as a measure of tubular viability.

Advantages of the renal slice technique include its versatility, correspondence with in vivo tissue, the general ease of preparation, its relative low cost, and the ease with which incubation conditions can be manipulated. Limitations to the use of renal slices exist, however, including the possibility of collapsed lumens, poor oxygenation, particularly if slices are too thick, the presence of some damaged tubules, which is more problematic if slices are too thin, and the presence of multiple nephron cell types, thus making it difficult to ascribe observed effects to specific cell populations.

Isolated Perfused Tubules

The techniques of isolating and perfusing individual nephron segments in vitro have been widely used in transport physiology studies since their development in the 1960s. Although nephron segments can be and have been obtained from many species, tubules are the easiest to dissect from the rabbit kidney without the use of digestive enzymes. Detailed procedures for the harvesting of renal tissue, dissection and identification of nephron segments, and the design of perfusion and collection pipettes are described by Zalups and Barfuss.[65] S1, S2, and S3 segments of the proximal tubule, cortical collecting ducts, and medullary and cortical ascending thick limbs of Henle's loop have all been isolated from rabbit kidney, and studied for their transport function and viability after exposure to various xenobiotics.

In most studies, perfusing and bathing solutions are simple electrolyte solutions supplemented with D-glucose and glutamine or glutamate, with pH adjusted to 7.4. When it is desirable to more closely mimic the in vivo state, isolated segments of the nephron can be perfused with an ultrafiltrate of rabbit plasma, made by passing the plasma through a 50 kDa cutoff filter. Thus, a great deal of control over the fluid environment can be achieved with this method. Another application of this model is to determine the transepithelial transport of solutes, including nephrotoxicants. Thus, depending on whether substrate is placed in the perfusate or bathing medium, lumen-to-bath or bath-to-lumen fluxes can be determined. Limitations in the application of this model for renal toxicology include the requirement for sophisticated and expensive equipment, the difficulty in preparing the nephron segments, the rather poor ability to isolate and perfuse nephron segments from species other than the rabbit, and a relatively limited life span for the model once nephron segments are isolated.

Isolated Tubular Fragments and Cells: Freshly Isolated and Primary Culture

Tubular segments derived from specific nephron cell populations can be isolated by microdissection. These preparations maintain their normal tubular architecture, function, and polarity. Because the isolation method is rather tedious and yield of tubules is typically low, other procedures, most notably treatment with collagenase, were developed. For example, perfusion of renal cortical tissue with buffers containing chelators such as EDTA and collagenase have been used to prepare tubule fragments from rats and rabbit. One problem was that these preparations comprised tubules from multiple nephron segments. Hence, procedures, such as density-gradient centrifugation in Percoll or Ficoll or electrophoresis were then applied to obtain enriched preparations of tubules from either proximal tubule or distal tubule.[66–72] Although less commonly used than proximal tubular suspensions as an experimental model, tubule fragments from the medullary thick ascending limb have also been used.[73–77]

In general, procedures for the isolation of proximal tubules involve either enzymatic digestion and separation or mechanical separation.[78] In the enzymatic methods, cortical tissue can be digested either by perfusion with a collagenase-containing, balanced salt buffer or medium or can be dissected, minced, and incubated with the collagenase-containing buffer. Either of these methods provides a high yield of highly enriched proximal tubules that are largely S1- and S2-derived. Tubules can then be used for either short-term studies of metabolism, transport, and toxicity, or can serve as seed material for primary culture.[79–82] Variations of culture and incubation conditions to increase oxygenation have also been used to improve the ability of rabbit proximal tubule primary cultures to maintain differentiated function.[83] Contamination with glomeruli can be minimized by including iron oxide in the initial perfusate, which collects in glomeruli and can be removed with magnets.

Individual epithelial cells, rather than tubule fragments, can also be isolated and used as either fresh suspensions for acute studies or as seed material for primary culture.[47] Isolation of single cells generally requires somewhat more vigorous enzymatic treatment than for nephron segments. As with methods to isolate tubules, once isolated, suspensions of cells require a secondary enrichment step to obtain highly enriched cells derived from a specific nephron segment. Hence, procedures such as Percoll density-gradient centrifugation[83,84] can be applied to enhance purity of a mixed cortical cell population (see following text).

The decision to use either tubule segments or single cells as a model depends on several factors. Firstly, the ease with which either tubule fragments or single cells can be prepared varies with species, so that the choice of model is partly dictated by the species of interest. As noted earlier, tubule fragments are readily isolated from rabbit kidneys. Although tubule fragments can also be isolated from rat, it is easiest to obtain single cells.[85] Isolation procedures have also been adapted to prepare suspensions of proximal tubular cells from human kidneys.[41,42,86] This is a significant advance because the use of cells derived from human kidneys eliminates the uncertainty involved in extrapolation of data from experimental animals to humans for risk assessment purposes. A second consideration is experimental design. One major difference between tubule fragments and isolated cells is that epithelial polarity is lost in the latter. Thus, all membrane surfaces have equal access to substrates in the

suspending medium. Additionally, collapse of the lumen in tubule fragments may occur, thus limiting access of substrate to the brush-border membrane.

Although single cells in suspension can be isolated from nephron cell types derived from multiple regions of the kidney, most studies have been performed with cells derived from the renal cortex and outer stripe of the outer medulla.[47,48,84,85] The basic procedure involves perfusion of the kidneys through the aorta below the renal arteries, first with a Ca^{2+}-free Hank's buffer containing EGTA and then with a Hank's buffer containing Ca^{2+} added back and collagenase. Because the inner medulla is poorly perfused, it can be removed intact with forceps, thus yielding a preparation that is predominantly of proximal tubular origin but also containing other cell types from the cortex and outer stripe of the outer medulla. Glomeruli, tubular fragments, and multicellular aggregates are removed by filtration through polypropylene mesh. The suspension of isolated renal cortical cells can then serve as a starting material for additional purification or enrichment steps to prepare multiple epithelial cell populations.[47,48,84]

Advantages of the single cell preparation include the high degree of homogeneity of the biological material, the equal access of all membrane surfaces to chemicals in the suspending medium (if membrane polarity is not important for the process being studied), that there are adequate amounts of material from a single animal to permit paired control and treated samples, and control of incubation conditions (e.g., temperature, pH, osmolarity, buffer and medium composition), thereby eliminating some of the uncertainty for mechanistic studies. Limitations of the procedure include potential damage to the cells during the isolation procedure, the limited life span of the cell suspensions (≤4 h), and the loss of membrane polarity (if membrane polarity is important for the process being studied). Although potential damage can occur just by the collagenase perfusion step, any damage is typically modest and the versatility of the procedure allows paired controls and treated samples to be used for incubation, thereby helping to ensure that observed responses are due to treatments and not to cell isolation artifacts. Moreover, cell viability is routinely checked upon completion of the cell isolation by both trypan blue exclusion and LDH leakage assays. In the first case, cells are mixed with trypan blue in saline and are viewed under a light microscope on a hemacytometer. Only those cells whose plasma membranes are damaged will take up the dye and stain blue; trypan blue is thus referred to as a vital dye. Cells are thus counted for the proportion of staining, which is typically 5% to at most 15% after isolation. Similarly, for the LDH leakage assay, cells are mixed with pyruvate and NADH; oxidation of NADH is measured by the decrease in absorbance at 340 nm and only those cells whose plasma membranes are damaged will either leak out LDH or allow NADH to enter and be oxidized. After recording the decrease in A_{340} for 2 min, the detergent Triton X-100 is added to fully solubilize the membranes, thereby providing complete access of the cellular LDH to the added NADH; A_{340} is recorded for another 2 min. Comparison of the slopes representing the rate of NADH oxidation (decrease in A_{340}) gives a measure of cellular viability. Typically, the slope of the two rates for control cells is between 0.05 and 0.15, indicating the presence of a high proportion of cells with intact cellular membranes and thus high viability. If measurements of trypan blue exclusion or LDH leakage fall outside of these typical ranges, the cells are not used for further studies.

As with the tubule fragments, specific nephron cell types in the cortical cell preparation can be enriched by a variety of methods. Although microdissection methods provide material of extremely high purity, the procedure is not particularly useful for most typical biochemical or toxicological studies, particularly those with cells from the rat or mouse, because of low yield of material. Additionally, such methods are fairly tedious and time consuming. The limited time frame for which the isolated cells are viable necessitates that they be used in studies as soon as possible after their isolation. Electrophoretic separation methods also suffer from the same limitations. Hence, any procedure used to obtain enriched populations of renal epithelial cells must satisfy four primary criteria[47]: (1) the procedure must be relatively rapid, (2) any material used in the separation process must be biologically inert, (3) final yield must be high enough to enable performance of a sufficient number of paired control and test incubations, and (4) purity must be high enough to enable unambiguous conclusions regarding the nephron cell type of origin.

Differences in cell density are one common means to separate different cell populations from one another. Although methods relying on cell density differences provide enrichment rather than absolute purification (because cell density is generally not a discrete property, but exhibits a range of values for a given cell population), many of the procedures readily satisfy the four criteria noted earlier. Procedures for application of density-gradient centrifugation with Percoll to separate proximal tubular and distal tubular cells from cortical cell suspensions from the rat are described in detail elsewhere.[47,84] Percoll is a liquid consisting of microscopic, carbohydrate-coated beads that spontaneously forms a continuous density gradient when placed in a centrifugal field. Colored, density marker beads are commercially available to calibrate the density gradients. Once distinct cell populations are obtained, marker enzymes are used to confirm identity and to assess the degree of enrichment. Markers include parathyroid hormone-sensitive adenylate cyclase, alkaline phosphatase, GGT, and glucose-6-phosphatase for proximal tubules and hexokinase and renal kallikrein for distal tubules.

A limitation with the use of suspensions of either tubule fragments or single cells is their short life span. To accommodate experiments that study processes that occur on longer time frames than the few hours that are possible with the freshly isolated tubules or cells and yet retain the use of a model that comprises material derived directly from the in vivo biological material, many investigators have established primary cultures of renal epithelial cells. The proximal tubule from rat, rabbit, or mouse has been the most common biological source for primary cell culture,[47,79–83,87–96] although investigators have also developed culture methods for thick ascending limb and distal tubular cells.[76,92,97] Although tissue from rodents is more

TABLE 30.6
Composition of Medium Used in Primary Culture of Proximal Tubular Cells from Rat or Human Kidney

Basal medium: DMEM/Ham's F12 (1:1)
HEPES (15 mM, pH 7.4)
$NaHCO_3$ (20 mM)
Antibiotics (only through Day 3): penicillin192 IU/mL), streptomycin sulfate (200 μg/mL),
Amphotericin B (2.5 μg/mL)
Bovine insulin (5 μg/mL)
Human transferrin (5 μg/mL)
Sodium selenite (30 nM)
Hydrocortisone (100 ng/mL)
Epidermal growth factor (100 ng/mL)
3,3′,5-Triiodo-DL-thyronine (T_3) (7.5 pg/mL)

readily obtained, investigators have applied essentially identical methods to grow primary cultures of human proximal tubular cells.[42,86,98–102] Regardless of their species of origin, primary cultures of proximal tubular cells are generally grown in a serum-free, hormonally defined medium, one version of which is shown in Table 30.6. Serum is omitted because it encourages or enables growth of fibroblasts, which comprise a very minor contaminant of the cell preparation but can readily overtake the culture and prevent growth of the proximal tubular epithelial cells. The disadvantage is that the cells do grow more slowly in the absence of serum. Importantly, the cell culture maintains their differentiated properties in the serum-free, hormonally defined medium.

A photomicrograph showing human proximal tubular cells grown in monolayer culture and exposed to either control conditions or to the nephrotoxicant *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) illustrates the morphology of primary cell cultures under both physiological and toxicological conditions (Figure 30.6). Control cells exhibit normal epithelial morphology, with a prominent nucleus and a generally cuboidal shape with close cell-to-cell contacts. In contrast, cells incubated with increasing concentrations of DCVC exhibit an increased number of intracellular vesicles and many cells with elongated shape, indicating cellular injury. At the highest dose of DCVC used (i.e., 500 μM), abnormal cellular shape is observed with numerous intracellular vesicles and vacuoles. We have shown that human proximal tubular cells incubated with a relatively low dose of DCVC (≤100 μM) for

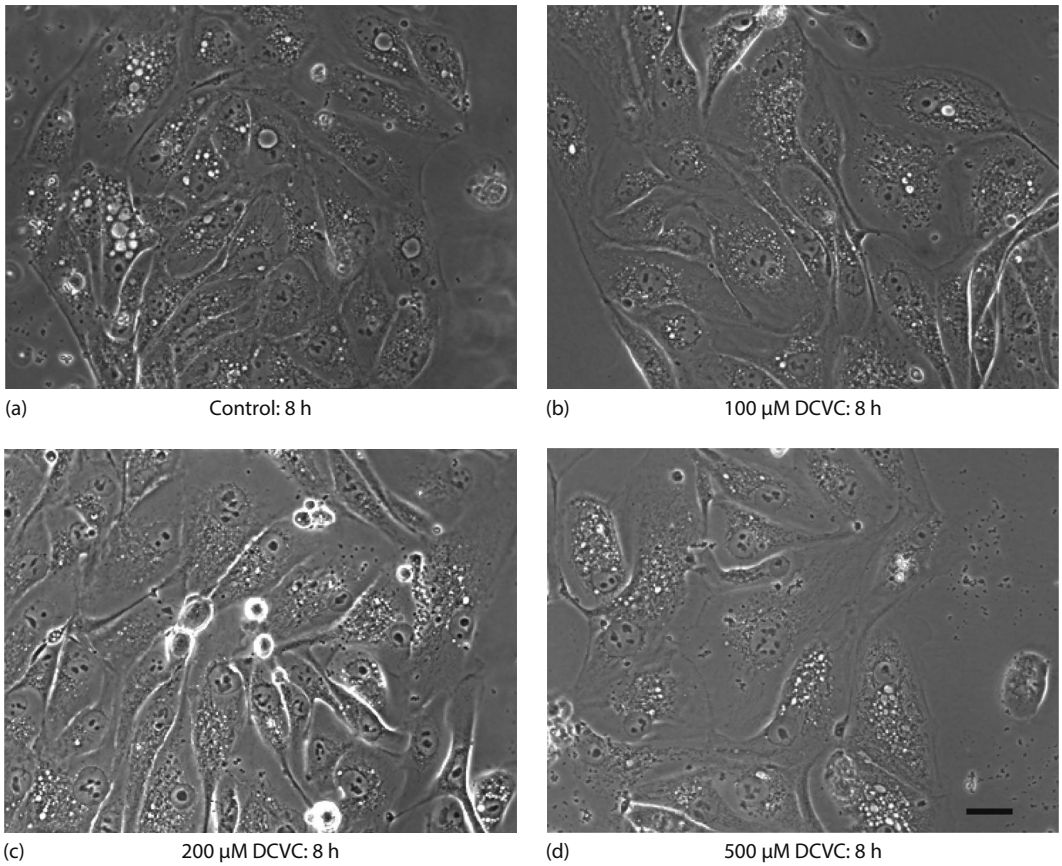

FIGURE 30.6 Photomicrographs of confluent primary cultures of hPT cells treated for 8 h with DCVC. hPT cells were cultured for 5 days in supplemented, serum-free, hormonally defined DMEM:F12 medium on 35 mm polystyrene dishes coated with vitrogen until they were confluent. Cells were then incubated for the indicated times up to 48 h (results for 8 h are shown) with either medium (= Control) (a) or 100 μM (b), 200 μM (c), or 500 μM DCVC (d). At the indicated times, photomicrographs were taken at 100× magnification on a Carl–Zeiss Confocal Laser Microscope. Bar = 5 μm.

a relatively short time period (≤8 h) undergo both apoptosis and enhanced proliferation whereas cells incubated with a higher dose of DCVC (≥100 μM) for a longer period of time (≥24 h) exhibit primarily necrosis.[103,104]

The use of primary cell cultures has many advantages. Inasmuch as cells grow in monolayers, plasma membrane polarity is maintained. Depending on the growth surface used, processes such as transepithelial transport can be studied. Many studies use renal cells grown on collagen-coated, plastic tissue culture flasks or dishes; cells will grow with their luminal membrane face up. Thus, although membrane polarity is maintained, components in media only have direct access to the luminal membrane. This problem has been solved by the use of semipermeable filters that are raised over the culture dish surface, thereby creating both luminal and basolateral compartments to enable media components to come in contact with both membrane surfaces. The only disadvantage of using such filters is that they are usually somewhat opaque, thus making it impossible to view the cells on the filter surface on a microscope. Another advantage is that the primary cultures are relatively simple to prepare. Because these cells remain viable in primary culture for up to 7 days (or longer if passaged), there are a large variety of assays and tests that can be done with them, including all the assays one can perform with the freshly isolated tubules or cells (e.g., acute cellular necrosis as determined by trypan blue exclusion or LDH leakage, metabolism, transport, cellular oxygen consumption, levels of key intracellular metabolites, e.g., ATP, glutathione), as well as assessments of apoptosis, repair and proliferation, gene expression, DNA damage, etc. Hence, the primary cell culture model is extremely versatile and, if cultured properly, should reflect very well the normal, in vivo biochemistry and physiology of the proximal tubule.[32,42,43]

If not passaged, primary cultures remain viable only until they become confluent, which is typically within 7 days. Hence, if longer-term studies are required, a different experimental model must be chosen, as will be discussed in the following section. In some cases, primary cell cultures can be passaged, thereby extending their usable lifetime to as much as several weeks.[99] In spite of the use of serum-free, hormonally defined media, such passaged cells often exhibit altered phenotypes with respect to drug metabolism, cellular energetics, and membrane transport properties.[42] This loss of differentiated function makes them a questionable model for use in assessing cell type–specific toxicity, but they may be useful for study of specific processes whose expression and/or function are retained.

Renal Cell Culture Lines

In contrast with primary cell cultures, cell lines are cells that have been transferred at least once to another culture surface (i.e., subcultured) and, more often, have been transferred multiple times. In some instances, cellular genetics have been altered so that the cells become immortalized and can thus be passaged indefinitely. Such immortalized cells are referred to as continuous cell lines. The process by which cells can be immortalized can be spontaneous or can be induced by various agents (e.g., mutagenic chemicals, radiation, viruses). Some continuous renal cell lines that have been used for studying renal cell function and toxicology are listed in Table 30.7.

TABLE 30.7
Selected Immortalized, Continuous Renal Cell Lines

Cell Line	Species	Presumed Cell Type of Origin
SGE_1	Wistar rat	Glomerulus
NRK-52E	Norway rat	Proximal tubule
$LLC\text{-}PK_1$	Hampshire pig	Proximal tubule
OK	American opossum	Proximal tubule
MCT	Mouse	Proximal tubule
JTC-12	Cynomolgus monkey	Proximal tubule
HK-2	Human	Proximal tubule
GRB-MAL	Rabbit	Medullary thick ascending limb
M-m TAL-lc	Mouse	Medullary thick ascending limb
MDCK	Cocker spaniel	Distal tubule and collecting duct
A6	African clawed toad	Distal tubule and collecting duct

The development of immortalized cell lines of epithelial cells generally requires transfection of short-term cultures with plasmid or viral DNA containing an immortalizing gene (e.g., an oncogene). Taub[105] has reviewed in detail procedures for obtaining such continuous cultures. Others[106,107] have also successfully developed immortalized cell lines from proximal tubular and distal tubular primary cell cultures using the SV40 virus as a transforming agent. The need to use an immortalizing agent is necessitated by the generally poor ability of primary cultures to undergo and survive multiple subculturing. Most of the short-term cultures have a limited doubling potential and undergo senescence and eventually die. It is unclear whether the limited doubling potential of normal cells is an inherent property or is due to imperfections in the tissue culture environment. For example, primary cultures of human proximal tubular cells can be subcultured up to five or six times, but it is important that cell density is not decreased by more than 50% in each passage or cells will not grow.[42,99]

There are several advantages and uses for continuous epithelial cell lines in renal toxicology studies. For example, examination of processes that occur over relatively long time periods can be studied in these cultures whereas the life span of primary cell cultures is insufficient for such studies. Other advantages are that these cell lines are relatively easy to use and produce highly reproducible results, without much of the difficulties inherent in the use of primary cultures. That said, however, the mere fact that the cell lines are immortalized indicates that they have undergone genotypic and phenotypic changes that make them different from the cell types from which they were derived. Hence, it is critical to validate that the processes under study are expressed and regulated properly. Even when the process under investigation is expressed in the cell line, other processes that are normally found in the in vivo cell type or in the kidney as a whole may not be expressed or not be regulated in the same manner. This

makes interpretations of physiological significance difficult and highlights a point made at the beginning of this chapter that multiple experimental models are needed to fully understand the processes of physiological, pathological, or toxicological importance. Nonetheless, immortalized cell lines have been invaluable for numerous studies of renal function and will continue to provide significant insight as long as the constraints and limitations to their use are kept in mind.

ASSAYS OF RENAL CELLULAR FUNCTION

Urinary Enzymes and Other Proteins

Measurements of urinary enzymes and other proteins were discussed earlier in the context of clinical and whole animal assessments of renal function and toxicity. In this section, the focus is on the measurement of renal enzymes and other proteins that can be used as specific biomarkers of renal function or toxicant exposure. This section will focus on biomarkers of effect that can be used to provide specific, mechanistic insight into pathological or chemically induced renal injury.

A recently discovered marker for renal injury that has been described in a series of studies by Bonventre and colleagues[55,57,108–110] is Kim-1. Kim-1 is a type 1 transmembrane protein that is not detected in normal kidney tissue but is expressed at very high levels in dedifferentiated proximal tubular epithelial cells of human and rodent kidneys after either ischemic or chemically induced injury. It appears to satisfy several of the criteria for being an ideal biomarker of effect for renal injury: Kim-1 is stable in urine for prolonged periods of time; it is specific to the kidneys; its expression increases markedly from a baseline of essentially zero; and its increased expression occurs early in the pathologic or toxic event, thus indicating a high degree of sensitivity. Both protein and mRNA expressions are increased in injured renal tissue. It also satisfies the criterion of being absent in normal kidney tissue and only being detectable in tissue that has been injured and/or is undergoing repair and proliferation.

Besides the biologic markers discussed earlier, several other markers have been suggested to be linked to cell injury. These and the ones discussed earlier may be divided into several categories[1]: (1) immunologic factors (e.g., humoral factors such as antibodies and antibody fragments, components of the complement cascade, and coagulation factors) (2) lymphokines (3) major histocompatibility antigens (4) growth factors and cytokines (e.g., platelet-derived growth factor, transforming growth factor, tumor necrosis factor, interleukins, etc.) (5) lipid mediators (e.g., prostaglandins, thromboxanes, leukotrienes) (6) extracellular-matrix components (e.g., collagens, laminin, fibronectin) (7) adhesion molecules (8) reactive oxygen and nitrogen species (9) transcription factors and proto-oncogenes (e.g., c-*myc*, c-*fos*, c-*jun*, c-Ha-*ras*, *Egr-1*) (10) tubule antigens (e.g., Tamm-Horsfall protein, brush-border enzymes, cystatin) (11) heat shock proteins and (12) endothelin. Validation is required, however, for many of these markers.

Assays of Cell Death: Necrosis, Apoptosis, and Autophagy

Most of our knowledge about mechanisms of renal cell injury comes from studies that either focus on or use preparations of proximal tubular cells. This is because the proximal tubule is the easiest nephron cell type to obtain in high purity and is the primary target site for the majority of drugs and other toxic chemicals that target the kidneys, due to the large array of plasma membrane carriers and drug metabolism enzymes present. Although most of the mechanistic information about cell death has come from in vitro studies, some information, particularly about target cell identity and involvement of hormonal, neuronal, or other extrarenal factors, has come from in vivo studies. Renal cells that are intoxicated by drugs or other chemicals can undergo several potential responses, including cell death, growth arrest, or proliferation and repair. It has become clear over the past several years of research, that for a very large number of toxic chemicals, exposure can elicit all three responses. The interplay between these responses is illustrated in Figure 30.7. Hence, as a response to a toxic insult, epithelial cells may either exhibit altered regulation of cellular function or cytotoxicity. The altered cells may either progress further to undergo neoplastic transformation or may be repaired. Cells experiencing cytotoxicity may either be repaired or, depending on exposure conditions, may undergo cell death by either apoptosis or necrosis. We have proposed such a scheme for trichloroethylene-induced nephrotoxicity.[103,104] While the kidney can repair itself after sublethal injury, it has been hypothesized that repeated cycles of sublethal injury followed by repair may lead to uncontrolled cell proliferation and neoplastic transformation.[38] The factors that determine which response occurs and predominates are not completely understood, but certainly include dose and time of exposure.

Cell death, both in general and for renal tubular epithelial cells in particular, can occur by two basic mechanisms, apoptosis or necrosis (oncosis).[111] Some basic features of necrosis

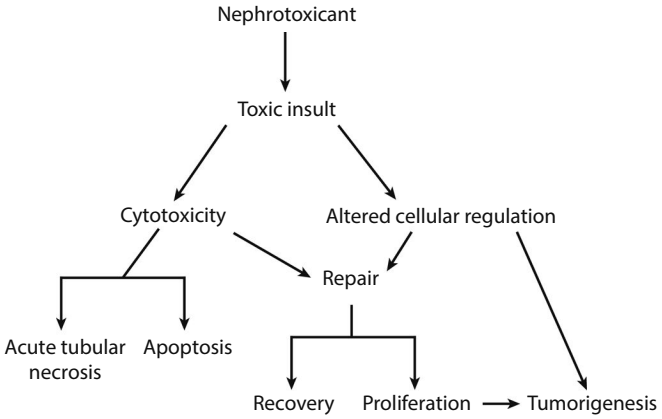

FIGURE 30.7 General scheme of renal cellular responses to nephrotoxicants.

TABLE 30.8
Comparison of Selected Features of Necrotic and Apoptotic Mechanisms of Cell Death

Parameter	Necrosis	Apoptosis
Cell shape	Swelling	Shrinkage
Membrane	Increased permeability	Intact
Energy dependence	No	Yes
Protein synthesis required	No	Yes
Toxicant dose	Relatively high	Relatively low
ATP depletion	Yes; early effect	Not until just before cell death
Second messengers	Not involved	Often involved
DNA damage	Random SS/DS breaks	Laddering
Nuclear damage	Chromatin damage, lysis	Chromatin condensation

and apoptosis are compared in Table 30.8. The many differences between the two forms of cell death are very dramatic. Thus, whereas apoptosis is a highly regulated, energy-dependent process, necrosis can be viewed as an uncontrolled, pathological response; whereas apoptosis involves events in single cells, necrosis typically involves large numbers of cells in a wide area of tissue and is often mediated in vivo by infiltrating macrophages and similar inflammatory mechanisms.

Cell death by necrosis is typically measured by assays for plasma membrane permeability, such as LDH release or trypan blue uptake (see preceding text). The LDH release assay method is shown schematically in Figure 30.8 and illustrates typical results for a control incubation (left panel), which exhibits a small amount of LDH release, and an incubation with a highly cytotoxic concentration of a chemical (right panel), which exhibits a large amount of LDH release. Results with trypan blue exclusion (or with another vital dye) would be similar to those with LDH release. Trypan blue exclusion is measured by mixing an aliquot of cells with the dye and applying it to a hemacytometer; this enables not only the assessment of viability by dye exclusion but also the determination of cell number. For routine assessments of cell viability, LDH release is recommended because of its simplicity, widespread use that has been validated in numerous studies with isolated cells from multiple tissues, and its lack of potential bias as can exist with the counting of stained cells under a microscope.

The LDH release assay was originally developed and used in suspensions of isolated cells.[85] With the advent of so many studies being conducted with either primary cell cultures or continuous cell lines, some variation to the basic method is needed, although the principles of the assay remain the same. For adherent cells attached to a stationery matrix, LDH activity is first measured in media as NADH oxidation at A_{340}. After removal of media and washing with phosphate-buffered saline (PBS), cells are solubilized with 0.1% (v/v) Triton X-100, and LDH activity is determined in the total cell extract. The fraction of LDH release is then used as an index of irreversible injury or necrosis, and is calculated according to the following:

$$\%\ \text{LDH release} = \left[\frac{\text{LDH activity in media}}{(\text{LDH activity in media} + \text{LDH activity in total cells})}\right] \times 100\%$$

In some cases, however, LDH release cannot be used as a measure of cell viability because the toxicant directly inhibits its LDH activity, such as occurs with inorganic mercury.[49,50] The likely mechanism of inhibition is direct binding to the

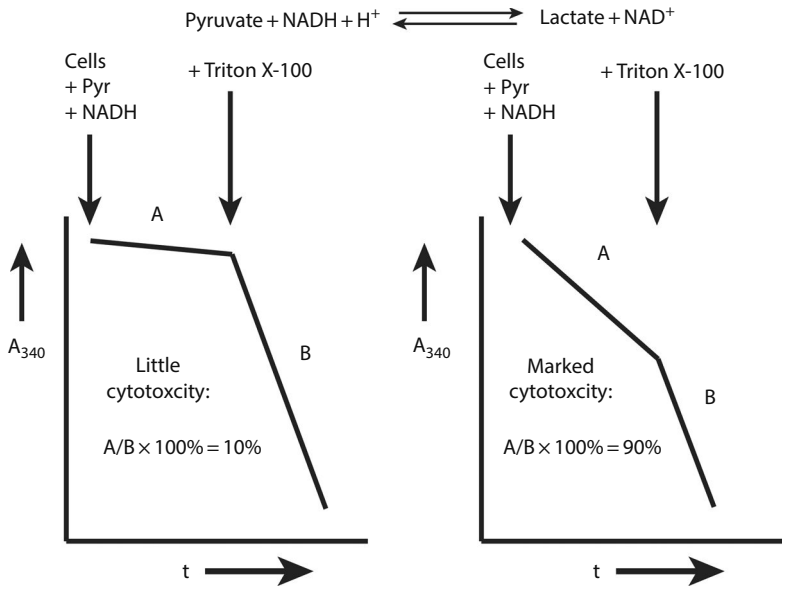

FIGURE 30.8 LDH release assay.

critical cysteinyl sulfhydryl group of LDH. Hence, any sulfhydryl-reactive chemical, or a chemical that is metabolized to a sulfhydryl-reactive species, will likely inhibit LDH, although potency will likely vary among compounds. In the situation in which LDH activity is inhibited, total LDH activity can be used as an estimate of cell viability, with activity calculated according to:

$$\text{Total LDH activity} = \text{LDH activity in media} + \text{LDH activity in total cells}$$

An additional assay that can be used to estimate cell viability is the MTT cell proliferation assay. MTT, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is reduced by live cells to a strongly pigmented, purple formazan product. After solubilization, the absorbance of the formazan product can be measured in a microplate reader at 570 nm. A kit is available from Molecular Probes (http://probes.invitrogen.com) that is designed as a high-throughput assay of cell viability and proliferation. The manufacturer cautions that because MTT can be reduced intracellularly by both various dehydrogenases and GSTs, MTT may not always be a reliable probe of cell viability in cells exposed to chemicals that affect these enzymes. Nonetheless, it has become a commonly used and convenient assay.

Cells undergoing apoptosis, in contrast to necrosis, exhibit several unique morphological features (cf. Table 30.8). Detection of these features has been used in several assays to detect apoptosis. One common method involves in situ histochemical staining of cells and light microscopy.[112] This method, called the TUNEL method (*t*erminal deoxynucleotidyltransferase [TdT]-mediated d*U*TP-biotin *n*ick *e*nd *l*abeling), relies on the in situ labeling of DNA breaks in individual nuclei in tissue sections processed by routine histopathology procedures. TdT specifically binds to 3′-OH ends of DNA that are exposed by proteolytic treatment. This is followed by the synthesis of a labeled polydeoxynucleotide molecule, formed by the use of TdT to incorporate biotinylated deoxyuridine into the sites of DNA breaks. Signal is amplified by avidin peroxidase, enabling detection by light microscopy.

Some forms of apoptosis can be detected by running agarose gels with ethidium bromide staining to detect DNA laddering that often, but not always, occurs in cells undergoing this form of cell death. The laddering is based on activation of endonucleases that cleave the DNA between nucleosomes, producing 200 bp multimers. While this is a useful method, it is not quantitative, and such DNA laddering is not obligatorily associated with apoptosis.

Several flow cytometry methods have been developed to analyze the cell cycle or to label cellular components with, for example, fluorescent tags, and then sort cells according to their content of the fluorescently labeled component. Advantages of methods using flow cytometry include their ability to identify and readily quantify multiple subpopulations of cells based on a large variety of parameters, and their ease of use. Flow cytometers can quantify the proportion of cells in G_0/G_1, S, and G_2/M phases of the cell cycle by the use of fluorescent DNA dyes. For example, one can easily identify cells in G_2/M phase as compared to those in G_0/G_1 phase because the former have twice the amount of DNA as the latter. Besides emitted light, the flow cytometer also analyzes scattered light from cells passing through the laser beam. Forward scatter and side scatter indicate cell size and granularity (density), respectively. Because apoptotic cells are typically condensed, the flow cytometer detects them as smaller, less fluorescent entities. Cells are stained with propidium iodide (PI), which is a DNA-intercalating agent like ethidium bromide. The analytical procedure used to detect the different cell populations is called fluorescence-activated cell sorting (FACS) analysis. An example of such an analysis is shown in Figure 30.9, where primary cultures of human proximal tubular cells were incubated with either medium alone (= Control) or with 50 μM DCVC.[103] A marked increase in the fraction of apoptotic cells is evident after incubation with DCVC.

Another flow cytometric assay for apoptosis involves assessment of the changes in plasma membrane morphology, which is an early effect involved in cells that undergo apoptosis. In such cells, the membrane phospholipid phosphatidylserine is translocated to the outer face of the plasma membrane, thereby exposing it to the extracellular environment. Annexin V is a 35–36 kDa Ca^{2+}-dependent, phospholipid-binding protein with a high affinity for phosphatidylserine. In measurement of apoptosis, Annexin V is conjugated to a fluorochrome such as fluoroscein isothiocyanate (FITC), enabling binding of Annexin V-FITC to be a sensitive probe of early apoptosis. Cells are stained with both Annexin V-FITC and a vital dye such as PI, are subjected to flow cytometry and FACS analysis, and are identified as early apoptotic (Annexin V-FITC positive and PI negative), late apoptotic or necrotic (Annexin V-FITC positive and PI-positive), or viable (Annexin V-FITC negative and PI-negative).

A number of other assays for apoptosis in renal epithelial cells are available, involving flow cytometry, Western blotting, or enzymatic assays. Examples include measurements of apoptosis-associated proteins, such as Bcl-2 family members, cytochrome c, and cleavage products of poly(ADP-ribose)-polymerase (PARP). For more details on mechanisms of apoptosis and assays used to quantify apoptosis, the reader is referred to recent reviews.[113] As a general rule, it is advisable to assess cell death, and apoptosis in particular, by more than one method and to study cellular responses over a long enough time course and a broad enough concentration range of test chemical.

Renal Cellular Function

Although measurements of cell death by necrosis or apoptosis provide end-points for screening potentially nephrotoxic chemicals, it is often important to assess the effects of chemical exposures or pathological states that are more subtle or that provide an indication of potential toxic effects prior to irreversible cell death. These would include

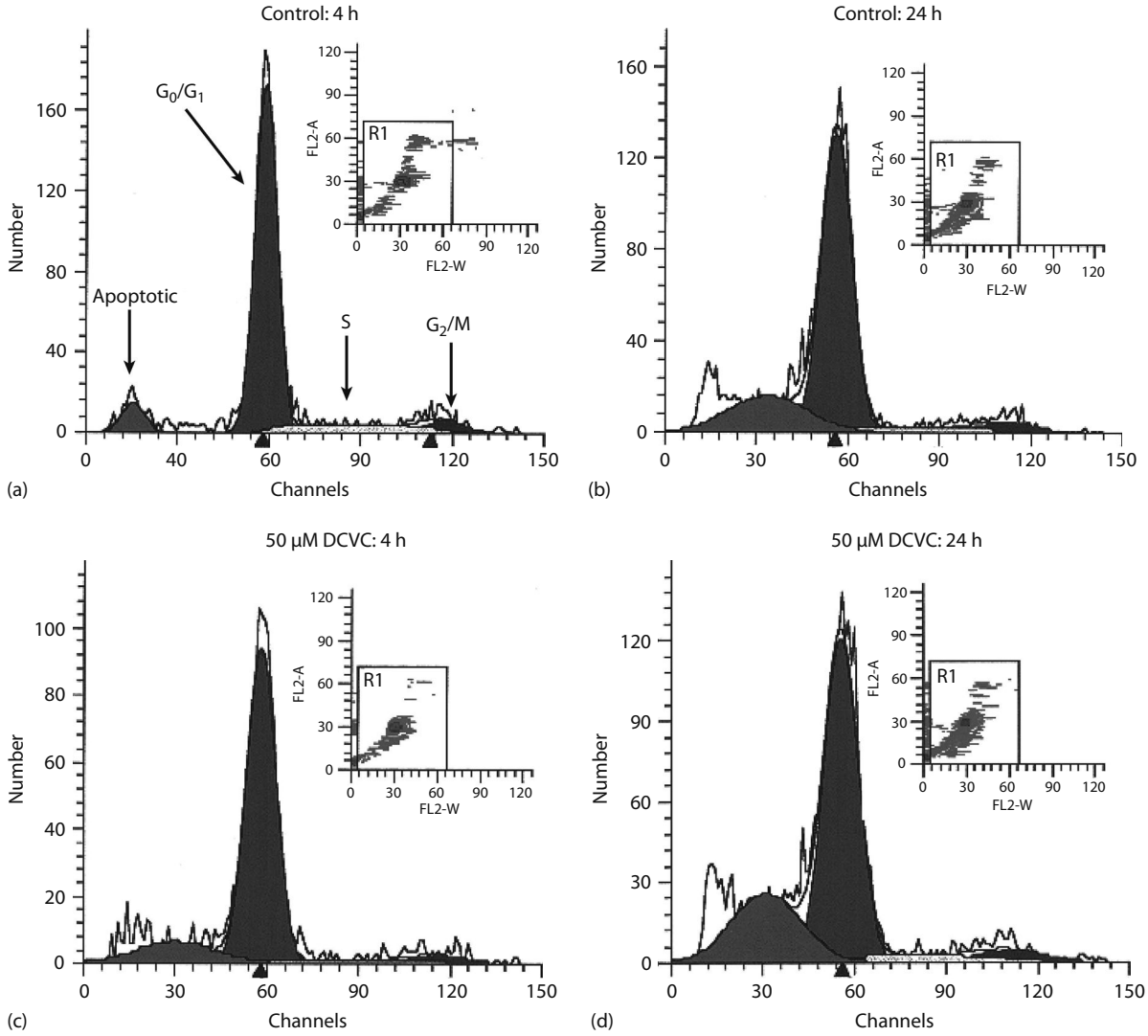

FIGURE 30.9 Flow cytometry analysis of PI-stained hPT cells treated for 4 or 24 h with 50 μM DCVC. Confluent hPT cells were grown on 35 mm polystyrene culture dishes and were treated with PBS (= Control) for 4 h (a) or 24 h (b) or 50 μM DCVC for 4 h (c) or 24 h (d). Cells were then harvested by trypsin-EDTA digestion, washed in sterile PBS, fixed overnight in ethanol, stained with PI, and then analyzed by flow cytometry with a Becton Dickinson FACSCalibur flow cytometer. Peaks from left to right represent apoptotic cells, cells in G_0/G_1, cells in S phase, and cells in G_2/M. Results are from a single preparation and are typical of those from six separate experiments. Note that G_0/G_1 peaks comprise the majority of cells in most incubations and are somewhat broad because the cells are confluent. The coefficient of variation (CV) for these peaks ranged between 3% and 18%, with approximately 80% of the peaks in samples exhibiting CV values of 5%–8%. Insets: Distribution of cells according to fluorescence intensity. Cells outside the box are those that were excluded from the analysis due to aggregation.

measurements of cellular respiration, active ion transport (e.g., $(Na^+ + K^+)$-stimulated ATPase activity), cellular redox status, and concentrations of key intracellular metabolites, such as ATP and GSH. Mitochondria are common and early targets for many nephrotoxic chemicals. Hence, sensitive assessments of cellular energetics and mitochondrial function should provide good indicators of agents whose effects can be mediated through this organelle.[114] One reason the mitochondria are such a critical and prominent intracellular target for nephrotoxicants is that they contain a large number of protein thiol groups that can become inactivated by oxidation or alkylation and many toxic chemicals are bioactivated to oxidants and reactive electrophiles.

Molecular Markers of Renal Cellular Repair, Regeneration, and Proliferation

As illustrated in Figure 30.7, renal epithelial cells can respond to toxicant exposures in a variety of ways, depending in part on dose and time of exposure. Other factors are surely important in determining whether the exposure leads to cell death, repair, or uncontrolled proliferation. There are a number of markers for repair and proliferation for the kidneys.[115] For example, the normal, differentiated proximal tubular cell expresses cytokeratins but not vimentin, which is a marker for endothelial cells. Renal proximal tubular cells that have either undergone neoplastic transformation or have dedifferentiated

and are undergoing repair and proliferation do express vimentin. Morphologically, dedifferentiated proximal tubular cells exhibit differences from the normal, differentiated state, such as the lack of a brush border and an elongated rather than a cuboidal shape. While the renal epithelium has regenerative capacity, the normal, terminally differentiated renal epithelium is nonproliferating, as evidenced by the flow cytometry FACS analysis shown in Figure 30.9, which shows that <10% of control cells are in S phase. Assays such as radiolabeled thymidine incorporation, 5-bromo-2′-deoxyuridine (BrdU) incorporation, and expression of proliferating cell nuclear antigen (PCNA) can be used to demonstrate repair and proliferation of the renal tubular epithelium.

Use of Genomics, Proteomics, and Metabolomics in Renal Physiology and Toxicology

Recent advances in genomics, proteomics, and metabolomics provide new opportunities for studying the molecular and cell biology of the renal cellular response to toxicant exposure and renal disease. Moreover, the increasing proportion of high-throughput assays creates both opportunities and challenges. In terms of opportunities, large amounts of data are obtainable that can uncover relationships that were previously unknown. In terms of challenges, however, these same large amounts of data generate a computational nightmare and can produce both false negatives and, more importantly, false positives. Nonetheless, a systems biology approach to understanding the influence of environment and organ and cellular function is now viewed by many as the only true way to provide a fundamental understanding of physiologic, pathologic, and toxicological processes.[116,117]

In the area of genomics, cDNA microarrays can be used to determine genes or classes of genes that are either upregulated or downregulated as a consequence of toxicant exposure. A threshold for a change to be significant (1.5-fold or 2-fold if being more conservative) is typically applied in the analysis. Patterns of gene expression changes can provide a signature for a chemical or class of chemicals that modulate renal function.

While microarrays are indicative of changes in mRNA expression, they may not translate into functional changes. Hence, the additional application of proteomics approaches can provide more detailed information on changes in both protein expression and posttranslational modifications of proteins (e.g., phosphorylation status), which can thus provide functional information.[118] Proteomics can also be used to determine the identity of proteins that are specifically modified by reactive species generated by xenobiotic metabolism. In this manner, detailed molecular information about how a toxicant produces its effects can be gained.

Metabolomics methods can also be used to provide insight into how nephrotoxicants produce their effects.[119,120] The general approach relies on measurements of intermediary metabolites in urine, using such techniques as ^{13}C-nuclear magnetic resonance spectroscopy. Just as cDNA microarrays can provide a signature for the effects of a toxicant on mRNA expression, metabolomics can provide a signature for the effects of a toxicant on pathways such as glycolysis, oxidative phosphorylation, and amino acid metabolism. Although none of these approaches can be readily performed in most laboratories, due to the requirement for specialized equipment, they are becoming more commonly used in toxicology and promise to open up new areas of investigation for the future.

While methods to modify the genetics of renal proteins in in vitro models, particularly established cell lines, by approaches such as cDNA transfection, antisense oligonucleotides, and small-interfering RNA (siRNA) treatment are well developed, similar methods in whole animals are in their early stages of development and application. Masereeuw and colleagues[121] demonstrated that intravenous administration of siRNA directed against Mrp2 resulted in selective silencing of Mrp2 expression in renal proximal tubules. These findings support the concept that intravenous administration of siRNAs can be a potential therapeutic tool for gene silencing in the kidneys.

HUMAN DISEASE AND RISK ASSESSMENT

Experimental Models of Renal Diseases

An important experimental approach to improve the understanding of disease etiology and to design improved treatments for human diseases is the development of experimental models that mimic various aspects of the disease under study. Genetically modified animals, such as transgenic mice,[122] have been used to study the functions of specific proteins. An interesting approach along these lines, designed to mimic conditions that exist in a variety of human renal diseases is the expression of human proteins in mice, creating so-called *humanized mice*.[123] Renal diseases that have been studied include diabetic nephropathy, polycystic kidney disease, various glomerular nephropathies, to name a few. Special rat strains are also available, such as the spontaneously hypertensive rat,[124] to study the role of renal function in specific pathologies. Experimental approaches used to study renal function and toxicity will be similar with these animals and normal animals. Caution must be exercised, however, in the interpretation of findings because a genetic modification may alter multiple processes. Hence, a complete characterization of these animals is necessary.

Extrapolation of Animal Data to Humans

Although experimental animals such as rats and mice are frequently used to study nephrotoxicity and renal function, humans are the ultimate species of interest. Laboratory animals or in vitro preparations derived from such animals are used instead of humans or tissue from humans for several reasons. First, the design of clinical studies cannot include investigator-initiated study of mechanisms of nephrotoxicity. It is only when humans are inadvertently exposed to a nephrotoxic chemical, such as might occur in an occupational

setting, an environmental contamination, or a poisoning, can information about toxicity be gleaned. Several limitations exist, however, with such studies. For example, exposure information is often incomplete and/or limited, requiring the use of assumptions and approximations. The level of control of experimental design that exists with animal studies is not possible with clinical studies. Second, although in vitro studies with renal tissue from humans are possible and allow the investigator to exert the same degree of control over exposure conditions as are available with in vitro studies from animal tissues, human tissue is not always available and is significantly more expensive than studies with animal tissues.

Nonetheless, mechanistic studies with renal tissue from humans are feasible and have many advantages. The use of human tissue for studies of chemically induced nephrotoxicity and renal function removes the need for interspecies extrapolation. While such extrapolations have been applied successfully for many chemicals, they have been much less successful for other chemicals. A prime example of a situation where rodent-to-human extrapolations have yielded equivocal results for human health risk assessments is that of trichloroethylene.[38] For halogenated solvents such as trichloroethylene, rodents appear to be poor surrogates for humans because of inherent differences that cannot be readily corrected. Hence, while rodent data are used, the uncertainty factors applied to the human health risk assessments are rather large compared to those used for many other classes of chemicals.

Future Considerations

While many advances have been achieved in recent years in the study of nephrotoxicity, several areas of research require continued development or refinement and many new techniques and methods are only beginning to be exploited for the study of renal function and toxicity. Examples of the former include the various cell culture models and biomarkers of effect, exposure, and susceptibility. Continued application of molecular biology techniques to cell culture can help improve the ability of these models to mimic the in vivo renal cell. Application of approaches such as siRNA technology to and the use of transgenic animals in renal toxicology should also improve our ability to define exposure conditions, understand susceptibility to renal cellular injury, and better define mechanisms of action. Although numerous markers of effect are available for the kidneys, most do not satisfy enough of the criteria to be truly useful as indicators of early stages of damage. Continued development of biomarkers that can indicate mild alterations in some parameter of renal function at early times postexposure is needed. Some recently developed markers, such as Kim-1, appear to satisfy the criteria needed for an effective biomarker.

Summary

When considering the kidneys as a target organ for chemically induced toxicity, many unique features are apparent that determine susceptibility. Four major factors are critical: (1) the high rate of renal blood flow, (2) the ability of the kidneys to concentrate chemicals in the intraluminal fluid, (3) the ability to actively reabsorb and/or secrete chemicals through the tubular epithelial cells with high activity, and (4) the presence of enzymes for the bioactivation of a protoxicant to reactive intermediates.

In choosing an experimental model with which to study renal function and toxicity, several questions should be asked first: (1) What is known about tissue and/or nephron cell type specificity? (2) What is known about metabolism? (3) What is known about transport? (4) Are extrarenal factors or processes required for the expression of nephrotoxicity? (5) What is the time frame over which the nephrotoxic response occurs? Choices of models using experimental animals (e.g., rat, mouse, rabbit) range from the whole animal to a range of in vitro model systems, including the isolated perfused kidney, isolated perfused tubules, renal slices, isolated tubular fragments, isolated cells, primary cell culture, and immortalized renal cell lines. Each model has distinct advantages and limitations. Depending on answers to the preceding questions, one should choose multiple models to address renal function and toxicity.

Once certain types of information about a putative nephrotoxicant are known, the model can then be chosen. The whole animal model is important for determining target organ specificity and the influence of extrarenal factors, including immunological and hormonal factors. Certain of the in vitro models have the advantage of maintaining tubular structure and epithelial membrane polarity. A critical issue in using many of the isolated tubule or cell models, including primary cell culture and immortalized cell lines, is how well-differentiated function is maintained. The importance of this issue is dependent on the process being studied, as some models that only express some of the normal, in vivo phenotype will be good models if the process being studied is retained. Several of the in vitro models have the added advantage of being amenable to being used for screening of potentially nephrotoxic chemicals. High-throughput assays, such as those that can use a plate reader, are available for many of the assays of renal cellular function and toxicity. The screening approach is particularly useful to eliminate candidate drugs in the earliest phases of investigation.

QUESTIONS

30.1 Describe procedures used to measure GFR.
30.2 Explain the biological significance of high-molecular-weight and low-molecular-weight proteinuria.
30.3 Explain how nephron heterogeneity determines the pattern of effects of nephrotoxicants.
30.4 Explain how tubuloglomerular feedback regulates GFR.
30.5 Describe the properties of a chemical that would be ideal for use in renal clearance measurements.
30.6 Compare and contrast whole animals and various in vitro models for their use in studies of nephrotoxicity and renal function in terms of their advantages and limitations.

30.7 Describe the properties of an ideal biomarker of effect for use in human health studies.

30.8 Describe the differences between necrotic and apoptotic cell death at both cellular and organ levels.

KEYWORDS

Kidney, Nephrotoxicity, Drug transport, Drug metabolism, Renal biomarkers, Human health risk assessment

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31 Gastrointestinal Toxicology

Robert W. Kapp, Jr.

CONTENTS

INTRODUCTION

The intensity of the effect of a toxicant on an organism is dependent primarily upon the relative effective concentration and the duration or persistence of the ultimate toxicant at a septic site of action. There are numerous portals of entry for toxicants. The oral exposure route is the primary route of exposure to all substances including toxicants. In order to assess the potential adverse effects of toxicants by oral exposure, it is necessary to understand the structure, function, and mechanisms involved in the adsorption, distribution, metabolism/biotransformation, and excretion of toxicants that enter

the gastrointestinal (GI) tract. This chapter focuses on the normal structure and function of the GI tract and how the process of digestion and absorption influences and is influenced by toxicants that enter the GI tract. For those toxicants, the intensity of the ultimate toxicant is determined by a number of factors that are part of the normal functioning of the GI tract including digestion, absorption, biotransformation, distribution, and elimination. The last part of this chapter describes the types of testing that can be performed to examine the effects of toxicants on various aspects of the normal functioning of the GI tract.

OVERVIEW OF THE GASTROINTESTINAL TRACT

All living cells require energy in the form of nutrients. Nutrients are substances that provide energy to basic body processes. In addition, nutrients are critical to the building of new or the replacement of old components for various body structures. Basically, nutrients are divided into two classes: macronutrients and micronutrients. In comparative terms, macronutrients are required in larger quantities than are micronutrients, which are required in much smaller amounts. The daily diet of most individuals consists of about 2–3 lb of food and 1.5 L of fluid containing perhaps 100,000 different substances in amounts varying from 1 or 2 L to as little as nanogram units. Of those 100,000 substances, only about 300 are classified as nutrients, and of that number, 45 are essential for the survival of the individual. Many of the consumed substances have value in preserving the food or add aroma or color to improve or enhance the taste or appearance of the food. Some of the substances are helpful in the digestion process, such as fibers and cellulose [2]. The vast majority of ingested food is broken down into more elemental units for use by the body. Table 31.1 provides a summary of the most common macronutrients and micronutrients consumed by humans.

The resulting breakdown products of these nutrient classes provide the organism with energy for the numerous life processes necessary to sustain the organism, such as active transport, protein and enzyme synthesis, and synthesis of structural molecules for cells. The minerals and vitamins can act as catalysts in thousands of body functions and are needed for the growth and maintenance of body structures. Water is necessary as a means to regulate body temperature, as a solvent, and as a lubricant, plus it plays a role in hydrolysis reactions.

Although single-cell organisms can obtain nutrients directly from their surroundings, multicellular organisms have developed highly specialized structures to collect, digest, and absorb these nutrients. The GI tract or alimentary canal is that specialized structure in multicellular organisms that essentially obtains nourishment by the ingestion of organic material, generally termed *holozoic nutrition*. This process of nutrition provides trillions of body cells with nutrients in order to function as described previously. Table 31.2 identifies the five basic stages of holozoic nutrition.

To complete these various stages of nutrition, the GI tract has specialized structures. The cell wall of the GI tract is composed of numerous tissue types that serve not only to break down foodstuff and fluid into absorbable components but also to create an environment for these components, as well as vitamins and minerals, to be absorbed into the bloodstream, assimilated, and then ultimately eliminated from the body. The GI tract is essentially a 25–30 ft series of hollow organs connected together to form a pathway that starts at the mouth and proceeds through the pharynx, esophagus, stomach, small intestines, large intestines, rectum, and anus. Although the lumen is found within the body, the contents within the lumen of the GI tract are considered to be external to the body. The digestive system functions to modify the ingested luminal contents so they can be absorbed from the lumen (exterior of the body) by the blood and lymph circulatory system (interior of the body). Traditionally, the digestion system is organized into two divisions: (1) the GI tract or alimentary canal, which includes the mouth, pharynx, esophagus, stomach, small intestine, large intestine, rectum, and anus, and (2) accessory structures that include teeth, tongue, salivary glands, pharynx, liver, gallbladder, and pancreas.

TABLE 31.1
Summary of Common Macro- and Micronutrients in Humans

Nutrient Classification	Nutrient Types	End Product
Macronutrients	Carbohydrates	Monosaccharides
	Lipids	Fatty acids and cholesterol and monoglycerides
	Proteins and nucleic acids	Amino acids, bases
	Water	Water
Micronutrients	Minerals	Calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium, zinc
	Fat-soluble vitamins	A, D, E, K
	Water-soluble vitamins	B_1 (thiamine), B_2 (riboflavin), B_3 (niacin), B_5 (pantothenic acid), B_6 (pyridoxine), B_7 (biotin), B_9 (folacin), B_{12} (cobalamin), C

Source: National Academy of Sciences, *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids*, National Academies Press, Washington, DC, 2005.

TABLE 31.2
Stages of Holozoic Nutrition

Stage	Function
Ingestion	Physical act of engulfing foodstuff into the alimentary canal
Digestion	Mechanical and chemical reductions of foodstuff to elemental particles
Absorption	Passive and active mechanisms involved in transporting the nutrients across mucosal cell membranes
Assimilation	The body's ultimate use of the absorbed nutrients
Egestion	The elimination of the unused and undigested GI tract contents

Sources: Data from Rothery, M., A level biology. Module 1., *Digestion*. Available online: www.mrothery.co.uk, 2012; Geissler, C. and Powers, H., Food and nutrients, Chapter 1, in: *Human Nutrition*, Churchill Livingstone Elsevier, London, U.K., 2011, pp. 3–24.

The major organs of the digestive tract and their relationship to one another are shown in Figure 31.1.

Each portion of the GI tract is highly specialized with respect to both structure and function. Accessory digestive organs are connected to the GI tract through a series of ducts, including the salivary glands, the pancreas, the liver, and the gall bladder. The teeth aid in the physical breakdown of foodstuffs that enter the mouth. The tongue assists in mastication and swallowing. The pharynx assists in sealing the respiratory tract during the swallowing reflex. The esophagus transports the ingested foodstuff to the stomach. The stomach serves to mix the foodstuffs with acidic gastric secretions and prepare the resulting mixture, which is now a semiliquid mass of partially digested food (termed *chyme*), for entrance into the small intestine. The small intestine is a 20–25 ft tube in which the vast majority of the digestion and absorption of chyme occurs. The large intestine absorbs some electrolytes and water and prepares the contents for expulsion through the rectum and anus [5].

HISTOLOGICAL ORGANIZATION OF THE GASTROINTESTINAL TRACT

To understand the GI tract, it is necessary to understand its microscopic as well as macroscopic structures. Throughout the length of the GI tract, a cross section of the tissue consists of a series of four concentric layers or tunics. The intestinal

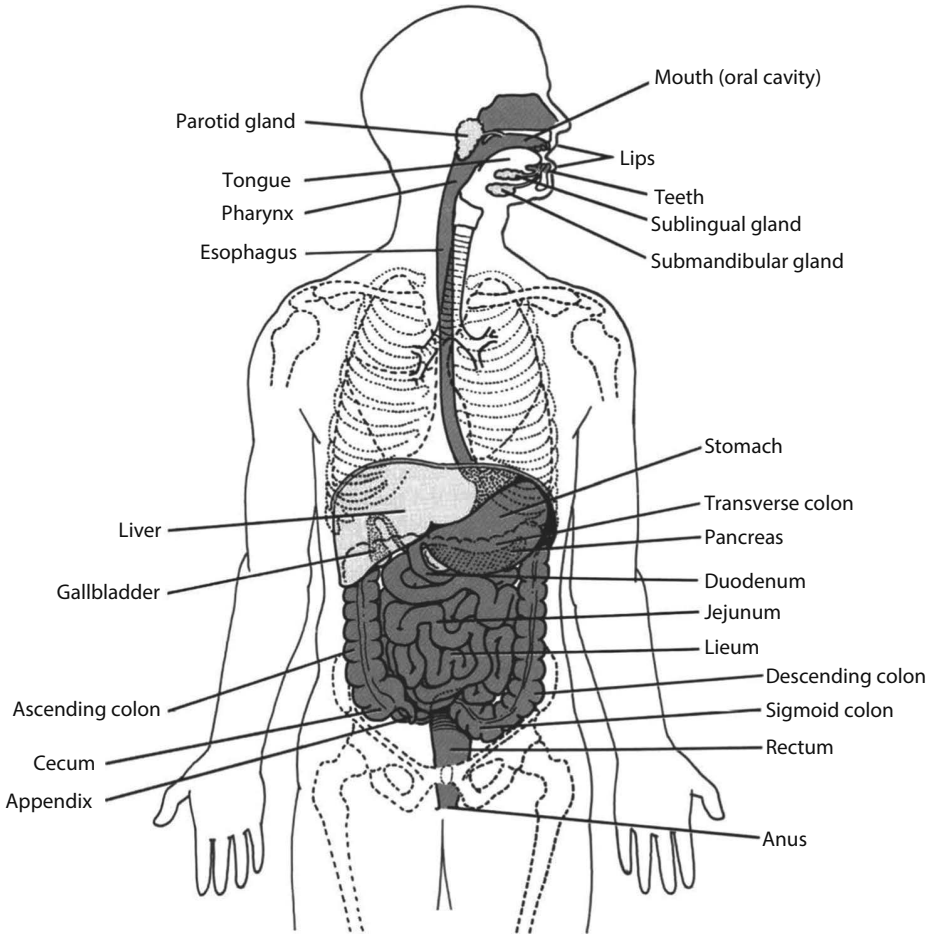

FIGURE 31.1 Overview of the digestive tract. (From Tortora, G.J. and Anagnostakos, N.P., The digestive system, Chapter 24, in: *Principles of Anatomy and Physiology*, 6th edn., HarperCollins: New York, 1990, p. 734. With permission.)

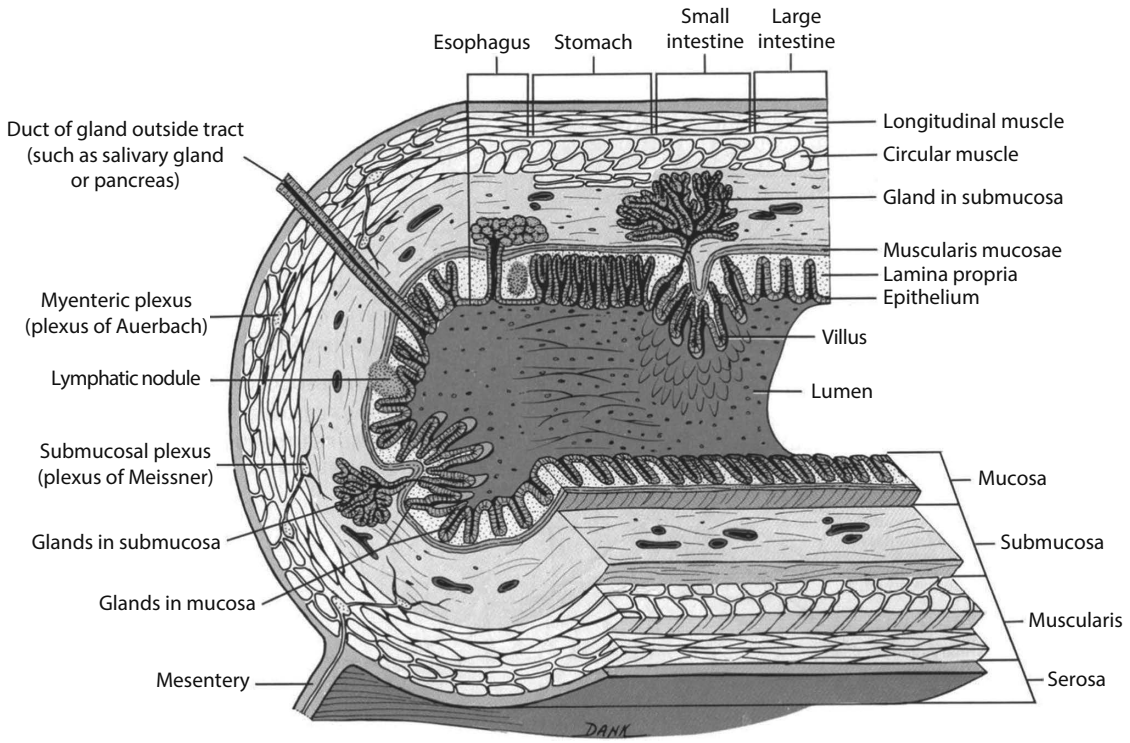

FIGURE 31.2 Representative sections of the GI tract. (From Tortora, G.J. and Anagnostakos, N.P., The digestive system, Chapter 24, in: *Principles of Anatomy and Physiology*, 6th edn., HarperCollins: New York, 1990, p. 735. With permission.)

wall varies in the various sections of the alimentary canal, reflecting the different roles in each locality; however, the basic structure remains the same (Figure 31.2).

Serosa

The outermost layer is the adventitia or serosa, which includes loose supportive tissue proximally and is contained by a thin membrane distally that covers the entire GI tract. Those portions located within the abdominal cavity are covered by a layer of squamous cells and an underlying connective tissue. The entire GI tube is suspended within the body cavity by a mesentery through which blood vessels, nerves, and lymphatics pass to the organ systems. It is part of the peritoneum and facilitates movement of the structure within the body cavity.

Muscularis

The next layer is the muscularis, which usually contains the inner circular layer of smooth muscle fibers and an outer longitudinal layer of smooth muscle fibers and the myenteric plexus. These muscle layers provide coordinated movements that allow for the mixing, digestion, and motility of ingested substances through the GI tract. The primary control of GI motility is centered in the myenteric plexus (plexus of Auerbach), which contains connections from both autonomic innervation connections, and is also found in the muscularis layer. The muscularis layer in the mouth, pharynx, and upper third of the esophagus has not only smooth muscles but also skeletal or striated muscles that produce the voluntary initial phase of swallowing.

Submucosa

The next layer, moving toward the lumen, is the submucosa, which is comprised primarily of loose connective tissue, lymphatic and blood vessels, glands, and the submucosal plexus. It is a very vascular layer and contains much of the plexus of Meissner, which is critical in controlling gland secretion and blood flow to the GI tract.

Mucosa

The innermost layer of the GI tract is the mucosa, which is in contact with the external environment and actually allows for the absorption of the nutrients. It is comprised of three additional subdivisions. Starting from the layer proximate to the submucosa, the muscularis mucosa includes a smooth muscle and elastic connective tissue layer which creates the folds in the mucous membrane that enhance the area of absorption throughout the GI tract. The next distinctive layer of the mucosa is the lamina propria, which is composed of loose connective tissue and contains blood vessels, lymphatics, and perhaps some glands. This layer supports the epithelial cells, provides the blood and lymph supply, and binds the layer to the muscularis mucosa. The final layer of the mucosa that lines the lumen of the GI tract and is in direct contact with nutrients is the epithelial layer. The structure and function of these epithelial cells vary along the course of the tract.

Depending on the location, the epithelial cells may be stratified squamous cells, such as in the esophagus, or secretory cells found in the stomach or absorptive cells in the small intestines. The functions of the stratified epithelium are secretion and protection. The functions of the simple epithelium, on the other hand, are secretion and absorption.

Cellular Absorption

Cell membranes are plasma barriers between the environment and the cell cytoplasm. The membrane is specialized in that it contains specific proteins and lipid components that allow it to perform its unique roles for that particular cell. Because the lumen of the GI tract is considered external to the body itself, a critical factor in the nutrient absorption process is crossing this mucosal epithelial cell membrane. The membranes are essential for the integrity and function of the cell. The functions of GI tract cell membranes include the following:

- Protection
- Transport into and out of the cell
- Enzymatic activity
- Providing receptors for signal transduction
- Providing intercellular adhesion proteins
- Cell-to-cell recognition via glycocalyx
- Attachment to the cytoskeleton and extracellular matrix

In 1972, Singer and Nicholson presented a fluid mosaic model for the gross organization and structure of the proteins and lipids of biological membranes in general [7]. In this model, the 7–9 nm thick lipid bilayer is a neutral, 2D structure with a mosaic of proteins embedded in it. Further, the phospholipids are amphipathic, meaning they are both hydrophilic and hydrophobic. Each phospholipid possesses a polar phosphate group at the head facing the outside of the membrane and two nonpolar fatty acids at the tail facing inward. Within model bilayers, lipids can exist in different phases: as gels or as liquid-ordered or liquid-disordered states (as described in the Singer–Nicholson model). See Figure 31.3 for the overall basic membrane structure. In the liquid-ordered state, saturated hydrocarbonic chains of phospholipids are tightly packed with cholesterol and glycolipids; thus, horizontal mobility in the membrane is limited. The cells that line the GI tract are similar in thickness (7–9 nm) and structure (phospholipid bilayer), with polar head groups consisting of phosphatidylcholine and phosphatidylethanolamine. These polar groups are arranged perpendicularly to the cell membrane and are located facing the inner and outer membrane surfaces. The zonula occludens or tight junction is the outer layer of the junctional complex between other epithelial cells that line the luminal surface of the digestive tube. The tight junction is a series of integral membrane proteins that form a fused ring around intestinal cells to help prevent molecules from passing between cells. Cell membranes display a tremendous complexity of lipids and proteins designed to perform the functions cells require. To coordinate these functions, the membrane is able to laterally segregate its constituents. The model predicts the interspersion of proteins and lipids that undergo dynamic rearrangement through Brownian motion. Recent study data show that the compartmentalization of membrane components can be as important for effective signal transduction as the fluidity of the membrane. Studies directed at the plasma membrane have provided evidence for the existence of distinct domains in the submicron range [8]. The term *lipid rafts* was coined based on studies of epithelial cell polarity and gained widespread popularity in the past years [9]. Analysis suggested that *rafts* consist of cholesterol and sphingolipids in the exoplasmic leaflet of the lipid bilayer and cholesterol and phospholipids with saturated fatty acids in the endoplasmic leaflet. Isolating

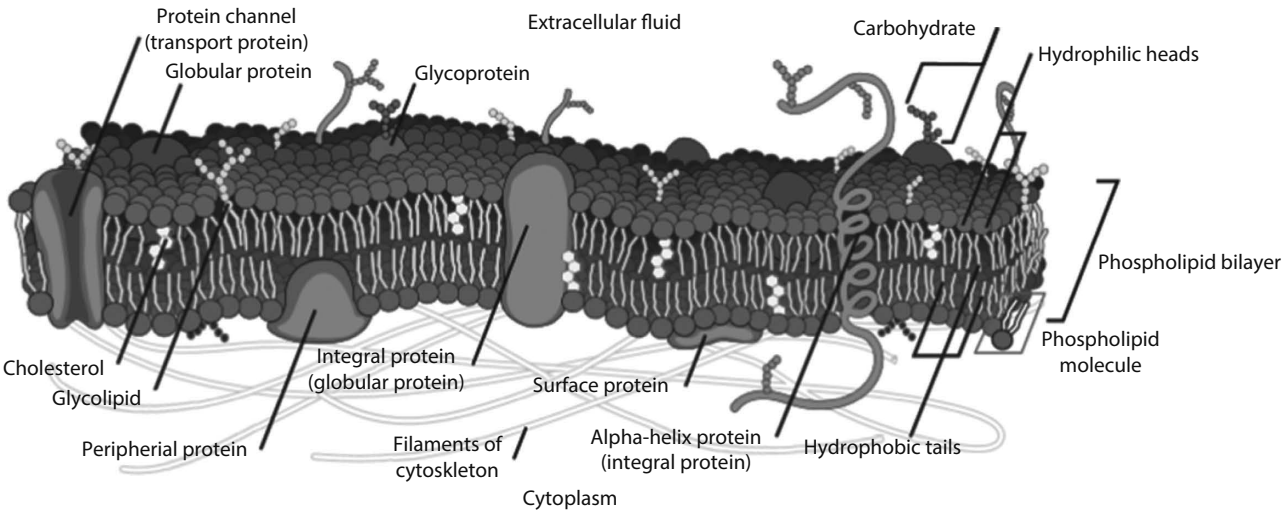

FIGURE 31.3 Schematic representation of the Singer–Nicolson membrane model. (From Wikipedia, Cell membrane detailed diagram, available online: http://en.wikipedia.org/wiki/File:Cell_membrane_detailed_diagram_en.svg, accessed March 5, 2014.)

lipid rafts as detergent-resistant membrane domains, followed by Western blotting, indicated that rafts indeed are efficient concentrators of various proteins, many of them active in cell signaling. This signaling capability is based on dynamic liquid–liquid immiscibility and underlies the raft concept of membrane subcompartmentalization. The roles of lipid rafts in cancer, in microbial pathogenesis, and in insulin resistance are not yet determined, but compelling evidence indicates the growing importance of membranes in health and disease. The *dynamically structured mosaic model* views the fluidity of the model as permitting the changes of the membrane architecture to the continuous dynamic restructuring of the clusters, which adapt to the needs of the cell and as determined by external factors [10–13].

Nutrients are absorbed across cellular membranes in the GI tract by five critical processes [14,15]:

1. *Simple passive diffusion* is the movement of substances through aqueous pores in cell membranes without the expenditure of energy. Most hydrophilic substances (molecular weight < 600) cross the membranes of the intestinal mucosa by passive transport via these aqueous pores in the mucosa. Small hydrophobic substances can also access the epithelial cell's aqueous pores by simple diffusion; however, as the size of the hydrophobic substance increases, it must cross through the lipid portions of the membrane. This movement is due primarily to the random motion that attempts to equalize the concentration of that substance on either side of the membrane. If higher concentrations of a substance exist on one side of a membrane, the law of mass tends to equilibrate the concentrations. The membrane is constructed of fat-soluble components that permit fat-soluble substances such as oxygen, nitrogen, carbon dioxide, and alcohols to easily diffuse across it.
2. *Filtration* occurs when water flows across membranes in large quantities because of hydrostatic or osmotic pressure. This water flow tends to pull some substances with molecular weights of 150–300 kDa through the same channels and tight epithelial junctions in the cells within the GI tract. The substances do not cross the membrane because of random motion, but rather they are forced through the membrane by the movement of the water. The transport of molecules via hydrostatic forces is also referred to as *solvent drag* or *bulk transport*. This type of nutrient absorption is more common in the kidney but does occur in the intestines [15].
3. *Facilitated diffusion* also known as facilitated transport or passive-mediated transport is the common mode of passive transport postulated for glucose, sodium ions, and chloride ions to migrate across the plasma membrane but to which the lipid bilayer of the membrane is virtually impermeable. In this case, the facilitated carriers are proteins that reside in the cell membrane. According to the alternating access model, the substrate binding site of a transport protein is alternately exposed to one or the other side of the membrane such that at no time is there an open and unrestricted permeation pathway through the transporter connecting the two fluid compartments separated by the membrane. The substance moves as if it were actively transported, except no energy is expended, and it is not moved against the concentration gradient. The carrier then returns to the outside of the membrane.
4. *Active transport* occurs when compounds require assistance in moving across the mucosal epithelial membrane. This is generally because the substance is large or insoluble or it is moving against a concentration gradient or otherwise defying the logics of physics in its direction of movement. Because these substances nonetheless do successfully traverse the membrane, a system of transport mechanisms has been suggested to explain the appearance of the sugars glucose and galactose; most amino acids; and minerals such as sodium, potassium, calcium, iron, chloride, and iodine, as well as some toxicants inside the mucosal epithelial cells. The divalent metal-ion transporter (DMT) assists in the GI tract absorption of metals, while the nucleotide transporter (nt) and peptide transporter (pept) are active in the transport of nucleotides and peptides, respectively. Certain characteristics exhibited by most active transport systems include the following: (1) the system is structurally specific for certain chemical features; (2) the transport system utilizes energy to function; (3) the system is competitive among other similarly structured chemicals that utilize the same transport system; (4) the system is transport-rate limiting, by either saturation or competition, and exhibits a transport maximum (Tm); and (5) the system moves substances against concentration and energy gradients. Generally, a substance being actively transported across the mucosal epithelial cell forms a membrane-bound complex on the low-concentration side of the membrane; subsequently, the substance then crosses the membrane through specific channels by the expenditure of energy, where it is released on the opposite, or high-concentration, side of the membrane.
5. *Vesicular transport*—most substances either diffuse or are actively transported into the mucosal cell membrane; however, some are ingested by a process known as vesicular transport, which includes both endocytosis (the movement of substances from extracellular spaces into intracellular spaces by engulfing them) and exocytosis (the movement of substances from the cell interior to extracellular spaces where there is transient vesicle fusion with the cell membrane at a structure called the porosome that dumps its contents out of the cell's environment). This process can be further broken down

into *pinocytosis* (or fluid-phase endocytosis), where the cell membrane engulfs dissolved substances and fluids, and *phagocytosis* (solid-phase endocytosis), where the cell membrane engulfs microscopically visible particles. The exocytic vesicles are created from a number of sources, including endosomes traversing the cell, while others are derived from the endoplasmic reticulum and Golgi apparatus expelling various substances from the intracellular space.

DIGESTIVE SYSTEM ORGANIZATION OVERVIEW

An average adult consumes about 1100 lb of food and about 150 gal of water each year. On average, approximately 10 L of chyme flows through the alimentary canal per day; about 100–150 mL of fluid remains in the feces and is egested through the anus [16,289]. The major subdivisions of the GI tract from the proximal to distal end include the mouth, where preparation of the nutrients occurs by mastication; the pharynx, which participates in swallowing; the esophagus, which functions as a conduit; the stomach, which serves as a reservoir for mixing and digestion and regulates content delivery; the small intestine, which serves as a digestive and absorptive organ; the large intestine, which absorbs water and electrolytes and processes the luminal contents for elimination; the rectum, which serves to accumulate and expel fecal material; and the anus, which controls defecation.

Digestion itself includes mechanical as well as chemical functions. The mechanical functions include chewing to reduce the size of the food to smaller particles, churning activities of the stomach that continue the reduction process, and finally the peristaltic action of the small and large intestines that propels food through the system. The chemical functions include a series of catabolic chemical reactions that ultimately break down protein into basic amino acids, convert carbohydrates into glucose, or convert fat into fatty acids and glycerol. As the food moves through the GI tract, it is mixed with various secretions that, combined with mechanical digestion, allow nutrients to pass through the walls of the epithelium cells into the body's blood and lymph capillaries.

Control of Digestive System Function

Because the digestive system is critical for survival of the organism, the entire GI tract is under robust control by electrical and chemical regulatory processes. Some of the signals originate from within the digestive tract and some originate external to the GI tract [17]. Some signals coordinate various sections of the digestive system, while others link the digestive system and the conscious brain. The central nervous system (CNS) and the GI system are in communication with each other. The CNS receives signals about gut tension, the chemical environment, and tissue conditions through afferent neurons from the GI tract [18]. The CNS, in turn, through efferent neurons, modulates the GI tract responses during the cephalic phases of digestion. The primary route of communication is through the vagal, splanchnic, and sacral nerve trunks [19].

The GI tract is unique among mammalian organs because it has its own nervous system—referred to as the *intrinsic* or *enteric nervous system* (ENS)—which is quite complex and is recognized as an integrative neuronal system separate from the CNS [20]. The ENS consists of nerve cell bodies that are embedded in the wall of the GI tract. There are about 100 million enteric neurons that are grouped in aggregates (termed enteric ganglia) that connect to one of two major ganglionated plexi in the GI tract: the myenteric or Auerbach plexus and the submucosal or Meissner plexus [21,22].

The GI tract is also affected by hormones produced in distant endocrine glands as well as many hormones produced within the GI tract itself [20,284]. The endocrine cells within the GI tract are collectively referred to as the *enteric endocrine system* (EES). The EES is scattered as single cells throughout the intestinal tract, located within the intestinal crypts and villi, and comprised 1% of the epithelial cell population [23]. There are at least 15 subtypes of enteroendocrine cells, which make up the EES that secrete multiple peptide hormones that assist in the control of physiological and homeostatic functions in the digestive tract, particularly postprandial secretion and motility [21].

Enteric Nervous System

The ENS can and does perform many of its tasks with little or no CNS control; however, normal digestive function requires coordination between the ENS and the CNS. These links are either parasympathetic connections (usually excitatory signals from the vagus and spinal nerves that stimulate motility and GI secretions) or sympathetic connections (usually inhibitory signals from the postganglionic fibers that reduce peristalsis and secretory activity), which permit signals from the CNS or the ENS to directly connect to the GI tract [21].

The entire length of the walls of the GI tract contains two complete networks of neurons. These neuronal networks include (1) the myenteric plexus (also known as the plexus of Auerbach), which is located between the circular and longitudinal layers in the muscularis and is active in the control of motility in the GI tract, and (2) the submucosal plexus (also known as the plexus of Meissner), which is located in the submucosa and is active in controlling blood flow and cellular secretions. Three basic types of neurons are located throughout the enteric plexuses. Sensory neurons respond to mechanical, osmotic, thermal, and chemical stimulation and provide the enteric plexus with a continuous and comprehensive status report on the state of the GI tract wall. Interneurons process signals from the sensory neurons and relay them to the enteric motor neurons. Motor neurons exert control over GI tract motility and some gastric secretions. These neurons secrete many neurotransmitters, but the primary excitatory neurotransmitter is acetylcholine; the primary inhibitory neurotransmitter is norepinephrine.

Enteric Endocrine System

The GI tract is the largest endocrine organ in the body, and it controls digestive function by producing hormones from many single-hormone-secreting cells that are located throughout the

mucosal of the stomach and small intestine. Over 25 hormones have been identified throughout the length of the GI tract. In 1969, Pearse [24] coined the term *amine precursor uptake and decarboxylation* (APUD) cells to describe cells dispersed throughout the body having a common ability to add and decarboxylate monoamines to bioactive amines. Characteristic amines and peptides have since been found widely distributed in not only APUD cells but also neurons located in different organs, thus giving rise to the concept of a common regulatory system termed the *diffuse neuroendocrine system* (DNES) [25,26]. APUD cells have been identified in many organs and include over 60 cell types, such as hypothalamus, adenohypophysis, pineal, parathyroid, thyroid, adrenal, placental, pancreas, GI tract, lung, urogenital tract, skin, carotid body, and sympathetic ganglia cells. These cell types have the common ability to produce biogenic amines by absorbing 5-hydroxytryptophan and L-dihydroxyphenylalanine and subsequently decarboxylating them.

Regulation of the GI tract and most other biological systems is very complex. Complicated interactions occur among the endocrine system, the CNS, the peripheral nervous system, and cells located throughout the organism. These systems communicate with neurotransmitters and neurohormones both locally and distally; thus, they appear to have regulatory roles through neurocrine, endocrine, and paracrine mechanisms. Paracrine hormones are those released by endocrine cells within the GI tract that can affect other local endocrine cells that are normally not targets for these hormones. Several peptides are active endocrine modulators of GI function and can act in a paracrine fashion as well. Some of the paracrine hormones include guanylin, histamine, motilin, neuropeptide Y (NPY), serotonin, somatostatin, substance P, uroguanylin, and vasoactive intestinal peptide. Studies have shown that immune cells produce regulatory peptides as well as antibodies that can affect cells locally and at other mucosal sites, as well as within the brain itself. This finding has led researchers to utilize the phrase *common mucosal immune system* (CMIS) [27,28]. Hence, there is an intricate and critical commonality among the endocrine, nervous, and immune systems, which act in concert to regulate the GI tract [29,282]. APUD and DNES cells are polypeptide-secreting cells that are able to act upon contiguous or nearby cells (paracrine), distant cells (endocrine), or neuronal cells (neurocrine) to regulate numerous signals. It has recently been suggested, however, that the term *diffuse neuroimmunoendocrine system* (DNIES) be used instead of DNES to more correctly describe the total integration of signaling mechanisms found in GI tract regulation [30,31]. Table 31.3 summarizes some of the most common hormones found in the alimentary canal.

Mouth and Tongue

Food enters the digestive system through the oral or buccal cavity, the outer opening of which is protected by the lips (or labia). The oral cavity is lined with mucous membranes and has as its lateral walls the cheeks; the hard palate, which forms the anterior roof, and the soft palate, which forms the posterior roof. The tongue constitutes the floor of the oral cavity and is a muscular organ that is attached underneath by the frenulum. The teeth (primarily the anterior incisors) begin the ingestion process by biting off suitable portions that can be retained in the mouth. When sufficient food is retained in the mouth, it is masticated by the molars and premolars and mechanically broken down into smaller particles by tearing, grinding, and chewing. The tongue is made up of six different muscles that allow for finite control during the mastication process. The process is aided by saliva, which is secreted from three pairs of salivary glands: parotid, submaxillary, and sublingual. A total of 1.5 L of saliva is secreted by these three glands every 24 hours [35]. Ordinarily, the mucous membranes produce only enough saliva to simply keep the mouth and pharynx moist by the parasympathetic nervous system; however, when food is introduced into the mouth, salivary secretions increase dramatically.

Food stimulates some of the 2000 taste bud receptors located primarily on the tongue. The taste buds not only provide a sense of taste to the brain but also send impulses to the salivary nuclei located in the medulla and pons. Impulses returning to the salivary glands from the salivary nuclei activate the increased secretion of saliva. Saliva contains two main types of protein secretions: (1) serous secretion by the parotid glands, which contains α-amylase and digests starches, and (2) mucous secretion by the submandibular and sublingual glands, which contains mucin for lubrication. Saliva itself is 99.5% water and 0.5% solutes [36]. The solutes include chlorides, bicarbonates, sodium phosphates, urea, uric acid, serum albumin, serum globulin, mucin, lysozyme, and α-amylase [37].

The saliva contents help liquefy the food for ease of passage through the GI tract. Ptyalin or salivary amylase initiates the breakdown of starches into glucose that also begins the chemical digestion process. Only a small portion (<5%) of the starch is actually digested in the mouth because of the quick transit time from the mouth to the esophagus and beyond. Bicarbonate ions tend to neutralize acids, and lysozyme helps to inhibit the bacteria present in many foodstuffs. Saliva also helps to cleanse the mouth and protect the teeth, as they are constantly bathed in fresh saliva. In addition to saliva, lingual lipase is secreted at the base of the tongue to initiate the process of fat digestion. It appears to have a minor impact on fat digestion [38].

Pharynx, Larynx, and Swallowing

The posterior exit of the mouth leads to the pharynx through a ring of palatine and lingual tonsils. The pharynx is the passageway for the foodstuff from the mouth to the esophagus and also serves as a conduit to the respiratory tract. Food movement must be coordinated to keep the respiratory tract clear of food as it passes on its way to the esophagus. The *swallowing reflex*, or *deglutition*, moves food into the esophagus by a combination of voluntary and involuntary muscle movements. The swallowing action moves food from the

TABLE 31.3
Enteric Hormones

Hormone	Location	Function
α-Amylase	Salivary glands and pancreas	Breaks down long-chain carbohydrates, yielding maltotriose and maltose from amylose or maltose, glucose, and *limit dextrin* from amylopectin.
Calcitonin receptor–like receptor (CALCRL)	GI tract	Receptor for peptide vasodilator; can function in the transmission of pain.
CCK	Duodenum (small intestine)	Control of pancreatic enzymes and bile secretion.
Galanin	CNS and duodenum	Implicated in many biologically diverse functions, including nociception, waking and sleep regulation, cognition, feeding, regulation of mood, and regulation of blood pressure.
Gastrin	Stomach	Control of gastric acid secretion.
Gastric-inhibitory peptide	Duodenum and jejunum (small intestine)	Control of gastric acid secretion and fatty acid metabolism.
Ghrelin	Stomach and pancreas	Promotes intestinal cell proliferation and inhibits its apoptosis during inflammatory states and oxidative stress and enhances the motility of GI tract.
Glucagon-like peptides	Ileum and the cecum, where proglucagon is cleaved into glucagon-like peptide 1 (GLP-1), GLP-2, and oxyntomodulin	GLP-1 lowers blood glucose levels and inhibits gastric and pancreatic secretions; GLP-2 may increase proliferation rate of GI tract cells; oxyntomodulin may inhibit gastric and pancreatic secretions and slow motility.
Guanylin	Small intestine	Activates production of cGMP; inhibits sodium absorption.
Ghrelin	Stomach	Control of growth hormone and energy balance.
Histamine	Small intestine	Activates acid secretion.
Insulin		
Leptin	Gastric chief cells and P/D1 cells in the stomach	Controls appetite and metabolism.
Motilin	Duodenum (small intestine)	Control of motility pattern in the small intestine.
Neuromedin U	Receptors located in CNS and GI tract	NmUR1 receptor located in the GI tract that regulates internal calcium concentration and inhibits cAMP production.
NPY	Small intestine	Stimulates appetite and increases fat storage.
Neurotensin	Endocrine cells and neurons in GI tract	Initiates smooth muscle contraction.
Peptide $YY_{3–36}$	Small intestine	Inhibits appetite and stimulates pancreatic and gall bladder secretions.
Secretin	Small intestine	Control of bicarbonate-rich secretions from the pancreas and liver.
Serotonin (5-HT)	Small intestine	Activates intrinsic motor neurons.
Somatostatin	Small intestine	Control of gastric secretions.
Substance P	Endocrine cells and neurons in GI tract	Activates pancreatic and bile secretion, increases motility, increases release of histamine.
Uroguanylin	Small intestine	Binds to guanylyl cyclase receptors and activates synthesis of cGMP.
Vasoactive intestinal peptide	GI tract	Control of musculature of GI tract; control of water and gastric acid secretions.

Sources: Data from Liddle, R.A., Gastrointestinal hormones and neurotransmitters, Chapter 1, in: *Sleisenger and Fordtran's Gastrointestinal and Liver Disease*, 9th edn., eds. M. Feldman, M.S. Friedman, and L.J. Brandt, Elsevier, London, U.K., 2010, pp. 3–25; Bowen, R., *Pathophysiology of the Digestive System*, Colorado State University, Ft. Collins, CO, 2006. Available online: http://www.vivo.colostate.edu/hbooks/pathphys/digestion/; Sternber, E.M., Interactions between the immune and neuroendocrine systems, in: *Progress in Brain Research*, Vol. 122, eds. Mayer, E.A. and Saper, C.B., Elsevier, New York, 2000, pp. 328–348; Ojeda, S.R. and Kovacs, W.J., Organization of the endocrine system, in: *Textbook of Endocrine Physiology*, 5th edn., eds. W.J. Kovacs and S.R. Ojeda, Oxford University Press, New York, 2012, pp. 3–20; Arulmani, U. et al., *Eur. J. Pharmacol.*, 500(1–3), 315, 2004; Bado, A. et al., *Nature*, 394(6695), 790, 1998.

rear of the mouth through the pharynx and past the larynx into the esophagus and ultimately into the stomach. It can be divided into three separate functions: (1) the voluntary stage, where the bolus is directed to the rear of the oral cavity and into the pharynx; (2) the pharyngeal stage, an involuntary stage where the bolus moves through the pharynx, past the larynx, and into the top of the esophagus; and (3) the esophageal stage, also an involuntary stage, where the bolus moves through the esophagus into the stomach.

When an appropriate mass of food is chewed and moistened, a bolus is moved toward the pharynx primarily by the voluntary action of the tongue and lips, which direct the food toward the pharynx. As the bolus moves out of the mouth, it produces pressure on the rear of the pharynx, which sends an impulse to the deglutition center in the medulla and pons in the brain stem. The returning impulses trigger a complicated set of involuntary muscular contractions and relaxations in the pharynx that elevate the soft palate, close off the nasopharynx,

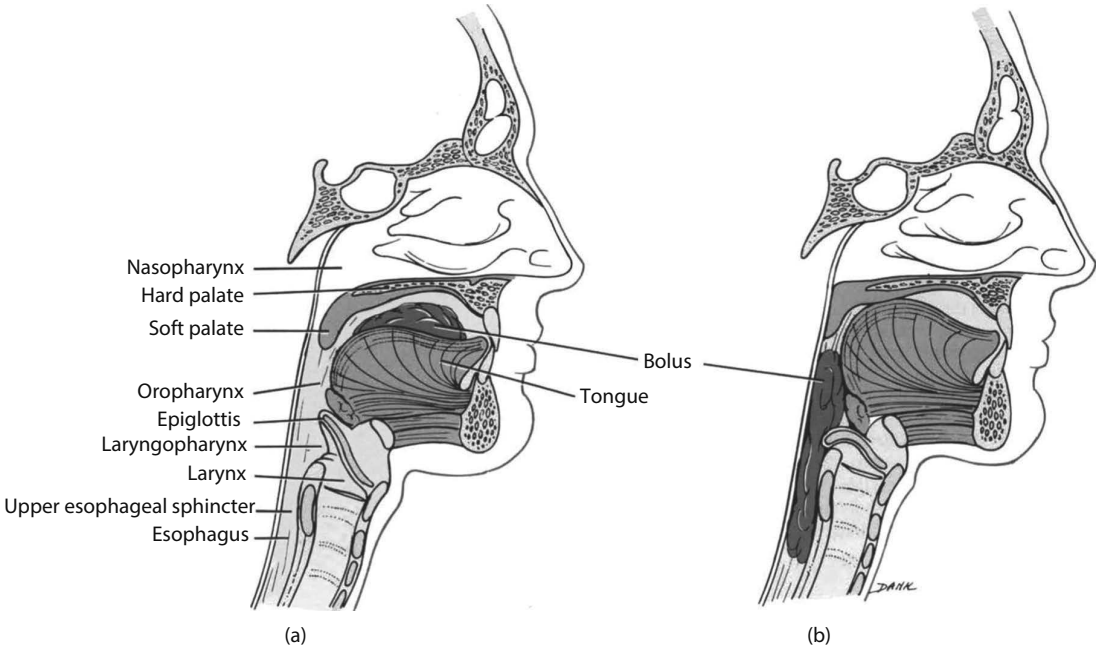

FIGURE 31.4 (a) Oral and (b) pharyngeal movements during deglutition, or the swallowing reflex. (From Tortora, G.J. and Anagnostakos, N.P., The digestive system, Chapter 24, in: *Principles of Anatomy and Physiology*, 6th edn., HarperCollins: New York, 1990, p. 743. With permission.)

and push the food downward toward the larynx. These contractions elevate the hyoid and larynx and move the epiglottis downward, closing the entrance to the trachea to prevent food from entering the respiratory tract. When the bolus has passed the larynx and entered the esophagus, the epiglottis reopens and breathing resumes. Figure 31.4 illustrates the swallowing process. A summary of the basic interactions and functions of the swallowing reflex is presented in Table 31.4.

Esophagus

The primary function of the esophagus is to simply transport masticated foodstuffs from the oral cavity to the stomach. A bolus of food leaves the oral cavity and continues to move through the cricopharyngeal sphincter located at the upper end of the esophagus that subsequently closes, preventing upward movement of the bolus. The esophagus is the third and final organ involved in the swallowing reflex process. Normally, this sphincter is contracted, closing the mouth of the esophagus. The esophagus is a muscle-walled tube about 10 inches long that connects the pharynx to the stomach. It passes behind the heart through the diaphragm and into the abdominal cavity. In its resting stage, the esophagus is collapsed. The upper third of the esophagus contains striated muscle, while the lower two-thirds contains smooth muscle. When the bolus has entered the esophagus, its movement is

TABLE 31.4
Summary of the Swallowing Reflex

Structure	Activity	Function	Control Status
Mouth/teeth/tongue	Tearing, mastication, and bolus manipulation	Preparation of appropriate-sized bolus; movement into the pharynx	Voluntary
	Secretion of mucus	Lubrication for passage into pharynx	Voluntary/involuntary
Pharynx	Triggers deglutination center in brainstem	Elevates the soft palate, closes off the nasopharynx	Involuntary
Larynx	Impulses received from brainstem	Elevates the hyoid and larynx and moves epiglottis over trachea	Involuntary
Esophagus	Receipt of bolus from pharynx	Opening of upper esophageal sphincter	Involuntary
	Initiation of peristalsis	Movement of bolus through esophagus	Involuntary
	Secretion of mucus	Lubrication for passage into stomach	Involuntary

Sources: Data from Tortora, G.J. and Derrickson, B., The digestive system, Chapter 24, in: *Principles of Anatomy and Physiology*, 13th edn., John Wiley, Hoboken, NJ, 2012, pp. 967–1023; Tamir, E., *The Human Body Made Simple*, 2nd edn., Churchill Livingstone, Edinburgh, U.K., 2002, pp. 71–92.

controlled by the smooth muscles in the wall of the esophagus; they contract in a rhythmic sequence and propel the bolus downward toward the stomach. These waves of muscular contractions are called *peristaltic waves* and are the mode of transit throughout the remainder of the GI tract. In the esophagus, the transit time is quite brief and no meaningful digestion occurs in the esophagus.

The lower end of the esophagus just above the level of the diaphragm has a sphincter called the *lower esophageal* or *gastroesophageal sphincter.* This structure helps separate the lumen of the esophagus from the lumen of the stomach and prevents reflux of the stomach contents backward into the esophagus. Unlike the rest of the GI tract, the esophagus does not have an outer serosa covering, which makes it susceptible to the spread of tumor cells. Blood is supplied from the inferior thyroid artery to the upper esophagus, the tracheobronchial arteries to the middle esophagus, and the left gastric artery to the middle esophagus. The esophagus is also drained of blood by three venous pathways: the upper third into the superior vena cava, the middle third into the azygous system, and the lower third into the portal vein.

Sensory stimulation of the esophagus is transported through the vagus nerve to the tractus solitarius. The thoracic sympathetic nerves also carry afferent nerve impulses to the esophagus. The parasympathetic nervous system is primarily derived from the vagus nerve, with supplemental impulses from the upper portion by the glossopharyngeal and spinal accessory nerves [40–42]. The peristaltic action in the esophagus is initiated by the presence of the bolus, which expands the esophageal walls; subsequently, stretch receptors are activated to contract behind the bolus and relax in front of the bolus. This occurs as one continuous wave that propels the bolus past the sphincter and into the stomach. The esophageal lumen is lined with stratified squamous epithelia, which are continually being renewed. As the squamous cells migrate toward the lumen, they change physical characteristics and are eventually sloughed off [43–45]. The outer lumen is protected by a coat of keratin that can resist degradation by the various proteolytic enzymes. Mucus-secreting glands located in the submucosal layer lubricate the esophagus and assist with the passage of the bolus. There are species differences in the amount of keratin present (e.g., dogs have less keratin than rodents do) and in the density of the mucous glands (e.g., rodents have none).

Stomach

The bolus continues from the lower esophageal sphincter into the stomach through an upper opening termed *cardia*. There is no circumferential muscle at the cardia; however, the anatomical structure of the area prevents the reflux of the stomach contents backward into the esophagus. The stomach is a J-shaped muscular structure with a capacity of about 2 L. It is located in the upper left anterior quadrant of the abdominal cavity. Much of the surface of the stomach is behind the lower rib cage. The stomach serves as a reservoir and as a digestive organ. It is divided into four distinct areas: the cardiac region, the fundus, the corpus, and the pyloric region [46]. The part of the corpus that is adjacent to the pyloric region is sometimes referred to as the *antrum* [47,48]. The structure and cellular composition of the tall columnar stomach epithelium remain constant throughout the different areas of the stomach. These cells contain mucus-producing apical vacuoles that produce a high-molecular-weight polymer consisting of a protein backbone with carbohydrate side chains that are held together by disulfide bonds [49]. This polymer forms a 1 mm thick secretory sheath that protects the mucosa from the acidic gastric fluids found in the stomach, most likely by retarding the normal diffusion of hydrogen ions [288]. These surface epithelia are renewed every 72 h by the isthmus of the gastric glands, where mitotic activity is increased and the new cells migrate toward the surface.

The major biochemical feature of the stomach is its acid-secreting properties. The stomach mucosa contains many folds, called *rugae*, which further contain narrow openings, termed *gastric pits*, which are formed as a result of mucosal cells penetrating the connective tissue downward into the lamina propria. The wall of the stomach is lined with gastric glands that are interspersed in the surface or in these gastric pits. The gastric pits contain, besides the previously noted mucous cells, three types of cells: parietal cells, which are found in the middle of the pits and produce hydrochloric acid and intrinsic factor; chief cells, also known as zymogenic cells, which are found at the bottom of the pits and produce pepsinogen, prochymosin, and gastric lipase; and enteroendocrine or G cells, which are found in the glands in the lower fundus or antrum and produce gastrin. During a meal, the stomach gradually fills up to a capacity of approximately 1–2 L. The secretions of the various cells types are controlled by impulses from the vagus nerve and by GI reflexes mediated through neural, autacoid, and endocrine signals [50,51]. Figure 31.5 shows the general organization of the stomach.

Hydrochloric acid does not directly function in the digestion process; however, it inhibits microorganisms and lowers the stomach acidity, which, in turn, inhibits emptying of the stomach. In addition, stomach pH is a critical determinant in the absorption kinetics of electrolytes and it activates pepsinogen. Carbohydrate digestion, which begins in the mouth upon exposure of the foodstuff to amylase, continues as the bolus passes into the stomach. The bolus is further broken down into acid chime in the lower third of the stomach that inhibits further carbohydrate breakdown. At this point, the enzyme pepsinogen initiates protein digestion. Pepsinogen is activated by hydrochloric acid cleaving off a portion of the molecule, thus creating the enzyme pepsin, which breaks off peptides from proteins. Intrinsic factor is a protein that binds vitamin B_{12} to enable absorption by the ileum of the small intestine. Table 31.5 presents a summary of mucosal secretions in humans.

When sufficient food enters the stomach, peristaltic movement, termed *mixing waves*, passes through the stomach every 15–25 s [37]. This movement combined with the stomach secretions reduces the foodstuff to a thin liquid termed *chyme*. The fundus section of the stomach shows little mixing movement and can function as a holding area for the chyme for up to an hour or more. During the time in the fundus,

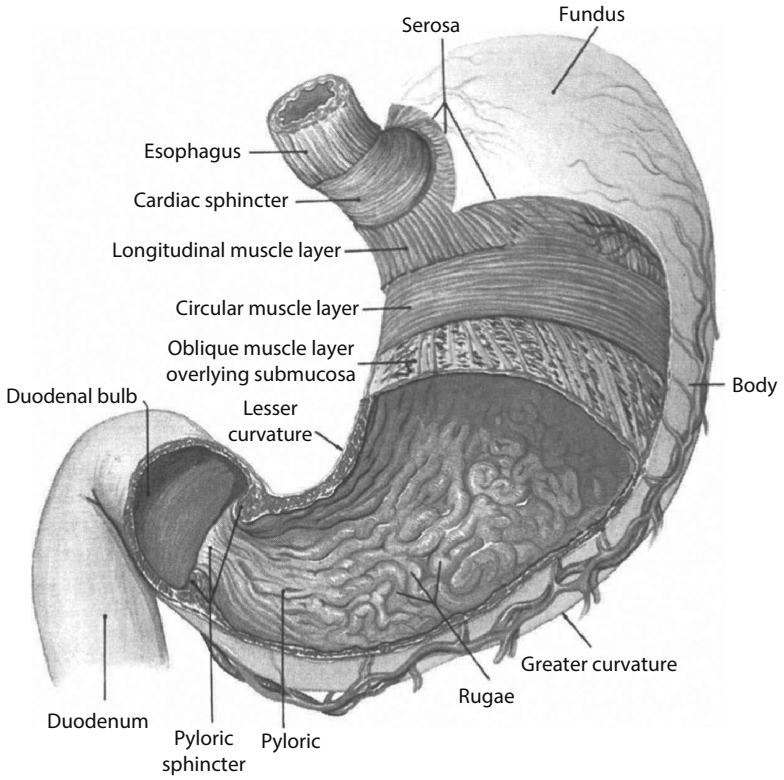

FIGURE 31.5 Structure and general organization of the stomach. (From Raufman, J.-P., Stomach: anatomy and structural anomalies, Chapter 64, in: *Textbook of Gastroenterology*, vol. 1, 4th edn., Yamada, T. et al. (eds.). Lippincott Williams & Wilkins, Philadelphia, PA, 2003, p. 1283. With permission.)

amylase digestion continues. The peristaltic wave action of the stomach walls propels the contents toward the pyloric sphincter. The pylorus is usually nearly closed and permits only very thin (and hence thoroughly digested) chyme into the duodenum of the small intestine. The larger contents are then turned back into the body of the stomach for further agitation and breakdown into smaller particles. Food remains in the stomach for variable periods of time depending on its size and composition. This period can be from 30 min to several hours or longer. The continuous peristaltic action of the stomach and the degree of contraction of the pyloric sphincter allow for the proper amount of chyme to enter the small intestine to allow proper absorption of nutrients. Little absorption occurs in the stomach because the stomach wall

TABLE 31.5
Summary of Stomach Mucosal Secretions

Structure	Activity	Function
Mucous cells	Secretion of mucus	Prevents mucosal wall digestion
Parietal cells	Secretion of hydrochloric acid	Converts pepsinogen to active form (pepsin); maintains pH at ~2 for optimal pepsin functioning
	Secretion of intrinsic factor	Necessary for vitamin B_{12} absorption (required for RBC formation)
	Secretion of hydrogen potassium ATPase	Necessary for the acidification of the stomach contents
Chief (zymogenic) cells	Secretion of pepsinogen	Provides pepsin precursor that breaks down protein chains into peptides for absorption
	Secretion of gastric lipase	Assists in the breakdown of certain triglycerides with short-chain fatty acids
	Secretion of prochymosin	Provides chymosin precursor that coagulates milk protein, allowing it to be retained longer in the stomach for more complete digestion
Enteroendocrine (G) cells	Secretion of gastrin	Stimulates further gastric secretion, closes lower esophageal sphincter, increases muscular activity in the stomach, and opens pyloric sphincter

Source: Data from Tortora, G.J. and Derrickson, B., The digestive system, Chapter 24, in: *Principles of Anatomy and Physiology*, 13th edn., John Wiley, Hoboken, NJ, 2012, pp. 967–1023.

is impermeable to most materials. However, there are a few exceptions because the stomach does absorb small amounts of electrolytes, water, alcohol, and acetosalicylic acid.

Regulation of Gastric Secretion

The secretion of gastric enzymes is controlled in three separate phases: (1) cephalic phase, (2) gastric phase, and (3) intestinal reflex [37,52]. The cephalic phase is stimulated by chemoreceptors and mechanoreceptors in the nasal and buccal cavities; these receptors send impulses from the cerebral cortex in the hypothalamus through the vagus nerve that cause the release of acetylcholine, gastric lipase, and gastrin-releasing peptides from interneurons in the stomach. These impulses arising from the sight, smell, or even thought of food act on the parietal cells and G cells. In this way, the body prepares gastric secretion levels in the stomach to receive the anticipated food.

The gastric phase is both nerve and hormonally controlled. When food reaches the stomach, it causes distention and stimulates nervous receptors in the stomach wall. Distention activates the long vagovagal reflexes and the short local reflexes, which, in turn, send impulse to the medulla and back to the stomach, which release acetylcholine [51]. The release of acetylcholine stimulates the flow of hydrochloric acid from parietal cells and gastrin from G cells. The presence of peptides will also stimulate the production of hydrochloric acid through gastrin release by direct action on the G cells. This phase controls about 80% of the secretions released from the stomach [52].

The intestinal phase accounts for a small amount of gastric secretion. The presence of chyme in the duodenum stimulates the mucosa to release enteric gastrin. This substance causes the circulating gastrin to increase acid production through activation of the G cells, but in limited amounts. A summary of the factors that influence the stimulation of gastric secretion is provided in Figure 31.6.

The inhibition of gastric secretion occurs when partially digested carbohydrates and lipids enter the duodenum and initiate the enterogastric reflex, which involves impulses sent from the walls of the duodenum to the medulla and back to the gastric glands in the stomach. Additional chemical regulatory actions that ensure that excess acid production does not accumulate in the stomach include the following [52]:

- *Lowered pH*—gastric secretion is inhibited at pH < 3.
- *Cholecystokinin* (*CCK*)—triggered by the presence of chyme lipids and carbohydrates, this hormone is secreted by the intestinal mucosa; it inhibits gastric secretions, decreases GI tract motility, stimulates secretions from the pancreas while relaxing the sphincter at the hepatopancreatic ampulla, and increases the ejection of bile from the gall bladder.
- *Gastric-inhibitory peptide*—in the presence of fatty acids in the duodenum, this hormone is secreted by the intestinal mucosa; it inhibits gastric secretions and decreases GI tract motility.

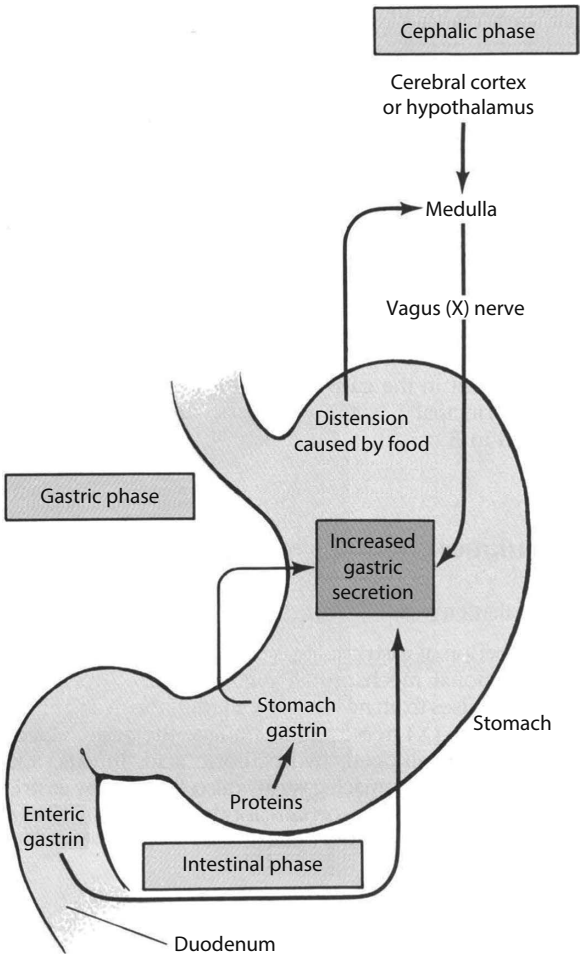

FIGURE 31.6 Schematic of the phases of gastric secretory regulation. (From Tortora, G.J. and Anagnostakos, N.P., The digestive system, Chapter 24, in: *Principles of Anatomy and Physiology*, 6th edn., HarperCollins, New York, 1990, p. 750. With permission.)

- *Secretin*—in the presence of acid, chyme, or fluids in the duodenum, this hormone is secreted by the intestinal mucosa; it inhibits gastric secretions, decreases GI tract motility, and stimulates secretions from the pancreas, liver, and small intestinal glands.

Small Intestine

The small intestine is where the digestion of fat, protein, and almost all carbohydrates is completed and much of the absorption of nutrients occurs. The small intestine begins at the pylorus of the lower end of the stomach and ends at the juncture of the large intestine, a 15–20 ft distance [37,53]. The tube averages approximately 1 in. in diameter and is subdivided into three segments. The duodenum connects to the stomach and is the shortest segment at about 10 in. The jejunum is about 8–10 ft in length and extends to the ileum. The ileum is about 10–12 ft in length and attaches to the large intestine at the ileocecal sphincter. Most digestion occurs in the duodenum, where digestive enzymes are added from the

pancreas, gallbladder, and glands in the wall of the duodenum. The jejunum and ileum primarily absorb the digested nutrients from the duodenum.

The entire small intestine is comprised of the four basic layers that make up the vast majority of the GI tract. There are some variations that permit the small intestine to finish the digestion process and initiate the majority of the absorption process. The small intestine mucosa contains gastric pits that are lined with glandular epithelium glands called the *crypts of Lieberkühn*. These glands secrete *intestinal juice*, which is a clear, yellow, alkaline fluid containing primarily water and mucus in amounts of up to 3 L/day. Additional enzymes such as maltose, α-dextrinase, sucrase, lactase, aminopeptidase, dipeptidase, ribonuclease, and deoxyribonuclease are added to the chyme in the small intestines. Many of these enzyme-producing cells digest the chyme on the cell surface rather than in the lumen of the intestine. This intestinal juice is quickly reabsorbed by the small intestinal villi, and that physical process brings the chyme into the proximity of epithelial cells that secrete intestinal enzymes. The submucosa contains duodenal Brunner's glands, which secrete alkaline mucus. As in the stomach, the mucus protects the mucosa from breakdown by the hydrochloric acid and helps to neutralize acid present in the chyme.

The structure of the small intestine is especially suited for digestion and absorption. Absorptive cells with finger-like projections, called *microvilla*, greatly expand the surface area of the membrane. The mucosa is arranged in a series of villa that protrude into the intestinal lumen and further increase the functional surface area. Each villus has a core of lamina propria. Embedded in this connective tissue are an arteriole, a venule, a capillary network, and a lymphatic vessel (lacteal), which allow nutrients to pass through the capillary wall directly into the lymphatic and cardiovascular systems. Additional projections, called plicae circulares, are located in the proximal end of the duodenum and terminate at about the middle portion of the ileum. These plicae circulares not only increase the surface area but also cause the chyme to swirl as peristalsis moves it through the small intestine, thus increasing the absorption process. These adaptations in surface area increase the absorptive surface area many times and provide an estimated 250–500 square yards of absorptive surface. Finally, located primarily in the lower part of the small intestine (the ileum) are 30–40 lymphatic nodules called Peyer's patches, which are part of the autoimmune system; they detect microorganisms and produce antibodies designed to fight infection [53,54]. In addition to its own enzyme secretions, the small intestine utilizes the fluid from three additional structures: (1) the pancreas, (2) the liver, and (3) the gallbladder. The contributions of each of these accessory organs are briefly summarized in the following.

Pancreas

The pancreas is a tubuloacinar gland that measures approximately 1 in. wide by 5 in. in length. The pancreas contains two types of glands. One type is hormonal in nature and consists of clusters of glandular epithelial cells called *pancreatic islets* (islets of Langerhans), which produce the hormones glucagon, insulin, and somatostatin. The remaining cells, called *asini*, comprise a vast majority of the organ; they represent the exocrine part of the pancreas and produce digestive enzymes or pancreatic juice. Up to 2 L of clear, colorless pancreatic juice is produced each day. The asini communicates directly with the intralobular ducts, which eventually collect into the major pancreatic duct or the duct of Wirsung. Generally, this duct unites with the common bile duct, which enters the upper end of the duodenum in a duct common to both organs, termed the *hepatopancreatic ampulla* or the *ampulla of Vater*. This digestive fluid consists of water, sodium, potassium, sodium bicarbonate, and various enzymes, including pancreatic amylase, trypsinogen, chymotrypsinogen, procarboxypeptidase, proelastase, pancreatic lipase, ribonuclease, and deoxyribonuclease. Trypsinogen, chymotrypsinogen, procarboxypeptidase, and proelastase are activated by various enzymes within the small intestine; they are produced in an inactive form in the pancreas so as not to break down and digest the pancreas itself. The pancreas is regulated by nervous and hormonal mechanisms. Impulses are also sent along the vagus nerve concurrent with the cephalic and gastric phases of gastric regulation noted previously, which stimulates the production of pancreatic juice. Hormonal regulation includes responses from chyme entering the duodenum that can release secretin or CCK. Secretin, which is produced by S cells of the duodenum in response to hydrogen ions, stimulates the production of pancreatic juice rich in sodium bicarbonate ions; CCK, which is produced by the I cells of the duodenum in response to peptides and fatty acids, stimulates the production of pancreatic juice rich in digestive enzymes [56,57]. The general structure and relative location of the pancreas are shown in Figure 31.7.

Liver

The liver is a triangular/cone-shaped organ located under the diaphragm; it weighs about 3 lb in the average adult. In addition to its digestive functions, the liver also participates in the detoxification of blood, synthesis of blood proteins, replenishment of erythrocytes, storage of glucose as glycogen, and the production of urea. The digestive functions include the production of bile. The liver consists of two main lobes—a smaller left lobe and a larger right lobe—both of which are made up of thousands of lobules separated by the falciform ligament. Contained within each lobule are an arteriole, a venule, and a biliary duct. Arteriole blood and venous blood are mixed in the canals of these lobules. Hepatocytes are in direct contact with the blood flowing in these canals. The biliary ducts in the canals serve to drain the bile from hepatocytes into the lobules. These lobules are connected to small ducts that connect with larger ducts to ultimately form the hepatic duct. The liver produces and secretes as much as 1 L of bile each day, and the hepatic duct transports the bile to the gallbladder and upper section of the duodenum. Bile is a watery green fluid with a pH of 7.6–8.6; it contains bile salts,

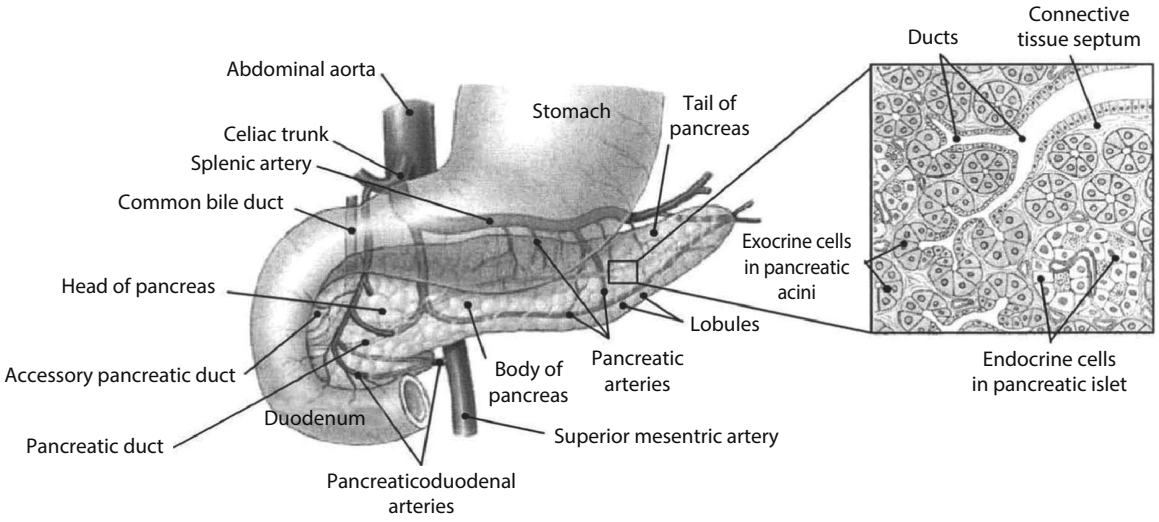

FIGURE 31.7 Structure and general organization of the pancreas. (From Martini, F.H., *Fundamentals of Anatomy and Physiology*, 7th edn., Pearson Education, Glenview, IL. Copyright © 2006 by Frederick H. Martini, Inc. With permission.)

bile pigments (primarily bilirubin), cholesterol, neutral fats, electrolytes, and a phospholipid called *lecithin*. Bile salts and lecithin emulsify fats, and the remaining fluid contents are excreted as waste. Bile salts are emulsifiers that break down fat globules into small (1 μm diameter) droplets that are more easily absorbed in the presence of pancreatic lipase. Absorption of cholesterol is enhanced by bile salts and lecithin. The liver is also regulated by nervous and hormonal mechanisms. Vagus nerve impulses concurrent with the cephalic and gastric phases of gastric regulation noted previously can double the production of bile. Hormonal regulation includes the release of secretin when chyme enters the duodenum. Secretin, which is produced by S cells of the duodenum in response to hydrogen ions, stimulates the production of bile. High blood concentrations of bile salts can also act to increase the rate of bile secretion [58,59]. The structure and general organization of the liver are shown in Figure 31.8.

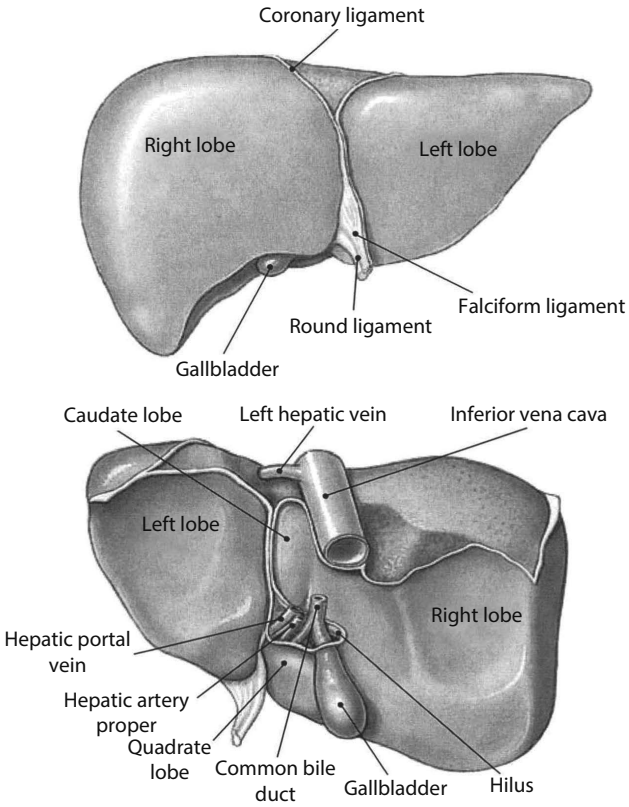

FIGURE 31.8 Structure and general organization of the liver. (From Martini, F.H., *Fundamentals of Anatomy and Physiology*, 7th edn., Pearson Education, Glenview, IL. Copyright © 2006 by Frederick H. Martini, Inc. With permission.)

Gallbladder

The gallbladder is a hollow, muscular, pear-shaped organ about 3–4 in. in length located immediately under the liver. The primary function of the gallbladder is to store, concentrate, and deliver bile from the liver to the small intestine. The gallbladder is normally relaxed and full of bile between meals. The gallbladder lumen mucosa contains simple columnar epithelial cells arranged in deep folds. The middle layer possesses smooth muscle instead of a submucosa that allows for the constriction process during the emptying of the organ. The outer layer consists of the visceral peritoneum. During the concentration of the liver bile, water and ions are absorbed through the mucosa. This organ delivers about 500 mL of bile to the duodenum each day. As noted in the sections on the pancreas, CCK, which is produced by the I cells of the duodenum in response to peptides and fatty acids, not only stimulates the production of pancreatic juice rich in digestive enzymes but also stimulates the contraction of the musculature of the walls of the gallbladder while simultaneously relaxing the sphincter at the mouth of the common bile duct, thus allowing the gallbladder to empty its contents into the duodenum [60,61]. The structure and general organization of the gallbladder and biliary ducts are shown in Figure 31.9.

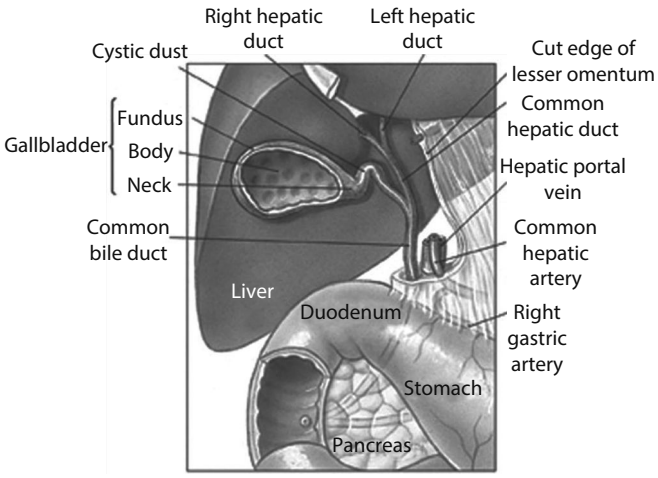

FIGURE 31.9 Structure and general organization of the gallbladder and biliary ducts. (From Martini, F.H., *Fundamentals of Anatomy and Physiology*, 7th edn., Pearson Education, Glenview, IL. Copyright © 2006 by Frederick H. Martini, Inc. With permission.)

Digestion and Absorption in the Small Intestine

The digestion process in the small intestine can be divided into the mechanics of movement and the chemical breakdown of foodstuffs. Movements in the small intestine include segmentation and peristalsis. Segmentation is a localized contraction that is not involved in the forward motion of the chyme through the GI tract. Segmentation simply forces the chyme into close proximity with the surface of the small intestine by contractions of 12–16 times/min [37]. Segmentation is primarily controlled by nerves in the intestinal walls that detect distention. Peristalsis, on the other hand, is a series of contractions and relaxations propelling the chyme through the GI tract. The peristalsis in the small intestine is very slow and deliberate, pushing the chyme through at a rate of about 1 cm/min and leaving the chyme in the small intestine for approximately 3–5 h. Peristalsis is similarly controlled by nerves in the intestinal walls that detect distention.

The chemical breakdown of the chyme begins as the article of food enters the GI tract in the mouth. The ptyalin in saliva initiates the conversion of starch to maltose. The stomach pepsin initiates the conversion of proteins to peptides. As the chyme enters the duodenum, it contains partially digested carbohydrates and proteins. The lipids are essentially undigested at this point in the process. As the chyme traverses the length of the small intestine, the digestive process is completed [62].

When the foodstuffs are prepared and digested as described earlier, the next critical phase is the absorption of the nutrients, which move across the epithelial cell lining of the GI tract and into the blood and lymphatic circulatory systems. It is estimated that about 90% of absorption occurs within the small intestine. The remainder occurs in the stomach prior to the foodstuff entering the small intestine or after the foodstuff leaves the small intestine and enters the large intestine. Absorption occurs in the villi by one of the following mechanisms: passive diffusion, facilitated diffusion, osmosis, or active transport. Roughly 10 L of fluid enters the small intestine and is absorbed each day. About 1–1.5 L is derived from saliva, 2–3 L is secreted by the stomach, about 2 L is secreted by the glands of the small intestine, about 1 L is contributed by the pancreas, and another liter enters as bile. Approximately 2 L is ingested as food and fluid each day. Of the 10 L, about 90% of the liquid is absorbed via osmosis by the blood capillaries of the villi in the small intestine [16,37,289]. The remaining 1 L is what actually passes through the ileocecal sphincter into the large intestine. The normal rate of absorption is approximately 200–400 mL/h.

It has been shown that the water moves in either direction across the intestinal mucosal cells to maintain an osmotic balance between the surface of the lumen, the cell interior, and the blood during uptake of elements of the chyme as well as electrolytes. Absorption in the small intestine is passive and requires the movement of solutes, so the rate of water uptake is a function of the solute absorption. Active transport of sugars and amino acids in the jejunum, in turn, causes passive movement of salt and water across the mucosal epithelial cells (cotransport). In the ileum, on the other hand, most of the water movement is by active transport via a specialized mechanism that pumps sodium into the lateral spaces within the cells. Water subsequently enters the lateral spaces from the cell (transcellular flux) or from the lumen (paracellular flux), resulting in an increased hydrostatic pressure and the transport of isotonic fluid into the extracellular space.

GI secretions and components of various ingested foods are sources of electrolytes for absorption in the small intestine. Intestinal transport of sodium has both active and passive mechanisms. Active transport can be independent or linked to the transport of other solutes such as sugar in a relationship referred to as cotransport. It is believed that cotransport accounts for most of the sodium absorption in the small intestine. The energy source for active sodium transport is the hydrolysis of adenosine triphosphate (ATP). Passive transport of sodium occurs primarily through the lateral spaces. Interestingly, in the presence of bicarbonate, sodium can be absorbed against an electrochemical gradient. It is suspected that a sodium–hydrogen exchange can account for this absorption. Chloride absorption is closely associated with sodium absorption by passive diffusion via the paracellular route, Na^+Cl^- cotransport, and Cl^-–HCO_3 exchange. Iodine and nitrate ion absorption is also closely related to sodium absorption and usually passively follows the sodium ions. Evidence suggests that these electrolytes can also be actively transported. Potassium absorption across the intestinal mucosal cells is determined by differences between two opposing unidirectional fluxes down the concentration gradient; it is passive in nature.

Gastric acid solubilizes calcium salts, which permits their being actively transported as the divalent cation. Parathyroid hormone in the presence of vitamin D (1,25-dihydroxycholecalciferol) increases the rate of Ca^{2+} absorption. Iron can be absorbed as heme iron, which is iron bound to hemoglobin, or as free Fe^{2+}. In the cytoplasm of the mucosal epithelial cells, the heme iron is broken down to the free Fe^{2+}, which

binds to apoferritin. As the free Fe^{2+} circulates in the blood, it binds to transferrin, which transports the free Fe^{2+} from the small intestine to the liver for storage.

Fat-soluble vitamins A, D, E, and K are incorporated into micelles and absorbed along with other lipids. Most water-soluble vitamins, including C and B complex (but not B_{12}, which requires intrinsic factor from the stomach), are absorbed by sodium-dependent cotransport or facilitated diffusion. Vitamin B_{12} absorption depends on gastric acid, pepsin, and intrinsic factor secreted from gastric parietal cells and the pancreatic duct cells. Intrinsic factor binds to and protects vitamin B_{12} from the digestion processes in the small intestine. Vitamin B_{12} binds to specific receptor sites in the mucosal epithelial cells of the ileum, where it enters by vesicular transport. Fat-soluble vitamins such as A, D, E, and K appear to be absorbed in micelles along with the dietary fats and are released into the lymphatic circulatory system. Water-soluble vitamins, such as the B vitamins and vitamin C, are absorbed across the GI tract by simple diffusion.

These digestion and absorption processes can also be categorized by type of substance entering the GI tract. The primary categories of foodstuffs include the following four major categories:

- Carbohydrates
- Proteins
- Lipids
- Nucleic acids

Carbohydrates

Generally, the digestion of carbohydrates occurs through the catalytic hydrolysis of α-1,4-glycosidic bonds situated between monosaccharides. The reaction is initiated by ptyalin, which is inactivated in the stomach by the acidic environment; however, it is completed by pancreatic amylase in the small intestine. These enzymes break down starches to the disaccharide maltose and to glucose polymers between three and nine molecules in length. It is believed that the rate of digestion of carbohydrate determines the place and form in which carbohydrate is absorbed [288]. Pancreatic amylase and ptyalin do not hydrolyze α-1,6-glycosidic bonds. As these maltose and other polysaccharides approach the lumen, they are exposed to maltose, sucrase, and lactase, which break down the disaccharides and glucose polymers to various monosaccharides such as glucose, fructose, and galactose. The branched polysaccharides are hydrolyzed by the action of the enzyme α-dextrinase. Table 31.6 provides a summary of the critical digestive processes of carbohydrates. Polysaccharides are not absorbable into the small intestine; hence, they must be reduced to monosaccharides, which are easily absorbed. Glucose and galactose are transported into cells by a sodium-dependent cotransport mechanism that transports glucose and sodium ions. The process uses energy generated by the sodium ion gradient to transport glucose and galactose into the epithelial cell. The transporter does not move sodium ions unless glucose is chemically bound to it. Once in the cell, fructose is converted from fructose to glucose. The resulting intracellular glucose from all sources is subsequently transported out of the cell into the extracellular space from that point by facilitated diffusion [63]. Table 31.7 provides a summary of the critical absorption mechanisms for carbohydrates found in the GI tract.

Proteins

Protein digestion is initiated in the stomach by the secretion of pepsinogen, which is activated to its active form—pepsin—in

TABLE 31.6
Summary of Carbohydrate Digestion

Enzyme/Digestive Fluid	Source	Process	Result
Ptyalin (α-amylase)	Salivary glands	Converts polysaccharides (starch) to disaccharides; the process is initiated in the mouth and is incomplete when the chyme reaches the small intestine.	Incomplete reaction to maltose (disaccharide); not absorbable in this form
Pancreatic amylase (α-amylase)	Pancreas	Completes the process initiated by the pytalin.	Maltose (disaccharide); not absorbable in this form
Maltase	Small intestine	Splits the disaccharide maltose into a monosaccharide.	Glucose (monosaccharide)
α-Dextrinase (isomaltase)	Small intestine	Hydrolyses 1,6-α-D-glucosidic bonds in dextrins and isomaltose produced after α-amylase degradation of starch and glycogen.	Glucose (monosaccharide)
Sucrase	Small intestine	Splits the disaccharide sucrose into two monosaccharides.	Glucose + fructose (monosaccharides)
Lactase (lactase–phlorizin hydrolase)	Small intestine	Splits the disaccharide lactose into two monosaccharides.	Glucose + galactose (monosaccharides)

Sources: Data from Nater, U.M. et al., *Int. J. Psychophysiol.*, 55(3), 333, 2005; Sibley, E., Carbohydrate assimilation, Chapter 17, in: *Textbook of Gastroenterology*, 5th edn., ed. T. Yamada, Wiley Blackwell Publishing, Hoboken, NJ, 2009, pp. 429–444; Wong, J.M.W. and Jenkins, D.J.A., *J. Nutr.*, 137, 2539S, 2007.

TABLE 31.7
Summary of Carbohydrate Absorption

Type of Nutrient	Type of Movement	Site of Transport	Destination
Maltose (disaccharide)	Not absorbable in this form	—	—
Glucose (monosaccharide)	Sodium-dependent active transport	Villi (small intestine)	Bloodstream to hepatic portal vein
Fructose (monosaccharides)	Facilitated diffusion	Villi (small intestine)	Bloodstream to hepatic portal vein
Galactose (monosaccharides)	Sodium-dependent active transport	Villi (small intestine)	Bloodstream to hepatic portal vein

Source: Data from Sibley, E., Carbohydrate assimilation, Chapter 17, in: *Textbook of Gastroenterology*, 5th edn., ed. T. Yamada, Wiley Blackwell Publishing, Hoboken, NJ, 2009, pp. 429–444.

the presence of hydrochloric acid. Interestingly, pepsin is the only enzyme capable of also digesting collagen. Additional inactive proteases are released by the pancreas in response to CCK. Trypsinogen is activated to trypsin, its active form, by enterokinase or enteropeptidase in the duodenum. Trypsin subsequently activates the additional pancreatic enzymes chymotrypsinogen and procarboxypeptidase to their active forms—chymotrypsin and carboxypeptidase. This protease activity converts proteins to one of the following: amino acids, dipeptides, or tripeptides. This breakdown of proteins is completed by the enzymes aminopeptidase and dipeptidase, both of which are secreted by enterocytes of the small intestine located on the lumen. Table 31.8 provides a summary of the critical digestive processes of proteins. In contrast to carbohydrates, which must be monosaccharides to be absorbed, the digested protein products of proteins can be absorbed as amino acids, dipeptides, and tripeptides. Once the dipeptides and tripeptides are transported into the intestinal cells, however, cytoplasmic peptidase hydrolyzes these peptides to amino acids. These substances are absorbed into and out of the epithelial cells by specific sodium-dependent amino acid cotransporters: neutral, acidic, basic, and imino [52]. Table 31.9 provides a summary of the critical absorption mechanisms of proteins.

Lipids

Lipids in the diet include triglycerides, neutral fats, cholesterol, cholesterol compounds, and phospholipids. As fats are ingested, they do not dissolve in water; fats tend to congeal into large masses, and this separation of lipids and water limits the effectiveness of the lipase–fat-digesting enzymes. Lipid digestion occurs primarily in the small intestine and involves the emulsification of fats by bile. Bile acids are derivatives of cholesterol synthesized within the hepatocytes in the liver. Interestingly, they are also facial amphipathic, meaning they possess both hydrophilic (water-soluble) and hydrophobic (fat-soluble) components. This amphipathic quality allows the bile acids to emulsify lipid aggregates and solubilize and transport lipids in an aqueous milieu. The process begins as triglycerides are broken down or emulsified in the duodenum by bile acids into monoglycerides and fatty acids [35,66]. Table 31.10 provides a summary of the critical

TABLE 31.8
Summary of Protein Digestion

Enzyme/Digestive Fluid	Source	Process	Result
Pepsin (derivative of pepsinogen)	Stomach (chief cells)	Initial breakdown of collagen and proteins into peptides	Peptide fragments
Endopeptidases (*hydrolyze interior peptide bonds*)			
Trypsin (derivative of trypsinogen)	Pancreas	Continues breakdown of proteins into peptides	Peptide fragments
Chymotrypsin (derivative of chymotrypsinogen)	Pancreas	Continues breakdown of proteins into peptides	Peptide fragments
Exopeptidases (*hydrolyze exterior peptide bonds*)			
Carboxypeptidase (derivative of procarboxypeptidase)	Pancreas	Continues breakdown of proteins into peptides	Peptide fragments
Aminopeptidase	Small intestine	Cleaves terminal amino acids at amino ends of peptides	Amino acids (dipeptides, tripeptides)
Dipeptidase	Small intestine	Cleaves two amino acid molecules joined by a peptide bond (dipeptide)	Amino acids (dipeptides, tripeptides)

Source: Data from Ganapathy, V., Protein digestion and absorption, Chapter 59, in: *Physiology of the Gastrointestinal Tract*, 5th edn., ed. L.R. Johnson, Elsevier, Amsterdam, the Netherlands, 2012, pp. 1595–1624.

TABLE 31.9
Summary of Protein Absorption

Type of Nutrient	Type of Movement	Site of Transport	Destination
Polypeptide fragments	Not absorbable in this form	—	—
Dipeptide α-amino acids (by cytoplasmic peptidase)	Sodium-dependent cotransport at lumen; facilitated diffusion to blood	Duodenum and jejunum	Bloodstream to hepatic portal vein
Tripeptide α-amino acids (by cytoplasmic peptidase)	Sodium-dependent cotransport at lumen; facilitated diffusion to blood	Duodenum and jejunum	Bloodstream to hepatic portal vein
Amino acids	Sodium-dependent amino cotransport (four types of amino acid carriers are neutral, acidic, basic, and imino)	Duodenum and jejunum	Bloodstream to hepatic portal vein

Source: Data from Ganapathy, V., Protein digestion and absorption, Chapter 59, in: *Physiology of the Gastrointestinal Tract*, 5th edn., ed. L.R. Johnson, Elsevier, Amsterdam, the Netherlands, 2012, pp. 1595–1624.

TABLE 31.10
Summary of Lipid Digestion

Enzyme/Digestive Fluid	Source	Process	Result
Lingual lipase	Salivary glands at the base of the tongue	Minor digestion of triglycerides to monoglycerides and fatty acids	Monoglycerides and fatty acids.
Bile acids	Hepatocytes in liver lobules	Emulsification of lipid aggregates; solubilization and transport of lipids	Breakdown of triglycerides into 1 μm globules; hydrophobic products of lipid digestion are solubilized in micelles.
Pancreatic lipases (pancreatic lipase, cholesterol ester hydrolase, phospholipase A_2)	Pancreas	Removes two of the three fatty acids from glycerol	Fatty acids, monoglycerides, cholesterol, and lysolecithin.
Lipoprotein lipase	Capillary endothelial cells	Breaks down triglycerides into fatty acids and glycerol	Removal of chylomicrons from the circulation system.

Sources: Data from Gimeno, R.E., *Curr. Opin. Lipidol.*, 18(3), 271, 2007; Milger, K. et al., *J. Cell. Sci.*, 119, 4678, 2006; Sun, W. et al., Intestinal lipid absorption, in: *Textbook of Gastroenterology*, 5th edn., ed. T. Yamada, Wiley Blackwell Publishing, Hoboken, NJ, 2009, pp. 445–463.

digestive processes of lipids. The short-chain fatty acids (<12 carbon molecules) are absorbed by the small intestine by simple diffusion. The long-chain fatty acids (>12 carbon molecules) and monoglycerides are dissolved and absorbed into the center of structures called micelles, which consist of 20–50 molecules of bile salt. As the micelle comes into contact with the intestinal cells, the fatty acids, cholesterol, and monoglycerides cross the luminal membranes into the intestinal cell wall, leaving the empty micelles in the chyme to repeat the process. Several transporter-mediated mechanisms have been postulated for the uptake of fatty acids such as CD36 [67] and FATP4 [68], among others [69,70]; however, some postulate that the transport is strictly passive diffusion [71]. It has been suggested that the mode of lipid uptake by the intestinal cells is dependent on the concentration of available fatty acids; that is, if the concentration of lipids is low and the body needs fatty acids, an active component appears to be a key factor in lipid transport. On the other hand, when the lipid concentration is high, most transport is postulated to be passive [77]. The bile acids are reabsorbed primarily in the ileum and returned to the liver in a cycle called *enterohepatic circulation*. Once the fatty acids and monoglycerides are in the epithelial cells, they are re-esterified to triglycerides, cholesterol ester, and phospholipids, which become coated with apoproteins. These newly formed structures, called *chylomicrons*, are transported out of the epithelial cells into the lymphatic system by exocytosis. Once in the circulatory system, the triglycerides in the chylomicrons are removed from the circulatory system as they pass through the liver. This removal is mediated by lipoprotein lipase, which is present in the capillary endothelial cells and breaks down the triglycerides into fatty acids and glycerol. To be mobile and functional in the blood, plasma lipids are combined with apoproteins. These structures are termed *lipoproteins*. There are several types of lipoproteins, depending on their specific composition of triglycerides, phospholipids, cholesterol, and protein. These lipoproteins include high-density lipoproteins (HDLs), low-density lipoproteins (LDLs), and very-low-density lipoproteins (VLDLs). Table 31.11 provides a summary of the critical absorption mechanisms of lipids.

Nucleic Acids

A wide variety of plant and animal foods contain DNA, RNA, nucleotides, and free nucleic acids. Once in the GI tract, DNA and RNA are broken down into free nucleic acids and nucleotides by various nucleases found in both intestinal and

TABLE 31.11
Summary of Lipid Absorption

Type of Nutrient	Type of Movement	Site of Transport	Destination
Short-chain fatty acids	Facilitated diffusion	Small intestine	Bloodstream to hepatic portal vein
Long-chain fatty acids, monoglycerides, cholesterol, and lysolecithin in micelles	Facilitated diffusion	Ileum	Bloodstream to hepatic portal vein
Chylomicrons	Exocytosis	Small intestine	Lymph vessels through the thoracic duct

Sources: Data from Gimeno, R.E., *Curr. Opin. Lipidol.*, 18(3), 271, 2007; Milger, K. et al., *J. Cell. Sci.*, 119, 4678, 2006; Sun, W. et al., Intestinal lipid absorption, in: *Textbook of Gastroenterology*, 5th edn., ed. T. Yamada, Wiley Blackwell Publishing, Hoboken, NJ, 2009, pp. 445–463.

pancreatic digestive juices. The pancreatic nucleases convert DNA and RNA into nucleotides along the length of the small intestines. DNA nucleotides include the following bases: adenine, thymine, guanine, and cytosine. RNA nucleotides contain the following bases: adenine, uracil, guanine, and cytosine. These nucleotides are further digested by enzymes produced by the small intestinal epithelial cells, including nucleosidase and phosphatase. Nucleotidase enzymes found in the membrane of the ileum epithelial cells further break down the nucleotides into pentoses, phosphates, and nitrogenous bases. Table 31.12 is a summary of the critical digestive processes of nucleic acids. Nucleotides can be partially absorbed in the duodenum and the jejunum. The nucleosides are absorbed in greater amounts than the nucleotides because they lack the phosphoric acids. Transport of nucleosides into the mucosal cell wall occurs by facilitated diffusion and specific sodium-dependent carrier-mediated transport mechanisms [78]. When the nucleic acids have been broken down into their component sugars, phosphates, and nitrogenous bases, they are absorbed directly into the epithelial cells by sodium-dependent active transport. Table 31.13 provides a summary of the critical absorption mechanisms of nucleic acids. Nutrients are absorbed along the length of the small intestine as shown in Table 31.14.

Large Intestine

The large intestine, also referred to as the colon, is the final part of the digestive tract; it measures about 5 ft in length and 2–3 in. in diameter and extends from the ileocecal sphincter at the end of the termination of the small intestine through the rectum, which connects to the anus. The large intestine is divided into several segments. Immediately below the ileocecal sphincter is the cecum, a saclike enlargement at the junction of the small and large intestines that measures about 2–3 in. in length. Attached to the cecum is a small tube called the *appendix*, which measures approximately a quarter of an inch wide and 2–3 in. in length; its function remains unclear [80]. Adjacent to the cecum on the right side of the abdomen is the ascending colon, which ascends to the bottom of the liver, where it turns left and forms the right colic flexure, which becomes the transverse colon. When the ascending colon reaches the end of the spleen, it turns downward and forms the left colic flexure, which becomes the descending colon. The next segment is the sigmoid colon, which descends into the pelvis and connects to the rectum. The last 8 in. of the large intestine comprises the rectum, which is a reservoir for waste. The final inch of the rectum is the anal canal, which ultimately becomes the anus. The anus has two sphincters, one that is involuntary and comprised of smooth muscle and a second external voluntary sphincter that is comprised of skeletal muscle. The anus remains closed except during the elimination of wastes [81]. The wall of the large intestine is comprised of the same layers as the rest of the GI tract; however, there are several differences. First, the muscularis layer consists of two layers, but the external layer of longitudinal muscles is thickened and forms three conspicuous

TABLE 31.12
Summary of Nucleic Acid Digestion

Enzyme/Digestive Fluid	Source	Process	Result
Ribonuclease	Pancreas and small intestine	Converts ribonucleic acid into nucleotides	Nucleotides
Deoxyribonuclease	Pancreas and small intestine	Converts deoxyribonucleic acid into nucleotides	Nucleotides
Nucleosidase	Small intestine epithelial cells	Converts nucleosides into purines and pyrimidines	Nucleosides, purines, and pyrimidines
Phosphatase	Small intestine epithelial cells	Catalyzes hydrolysis of esters of phosphoric acid	Phosphate
Nucleotidase	Ileum epithelial cells	Converts nucleotides to sugars, pentose, phosphates, and nitrogenous bases	Sugars, pentose, phosphates, and nitrogenous bases

Source: Data from Farrell, J.J., Digestion and absorption of nutrients and vitamins, Chapter 100, in: *Gastrointestinal and Liver Disease*, 9th edn., eds. M. Feldman, L.S. Friedman, and L.J. Brandt, Saunders Elsevier, Philadelphia, PA, 2010, pp. 1695–1734.

TABLE 31.13
Summary of Nucleic Acid Absorption

Type of Nutrient	Type of Movement	Site of Transport	Destination
RNA nucleotides	Sodium-dependent active transport	Duodenum and jejunum	Bloodstream to hepatic portal vein
DNA nucleotides	Sodium-dependent active transport	Duodenum and jejunum	Bloodstream to hepatic portal vein
Sugars, pentose, phosphates, and nitrogenous bases	Sodium-dependent active transport	Duodenum and jejunum	Bloodstream to hepatic portal vein

Source: Data from Farrell, J.J., Digestion and absorption of nutrients and vitamins, Chapter 100, in: *Gastrointestinal and Liver Disease*, 9th edn., eds. M. Feldman, L.S. Friedman, and L.J. Brandt, Saunders Elsevier, Philadelphia, PA, 2010, pp. 1695–1734.

TABLE 31.14
Summary of Small Intestine Nutrient Absorption

Region of Small Intestine	Nutrient Absorbed
Duodenum	Calcium
	Galactose
	Glucose
	Iron
	Magnesium
Jejunum	Amino acids
	Chloride
	Fatty acids
	Fat-soluble vitamins
	Fructose
	Galactose
	Glucose
	Potassium
	Sodium
	Sugars
	Water-soluble vitamins
Ileum	Alcohols[a]
	Amino acids
	Bile salts
	Chloride
	Fatty acids
	Potassium
	Sodium
	Some small peptides
	Vitamin B_{12}
	Water

Source: Adapted from Bowen, R., The small intestine: Introduction and index, Colorado State University, Ft. Collins, CO, 2006. Available online: http://www.vivo.colostate.edu/hbooks/pathphys/digestion/smallgut/index.html.

[a] Also absorbed in the stomach and oral cavities.

longitudinal bands called *teniae coli*. See Figure 31.10 for the overall structure and general organization of the large intestine. Together, the three bands run the length of the large intestine. By virtue of their tonic contractions, the walls of the colon pucker into pocket-like sacs called *haustra*, which give the colon its segmented appearance. Epiploic appendages or fat-filled bags of visceral peritoneum are attached to the teniae coli. Their function remains largely unknown.

Digestion

After the small intestine absorption is completed, the remaining fluid passes into the cecum of the large intestine. The ileocecal sphincter acts as a governor because it is normally slightly contracted and slows the passage of chyme from the small intestine to the cecum. Not only does this sphincter control the rate of chyme passage, but it also prevents the reverse flow of chyme back into the ileum. Interestingly, when the chyme reaches the colon, the primary mechanism of digestion is by bacterial action. The secretion of glands in the mucosal epithelial cell wall is a protective measure against potential damage from bacterial activity. The bacteria ferment carbohydrates, further break down amino acids, decompose bilirubin, and synthesize some B complex vitamins as well as vitamin K.

Bacteria

Bacteria are present throughout the length of the GI tract; however, a significant bacterial concentration is present in the large intestine. A critical step in the preparation of chyme for elimination is the fermenting action of bacteria normally present in the colon; therefore, in addition to the actions of the digestive juices and mixing action of the contents, most of the final phase of digestion in the large intestine depends on the symbiotic activity of bacteria in the system. Few bacteria are present in the gut at birth; however, rapid colonization occurs shortly after birth. It has been estimated that the average adult has 10^{14} intestinal flora, which equates to about 4–5 lb that is critical to the digestive process. The large intestine contains as many as 400 different species of bacteria, and an individual's flora is immunologically unique to that person [80]. The stomach has the least amount of microflora ($<10^3$ CFU/mL), primarily because the low pH destroys many organisms; in contrast, almost the entire digestion process can be attributed to the large number ($>10^{14}$ CFU/mL) of anaerobic bacteria present in the large intestine [80]. Table 31.15 provides a summary of the various types of microflora found in the GI tract from the stomach through the large intestine.

Most of the final phase of digestion in the large intestine depends on the symbiotic activity of bacteria in the system. The normal microflora digest materials that enter through the ileocecal sphincter and maintain a barrier to infections that could develop from hostile organisms. This barrier consists of an overwhelming number of resident microflora that give invaders little space or food, as well as a by-product of

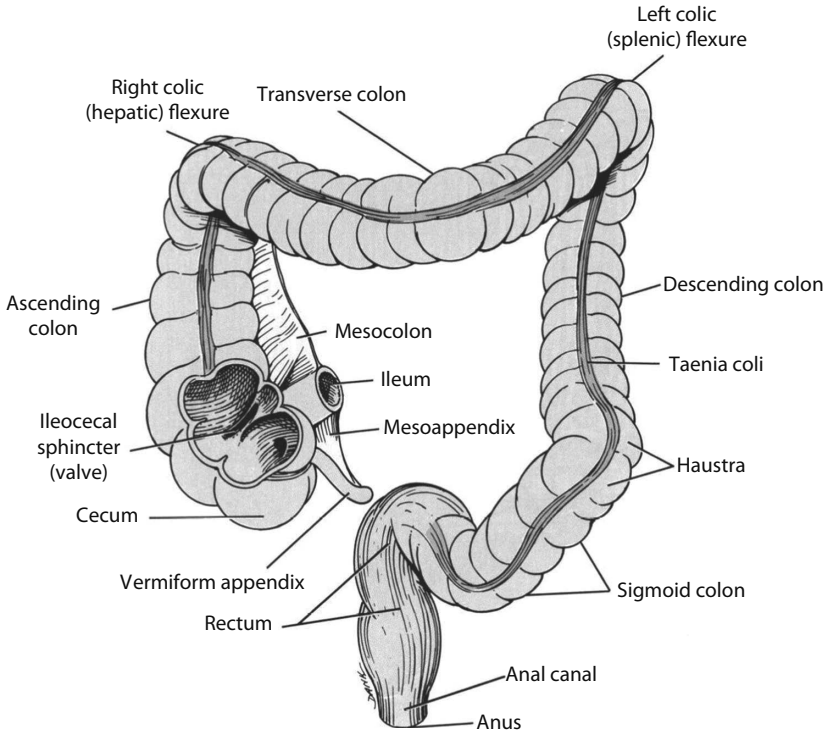

FIGURE 31.10 Structure and general organization of the large intestine. (From Tortora, G.J. and Anagnostakos, N.P., The digestive system, Chapter 24, in: *Principles of Anatomy and Physiology*, 6th edn., HarperCollins, New York, p. 760. With permission.)

metabolism that tends to reduce the reproductive capacity of foreign microflora. When foreign bacteria enter the GI tract, the resident bacteria are immunologically activated and help prevent colonization by the invading organisms. Normal intestinal bacteria help to prevent infections because the normal bacteria occupy all of the available niches for bacteria, thus depriving the invading bacteria of suitable places to begin reproduction. Further, the robust occupying bacteria will utilize available food, which keeps the invading flora from multiplying. Finally, some of the normal bacteria produce antibacterial chemicals (called bacteriocins) as a result of their metabolism. This by-product can generate a local antibiotic effect that hinders the reproduction of invading flora. Secretions in the large intestine consist primarily of alkaline mucus that protects the epithelium and neutralizes acids produced by bacterial metabolism. The most common include *Enterobacteriaceae*, *Bacteroides*, *Bifidobacterium*, *Streptococcus faecalis*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, *Ruminococcus*, *Clostridia*, and *Lactobacillus*. The environment of the large intestine is primarily anaerobic, which favors obligate anaerobes. Table 31.16 provides a summary of bacterial action in the large intestine.

Motility

When the chyme enters the cecum, the peristaltic action is triggered by distention of the wall in the colon; however, it occurs at a rate much slower than in the small intestine. The movement of fecal contents from the cecum to the rectum for elimination can take several days. The movements of the cecum and ascending colon are more vigorous and promote mixing to hasten the absorption process; however, the movements of the descending and sigmoid colon and rectum are slower and more deliberate to permit the formation of stool, which collects in the rectum. Although the origin of these coordinated contractions is not well understood, they appear to be dependent on a combination of internal rhythmic contractions and neural, paracrine, and humoral factors. Following a meal, colonic motility increases significantly, due to signals propagated throughout the ENS from the walls of the stomach, which triggers the gastrocolic reflex. Several times a day, mass movements push feces into the rectum in this manner. In addition, the presence of fats in the duodenum appears to trigger another mass peristalsis reflex, termed the duodenocolic reflex, through the ENS. The general state of the large intestine musculature also depends upon the distention of the colon walls that can initiate contractions within the colon. The large intestine has six types of movement as summarized in Table 31.17.

Absorption

Of the about 1 L of fluid that enters the large intestine each day, approximately 90% is reabsorbed, leaving only about 100 mL to be excreted with the feces. When the chyme reaches the cecum of the large intestine, most of the digestion and secretion have been completed. The primary functions of the large intestine are the absorption of water, vitamins, and electrolytes; the formation of feces; and movement of the feces to the anus for elimination. The intestinal mucosal of

TABLE 31.15
Microflora Found in the GI Tract

Location	Concentration (CFU/mL)	Types of Bacteria
Stomach	$<10^3$	Gram-positive aerobic microorganisms (e.g., *Streptococcus*, *Staphylococcus*, *Lactobacillus*)
Duodenum	10^3–10^4	Gram-positive microbes ↓↓↓ Gram-negative microbes ↑
Jejunum	10^4–10^5	Gram-positive microbes ↓↓ Gram-negative microbes ↑↑
Ileum	10^6–10^7	Gram-positive microbes ↓ Gram-negative microbes ↑↑↑
Large intestine	$>10^{14}$	Gram-negative anaerobic microbes (e.g., *Bacteroides*, *Bifidobacterium*, *Fusobacterium*, *Clostridium*, *Eubacterium*)

Sources: Data from Ringler, D.H. and Daibich, L., Hematology and clinical biochemistry, in: *The Laboratory Rat*, Vol. 1: *Biology and Disease*, eds. H.J. Baker et al., Academic Press, New York, 1979, pp. 105–121; Wright, E.M. et al., Sugar absorption, Chapter 58, in: *Physiology of the Gastrointestinal Tract*, 5th edn., Vol. 2, eds. L.R. Johnson, Elsevier, London, U.K., 2012, pp. 1583–1594; Onderdonk, A.B., The intestinal microflora and intra-abdominal sepsis, in: *Medical Importance of the Normal Microflora*, ed. G.W. Tannock, Kluwer Academic Publishers, Dordrecht, the Netherlands, 2010, pp. 164–176; Chen, Z. and Davidson, N.O., Genetic regulation of intestinal lipid transport and metabolism, in: *Physiology of the Gastrointestinal Tract*, 5th edn., Vol. 2, ed. L.R. Johnson, Elsevier, London, U.K., 2012, pp. 1643–1662; Guarner, F. and Malagelada, J.R., *Lancet*, 361(9356), 512, 2003; Todar, K., *Todar's Online Textbook of Bacteriology*, Department of Bacteriology, University of Wisconsin–Madison, Madison, WI, 2012. Available online: http://www.textbookofbacteriology.net/.

TABLE 31.16
Bacterial Action in the Large Intestine

Category	Action	Result
Carbohydrates	Fermentation	Release of hydrogen, carbon dioxide, methane gas
Proteins	Breakdown to remaining proteins	Release of amino acids
Amino acids	Breakdown to basic components	Release of indole, hydrogen sulfide, fatty acids, and skatole
Bilirubin	Breakdown to basic components	Simple pigments
Soluble fiber	Fermentation	Lubricating gel to maintain stools in soft and flexible consistency to assist in elimination
Bacterial synthesis	Synthesis of vitamins	Creation of vitamin K and some B complex vitamins, including riboflavin, nicotinic acid, folic acid, and biotin
Bacterial synthesis	Synthesis of nutrients	Bacterial self-supporting creation of nutrients for their own food supply
Immunological responses	Activation upon presence of foreign bacteria	Prevents colonization by invading pathogens

Sources: Data from Ringler, D.H. and Daibich, L., Hematology and clinical biochemistry, in: *The Laboratory Rat*, Vol. 1: *Biology and Disease*, eds. H.J. Baker et al., Academic Press, New York, 1979, pp. 105–121; Wright, E.M. et al., Sugar absorption, Chapter 58, in: *Physiology of the Gastrointestinal Tract*, 5th edn., Vol. 2, eds. L.R. Johnson, Elsevier, London, U.K., 2012, pp. 1583–1594; Onderdonk, A.B., The intestinal microflora and intra-abdominal sepsis, in: *Medical Importance of the Normal Microflora*, ed. G.W. Tannock, Kluwer Academic Publishers, Dordrecht, the Netherlands, 2010, pp. 164–176; Chen, Z. and Davidson, N.O., Genetic regulation of intestinal lipid transport and metabolism, in: *Physiology of the Gastrointestinal Tract*, 5th edn., Vol. 2, ed. L.R. Johnson, Elsevier, London, U.K., 2012, pp. 1643–1662; Guarner, F. and Malagelada, J.R., *Lancet*, 361(9356), 512, 2003; Todar, K., *Todar's Online Textbook of Bacteriology*, Department of Bacteriology, University of Wisconsin–Madison, Madison, WI, 2012. Available online: http://www.textbookofbacteriology.net/; Macfarlane, G.T. and Macfarlane, S., *Curr. Opin. Biotechnol.*, 18(2), 156, 2007.

the large intestine absorbs water, sodium, and chloride and secretes potassium and bicarbonate.

Water is absorbed in the large intestine in response to an osmotic gradient. Absorption of water in the large intestine is passive and requires the movement of solutes (primarily sodium); thus, the rate of water uptake is a function of the solute absorption. The mechanism responsible for generating this osmotic pressure involves sodium ions being transported from the lumen across the epithelium because of the sodium pumps and the ability of the luminal membrane to absorb sodium. In fact, the colonic epithelium is very effective at absorbing water and sodium (95% retention rate). Absorption in the colon is enhanced by the hormone aldosterone. This uptake of water serves to concentrate the contents of the large intestine to a semisolid form. Sodium and subsequently water are absorbed primarily in the cecum, the ascending colon, and, to a lesser extent, the transverse colon. Bicarbonate is secreted against a concentration gradient, and chloride absorption is linked to both bicarbonate secretion and sodium transport. The resulting secretion of bicarbonate ions into the lumen aids in neutralization of the acids generated by microbial fermentation in the large intestine. Potassium, on the other hand, is passively transported and can be absorbed if the potassium concentration is greater than 15 mEq/L or secreted if the potassium concentration is less than 15 mEq/L. Under normal circumstances, the luminal concentration of potassium is less than 15 mEq/L, which favors secretion.

Vitamins are important to the body as cofactors or coenzymes in numerous metabolic pathways. Some critical vitamins produced by intestinal bacteria are also absorbed in the large intestine. Approximately 50% of the daily requirement

TABLE 31.17
Muscular Action of the Large Intestine

Type of Motion	Description	Function
Haustral churning	Haustrum pouch relaxes and fills to capacity and then, as it becomes distended, contracts and moves the contents to the next haustrum.	This action provides a mixing motion to the contents that facilitates fluid extraction, creates larger masses, and helps advance the contents through the colon.
Peristalsis	A wave motion is produced by joint muscular coordination between the interior and exterior muscles of the muscularis layer.	This wave motion propels the contents forward through the colon; slower than similar action in the small intestine.
Mass peristalsis reflexes		
Gastrocolic reflex	As foodstuffs enter the stomach, a reflex is triggered at the midtransverse colon that initiates a strong mass peristaltic action.	The strong wave action forces any latent contents in the lower large intestine into the rectum to make room for the new stomach contents.
Duodenocolic reflex	As fats enter the duodenum, a reflex is triggered at the midtransverse colon that initiates a strong mass peristaltic action.	The strong wave action forces any latent contents in the lower large intestine into the rectum to make room for the new stomach contents.
Defecation reflex	As colon contents move into the rectum, a signal is sent to the brain.	This signal tells the conscious brain that the rectum is full and defecation must occur.
Cooperative abdominal effort	As defecation occurs, voluntary and involuntary abdominal muscles are contracted.	This cooperative action of the abdominal muscles assists in pushing the waste from the rectum and anus.

Sources: Hasler, W.L., Motility of the small intestine and colon, Chapter 11, in: *Textbook of Gastroenterology*, 5th edn., ed. T. Yamada, Wiley Blackwell Publishing, London, U.K., 2009, pp. 231–263; Bharucha, A.E. and Brookes, S.J.H., Neurophysiologic mechanisms of human large intestinal motility, Chapter 36, in: *Physiology of the Gastrointestinal Tract*, 5th edn., Vol. 2, ed. L.R. Johnson, Elsevier, London, U.K., 2012, pp. 977–1022.

for vitamin K is produced by bacteria found in the large intestine. Pantothenic acid (vitamin B_5), which is necessary in the production of steroids and some neurotransmitters, is also produced by intestinal bacteria. Finally, biotin, a necessary cofactor in glucose metabolism, is also produced by intestinal bacteria. Although each of these is generated by various intestinal bacteria, the resulting vitamins are absorbed primarily in the cecum and ascending colon. Organic wastes from the breakdown of heme produce bilirubin, which is broken down into urobilinogens and stercobilinogens. Ultimately, these are converted to urobilins and stercobilins, respectively. Bacterial activity on these latter substances in the large intestine generates

- Ammonium ions (NH^{4+})
- Hydrogen sulfide (H_2S), which produces a characteristic odor
- Indole, which produces characteristic odor
- Skatole, which produces a characteristic odor

Ammonia and some other organic wastes are absorbed by the large intestinal mucosal epithelial cells and enter the hepatic portal circulatory system, where they are processed by the liver and ultimately eliminated by the kidneys. Indigestible polysaccharides are also eventually deposited in the large intestine. The resident bacteria begin to break down these substances and utilize them as a source of nutrients.

The large intestine has a delicate lining that is moisturized by mucus and also by a gel that is a by-product of the bacterial fermentation process. Despite the many similarities in the cellular structure of the mucosa in the large and small intestines, clearly the most obvious difference is that the mucosa of the large intestine lacks absorptive villi. The mucosal epithelial walls of the large intestine have numerous crypts that extend deep into and open onto a flat luminal surface. The stem cells that support the process of renewal of the epithelium are located in the crypts. These cells divide to populate the crypt and surface epithelium. Mucus-secreting goblet cells are also much more abundant in the colonic epithelium than in the small intestine. These cells secret mucus in response to parasympathetic stimuli and the presence of lumen contents. The lining of the large intestine has numerous functions, as summarized in Table 31.18.

The chyme remains in the colon for 3–24 h. During that time, numerous processes occur. In addition to the muscular activity and the bacterial actions noted previously, several cellular exchanges take place, as summarized in Table 31.19. After 90% of the water is absorbed and completion of a myriad of additional actions in the large intestine, the chyme is transformed into a semisolid called *feces*, consisting of residual water, inorganic salts, bacteria, products of bacterial decomposition, mucus cells, cell fragments from the GI tract mucosa, and any undigested food. Under normal physiological conditions, normal feces consist of approximately 75% water and 25% solids. The characteristic brown color of feces is due to stercobilin and urobilin, both of which are produced by bacterial degradation of bilirubin. The characteristic fecal odor is a result of gases produced by bacterial metabolism, including skatole, mercaptans, and hydrogen sulfide.

Defecation Reflex

As described previously, mass peristalsis moves the fecal material from the transverse, descending, and sigmoid colon into

TABLE 31.18
Function of the Lining of the Large Intestine

Function	Location	Result
Protection	Nerve endings in wall of large intestine	Permits intact neural messages between the CNS and the colon
Lubrication	Entire large intestinal wall	Promotes stool transit
Facilitation	Movement of fluids through the wall of the large intestine	Optimizes nutritional uptake of vitamins and electrolytes
Bacterial growth	Large intestine wall	Promotes growth of healthy intestinal bacteria, which break down waste, create additional lubrication, cleanse the colon, and protect the colon against infection

Sources: Turner, J.R. and Madara, J.L., Epithelia: Biological principles of organization, Chapter 8, in: *Textbook of Gastroenteroelogy*, 5th edn., ed. T. Yamada, Wiley Blackwell Publishing, London, U.K., 2009, pp. 169–186; Furness, J.B., Sensory neurons of the gastrointestinal tract, Chapter 3, in: *Textbook of Gastroenteroelogy*, 5th edn., ed. T. Yamada, Wiley Blackwell Publishing, London, U.K., 2009, pp. 40–55.

TABLE 31.19
Cellular Exchanges of the Colon

Function	Process	Location	Result
Secretion	Secretion of mucus by goblet cells	Goblet cells located in tubular intestinal glands	Secreted mucus lubricates and protects the colon lining and nerve tissues.
Fluid absorption	Secondary to solute absorption, passive diffusion into epithelium cells	Primarily occurs in the columnar cells of the cecum and ascending colon	Approximately 80% of the water entering the colon is absorbed; maintains water balance.
Electrolyte absorption	Passive diffusion via sodium channels by columnar cells	Columnar cells located in tubular intestinal glands	Na^+, Cl^-, and K^+ are absorbed; maintains water balance.

Source: Keely, S.J. et al., Electrolyte secretion and absorption: Small intestine and colon, Chapter 14, in: *Textbook of Gastroenterology*, 5th edn., ed. T. Yamada, Wiley Blackwell Publishing, London, U.K., 2009, pp. 330–367.

the rectum. When distention at 25% or more is detected in the rectal wall, a stimulus is sent from pressure-sensitive receptors that initiate the defecation reflex, which empties the rectum. When distention is detected, receptors send nerve impulses to the spinal cord and then back through the parasympathetic nervous system to the descending colon, sigmoid colon, rectum, and anus. This impulse contracts the rectum and increases the pressure along the diaphragm and abdominal muscles, forcing the internal sphincter open and ultimately expelling the feces through the rectum and out of the anus. The external sphincter is striate muscle and is voluntarily controlled. If it is relaxed, the defecation process is completed. If it is voluntarily constricted, defecation can be temporarily postponed. As described previously, the voluntary cooperative abdominal and diaphragm effort increases the pressure inside the abdomen, which creates a backup into the sigmoid colon until the next mass peristalsis reflex occurs once again, stimulating the pressure-sensitive receptors that signal the CNS of the need to defecate.

EXPOSURE TO TOXIC SUBSTANCES

Because the GI tract is the normal portal of entry for foodstuff, exposure to any kind of ingestible toxic agent is possible, and the degree of exposure can be high. A toxicant can enter the body via water, foodstuff, pharmacological agents, or even inhaled substances that are subsequently swallowed via the resulting secretions from the lungs, trachea, or oral cavity. Toxicants enter the body by the same absorption pathways as necessary nutrients; they pass through the GI tract mucosa membranes and subsequently make their way into either the blood or lymphatic system. These substances may exert toxic events locally within the GI tract itself or at a distant site, depending on the nature of the ingested material, the rate of absorption, the biochemistry of the toxicant, and the physiology of the target organ. The degree of toxicity depends on the concentration and duration of exposure of a toxicant at the sensitive site. Several factors can alter the extent of potential toxicity when a toxicant has gained entrance into the lumen of the GI tract, including poor absorption from the GI tract, intestinal and hepatic first-pass effects as mediated by biotransformation, and the rate of incorporation of lipid-soluble substances into micelles. A toxicant can interact with the specific moieties at the site of action, or it can alter the microenvironment in such a way that the resulting action is toxic to the functioning of the specific site. The critical factors affecting the degree of toxicity are summarized in Table 31.20.

Biotransformation

The process by which substances are changed into different chemicals by enzyme reactions in the organism is called *biotransformation*, and it can be an important defense

TABLE 31.20
Factors Affecting Degree of Toxicity

Factor	Location	Effect
Chemical characteristics of toxicant	Lumen of GI tract	Lipophilicity, molecular weight, pH, and pK_a; affect extent and rate of absorption
Dissolution rate of chemical	Cell wall of GI tract	Absorption rate due to particulate size
Hydrochloric acid concentration	Stomach	Hydrolysis of toxicant into another compound
Dilution in gastric secretions	Lumen of GI tract	Absorption rate due to competition and concentration
Dilution in food content	Lumen of GI tract	Absorption rate due to competition and diluted concentration
Time of residence at absorption site	Lumen of various GI tract locations	Absorption rate due to time at absorption site
Microflora	Lumen of GI tract	Chemical changes to the toxicant that alter its absorption characteristics
Biotransformation	Lumen of GI tract, liver	Clearance of toxicant
Fecal excretion	Large intestine	Clearance of toxicant

Sources: Monosson, E., Absorption of toxicants, in: *Encyclopedia of Earth*, ed. J. Cutler, 2011. Available online: http://www.eoearth.org/article/Absorption_of_toxicants; Ding, X. and Kaminsky, L.S., *Annu. Rev. Pharmacol. Toxicol.*, 43, 149, 2003; Burks, T.F., Pathophysiological mechanisms of gastrointestinal toxicants, Chapter 8, in: *Comprehensive Toxicology*, 2nd edn., vol. 10, Elsevier, London, U.K., 2010, pp. 117–144; International Programme on Chemical Safety, Environmental Health Criteria 6, Principles and Methods for Evaluating the Toxicity of Chemicals, Part I, World Health Organization, Geneva, Switzerland, 1978. http://www.inchem.org/documents/ehc/ehc/ehc006.htm, accessed March 6, 2014.

mechanism. This is possible because some toxicants and even some body wastes are changed into less toxic substances or are simply prevented from forming toxic derivatives. A chemical can be toxic in its original form as it enters the body, or toxic derivatives of a nontoxic parent compound can be produced by biotransformation. Biotransformation occurs in GI tract mucosal cells, in the liver, and, to a lesser extent, at various other places throughout the circulatory system.

Biotransformation reactions can be divided into two basic types of reactions: phase I and phase II. Generally, in phase I reactions, the structure of the toxicant is changed in such a way that it is readied for additional changes during the next step in the process. Toxicants are neutralized in three ways during phase I: (1) the chemical structure is changed so it is hydrophilic, (2) the toxicant is broken down into two or more less toxic substances, or (3) the toxicant is transformed into an activated form that can be detoxified by other enzymes. Most of the phase I reactions are mediated by the cytochrome P450 enzymes, which primarily oxidize lipophilic toxicants and are responsible for adding polar groups to the molecule. Interestingly, the creation of these activated toxins can result in a more toxic derivative, so it is critical for the activated toxin to move forward to phase II reactions [97]. Seven major biochemical reactions occur in phase II: glutathione conjugation, amino acid conjugation, methylation, acetylation, sulfation, glucuronidation, and sulfoxidation. Each of these reactions transforms specific types of activated toxins by adding a molecule to the activated toxicant. A brief overview of the critical factors is summarized in Tables 31.21 and 31.22.

TABLE 31.21
Phase I Reaction Summary

Type of Reaction	Specific Function
Oxidation	Aromatic and aliphatic hydroxylation; epoxidation; N-hydroxylation; O-, N-, and S-dealkylation; S-oxidation; dechlorination; oxidative desulfuration; amine oxidation; dehydrogenation.
Reduction	Azoreduction, nitroreduction, carbonyl reduction.
Hydrolyses	Esterases, amidases, proteases.
Mixed function oxidase system	In many of the preceding reactions, the substrate binds to cytochrome P450, transferring electrons and oxygen in the following general reaction: $SH + NADPH + H^+ + O_2 \rightarrow SOH + NADP^+ + H_2O$.

Sources: Parkinson, A. and Ogilvie, B.W., Biotransformation of xenobiotics, in: *Casarett & Doull's Essentials of Toxicology*, 7nd edn., ed. C.D. Klaassen, McGraw-Hill Companies, New York, 2008, pp. 161–304; Renwick, A.G. and George, C.F., Metabolism of xenobiotics in the gastrointestinal tract, in: *Xenobiotic Metabolism in Animals: Methodology*, *Mechanisms*, *and Significance*, eds. D.H. Huston et al., Taylor & Francis, London, U.K., 1989, pp. 13–40; Kleinschmidt, K.C. and Delaney, K.A., Biochemical and metabolic principles, Chapter 12, in: *Goldfranks' Toxicologic Emergencies*, 9th edn., eds. L.S. Nelson, N.A. Lewin, M.A. Howland, R.S. Hoffman, L.R. Goldfrank, and N.E. Flomenbaum, McGraw-Hill Companies, New York, 2011, pp. 170–188.

Note: Phase I reactions tend to make the substrates more polar and more readily excreted from the organism by exposing the functional group and readying it to be changed further during phase II. Some of these reactions result in bioactivation of the substance.

TABLE 31.22
Phase II Reaction Summary

Type of Reaction	Specific Function
Glucuronidation	Diphosphate glucuronosyl transferase forms glucuronides with activated glucuronic acid, which can catalyze the conjugation of a substance with a polar group.
Sulfation	Sulfotransferase activates sulfate, which can react with a polar compound becoming more water soluble.
Glutathione conjugation	Glutathione *S*-transferase catalyzes the formation of strong electrophiles such as epoxides, haloalkanes, nitroalkanes, alkenes, and aromatic halo and nitrocompounds.
Epoxide hydrolase	The reaction proceeds through the activation of water that detoxifies the epoxides of hydration.
Acetylation	Acetyl CoA is attached to aromatic amines and sulfonamides.
Methylation	Several methyltransferases are utilized to increase lipophilicity.
Amino acid conjugation	Proceeds by two pathways: (1) the COOH group conjugated with NH_2 requires CoA activation and (2) aromatic NH_2 or NHOH conjugated with COOH requires ATP activation.

Sources: Parkinson, A. and Ogilvie, B.W., Biotransformation of xenobiotics, in: *Casarett & Doull's Essentials of Toxicology*, 7nd edn., ed. C.D. Klaassen, McGraw-Hill Companies, New York, 2008, pp. 161–304; Renwick, A.G. and George, C.F., Metabolism of xenobiotics in the gastrointestinal tract, in: *Xenobiotic Metabolism in Animals: Methodology*, *Mechanisms*, *and Significance*, eds. D.H. Huston et al., Taylor & Francis, London, U.K., 1989, pp. 13–40; Kleinschmidt, K.C. and Delaney, K.A., Biochemical and metabolic principles, Chapter 12, in: *Goldfranks' Toxicologic Emergencies*, 9th edn., eds. L.S. Nelson, N.A. Lewin, M.A. Howland, R.S. Hoffman, L.R. Goldfrank, and N.E. Flomenbaum, McGraw-Hill Companies, New York, 2011, pp. 170–188.

Note: Phase II reactions are generally the result of the formation of compounds less biologically reactive and are conjugated with endogenous substances in order to make them more water soluble. The final product can be eliminated from the body either in the urine or in the bile.

The metabolic activity of the small intestine is referred to as *intestinal first-pass effect*. The small intestine is considered an absorptive organ, but it also has the ability to metabolize drugs via many pathways involving preconjugation (phase I) and conjugation (phase II) reactions [98–103]. Virtually all of the drug-metabolizing enzymes present in the liver are also found in the small intestine; however, the enzyme levels are considerably lower in the small intestine than in the liver [104]. Another factor that plays a critical role in the amount of nutrient or toxicant absorbed is referred to as the *hepatic first-pass effect*. In this case, orally ingested nutrients or toxicants are absorbed by the intestinal mucosal cells, enter the capillaries and veins of the GI tract, and are transported by the portal vein directly to the liver before entering the general circulation of the body. The absorbed substance is exposed to the liver before its first pass through the body. Because most toxicants are lipophilic and nonpolar and have low molecular weights, they are easily absorbable through the GI tract mucosal cells and yet are difficult to eliminate and can accumulate throughout the body to toxic levels. Most lipophilic toxicants are difficult for the body to eliminate, and they accumulate to toxic levels. Many lipophilic toxicants are biotransformed into hydrophilic metabolites that do not easily enter the membranes of the target tissues. Endogenous materials such as bilirubin are also biotransformed into hydrophilic derivatives that are excreted into the bile and ultimately eliminated in the feces. This process is termed *detoxication*, and the general pathways are summarized in Table 31.23. When a toxicant is biotransformed to another compound whose derivative can be more toxic than the parent compound, this process is known as *bioactivation*, and it can be very toxic to the organism. This process, also referred to as *toxication*, has several pathways depending on the chemical (see Table 31.24).

Factors Affecting Absorption

The rate at which a toxicant is absorbed across the mucosal cell wall in the GI tract is a function of a number of physical and chemical factors. Some toxicants may directly increase the permeability of the intestinal mucosal cell wall, such as ethylenediaminetetraacetic acid (EDTA). Also, the longer the exposure time to the mucosal membranes, the higher the rate of absorption, so another factor affecting the rate of toxicant absorption is the rate of peristalsis. Agents such as laxatives or sedatives work indirectly on the rate of absorption by speeding or slowing the rate of motility through the GI tract. If the substance is subject to a high hepatic clearance (i.e., it is rapidly metabolized by the liver), then a substantial fraction of the absorbed substance is extracted from the blood and metabolized and excreted as bile before it reaches the systemic circulation. The bile is then reintroduced into the GI tract and eventually eliminated through the feces. The consequence of this phenomenon is a significant reduction in bioavailability, which can be an excellent protective mechanism if the material is a toxicant and can make therapeutic treatment difficult if, in fact, the substance orally ingested is part of a drug therapy regimen [14].

With a few notable exceptions, most substances (toxicants and nutrients alike) are poorly absorbed from either the oral cavity or the esophagus. This is due primarily to the fact that the transit time in these two sections is relatively brief. In addition, these structures have no villi or similar such absorbing cells. Exceptions include nicotine from tobacco

TABLE 31.23
Summary of Common Detoxication Pathways

Detoxication Pathway	Description	Result
Nucleophiles	Hydroxylated nucleophilic groups are conjugated by sulfation, glucuronidation, or methylation; thiols are conjugated by methylation or glucuronidation; and amines and hydrazines are conjugated by acetylation or oxidation.	Reduction in the rate of nucleophilic conversion to free radicals and/or electrophilic quinines and quinone imines
Electrophiles	Electrophilic toxicants are conjugated by conjugation with thiol nucleophile glutathione.	Reduction in the rate of electrophilic conversion to reactive free radicals
Free radicals	Superoxide anion radical is converted by superoxide dismutase (SOD), glutathione peroxidase (GPO), and catalase (CAT) to HOOH and H_2O.	Reduction in the amount of superoxide anion radical available for conversion to free radicals such as peroxynitrite, nitrosoperoxy carbonate, and carbonate anion radical
No functional groups	Functional groups such as hydroxyl or carboxyl are first added to the substance by the cytochrome P450 enzymes, and then a transferase adds a glucuronic acid, a sulfuric acid, or an amino acid to the functional group.	Toxicants rendered much less active or more easily eliminated
Proteins	Intra- and extracellular proteases interact with polypeptides.	Inactivation of toxic polypeptides

Sources: Data from Gregus, Z., Mechanisms of toxicity, Chapter 3, in: *Casarett & Doull's Toxicolog: The Basic Acience of Poisons*, 7th edn., ed. C.D. Klaassen, McGraw-Hill Companies, New York, 2008, pp. 45–106; Sreedharan, R. and Mehta, D.L., *Pediatrics*, 113(4), 4, 2004.

TABLE 31.24
Summary of Common Toxication Pathways

Toxication Pathway	Description	Result
Nucleophile formation	Nucleophile activation is rare; some nucleophiles such as HCN and CO are reactive as nucleophiles, but many can be further activated to electrophiles.	These reactions increase the rate of nucleophile conversion to free radicals or electrophilic quinines and quinone imines.
Electrophilic formation	Electrophiles are often produced by adding an oxygen atom to a molecule that removes electrons and results in an electronic-deficient structure that is electrophilic.	These electrophiles are electron deficient and are reactive, creating polarized substances that are frequently catalyzed by cytochrome P450.
Free radical formation	Free radicals contain one or more unpaired electrons and are formed by the addition of an electron, loss of an electron, or hemolytic fission of a covalent bond.	Free radicals can increase the amount of superoxide anion radical available for conversion to free radicals such as peroxynitrite, nitrosoperoxy carbonate, and carbonate anion radical.
Redox-active reactants	Conversion of superoxide anion radicals to hydroxyl radicals by SOD and catalyzed by transition metal ions.	Reactive metabolites such as some electrophiles and neutral or cationic free radicals can be activated by conversion to electrophiles, while free radicals with extra electrons can produce hydroxyl radicals upon hemolytic cleavage.

Source: Data from Gregus, Z., Mechanisms of toxicity, Chapter 3, in: *Casarett & Doull's Toxicolog: The Basic Acience of Poisons*, 7th edn., ed. C.D. Klaassen, McGraw-Hill Companies, New York, 2008, pp. 45–106; Sreedharan, R. and Mehta, D.L., *Pediatrics*, 113(4), 4, 2004.

products, alcohol, and nitroglycerin, which is immediately absorbed when placed sublingually for treatment in certain heart conditions.

As the toxicant passes into the stomach and on through the small intestine, it can be absorbed by one and more of several mechanisms. Although no known mechanisms specifically transport only toxicants into the body, many of existing pathways for nutrient absorption in the GI tract are also used by foreign substances to gain entry into the body. Generally, if a toxicant is an organic acid or base, it most likely will be absorbed via simple diffusion. By virtue of the structure of these substances, one can assume that an organic acid exists primarily in lipid-soluble form in the stomach and in the ionized form in the small intestines. Based on that assumption, an organic acid would be absorbed more readily in the acid stomach than in the neutral small intestine. Organic bases, on the other hand, would be absorbed more readily in the neutral small intestine rather than the acidic stomach; however, because the pH is about neutral in the small intestine (pH 5.0–8.0), both weak acids and bases are nonionized and readily absorbed by passive diffusion. Significant exceptions to this situation do arise, primarily due to the large surface

area of the small intestine, varying blood flow rate, and law of mass action maintaining a gradient. It should be noted, however, that small lipid-soluble substances generally enter the mucosal epithelial cells by passive diffusion.

In addition, a variety of active transport and facilitated mechanisms are responsible for transporting nutrients, including monosaccharides, amino acids, and minerals, into the mucosal epithelial cells. A xenobiotic transport mechanism that has been identified features multidrug resistance (MDR) proteins or P-glycoprotein. Unfortunately, this transport system moves some chemotherapeutic agents out of some tumor cells, rendering these agents less effective, but it also transports some other potentially toxic chemicals out of cells, thus protecting the organism from toxic doses. This mechanism, then, functions to move certain xenobiotics out of the cell and reduce the net amount of GI absorption of some toxic chemicals. MDR-associated proteins (MRPs) have been shown to transport glucuronides and glutathione metabolites out of cells as well [14]. The iron transport mechanism also absorbs toxins such as thallium, and manganese and cobalt both utilize and compete for access to the iron transport system. The calcium transport mechanism absorbs lead, and the pyrimidine transport system absorbs 5-fluorouracil. These toxicants are actively transported into the mucosal epithelial cells of the GI tract, but the vast majority of toxicants enter by simple passive diffusion. Low molecular weight, lipid-insoluble compounds are absorbed through aqueous membrane pores at the tight junctions in the membrane via passive diffusion. Another factor in the absorption of toxicants is that particulate toxicants can be absorbed via vesicular transport, either by pinocytosis or by phagocytosis.

In addition, another phenomenon has been identified as a rate-limiting barrier to the absorption of toxicants in the small intestine: the unstirred water layer. Normally, the intestinal mucosal cell membranes are separated from the aqueous phase in the lumen by an unstirred water layer. This layer surrounds the villi and becomes a significant rate-limiting step with regard to the entry of all substances (nutrients and toxicants alike) into the mucosal cells. This water layer is not well mixed, and the solute molecules in the chyme must diffuse across the unstirred water layer to gain access to the membrane of the mucosal cells [107]. Micellar solubilization, on the other hand, greatly enhances the number of molecules that are available for uptake by the mucosal cells in the lumen. Tso et al. [77] suggest that micellar molecules are in equilibrium with molecules in the unstirred water, so the momeric fatty acids, monoglycerides, and amino acids cross the microvillar membrane. The large, polar bile salts are not absorbed well by intestinal mucosa, so the bile salts remain in the chyme to participate in forming more mixed micelles as they cycle within the intestine. The integration of segmentation and propulsion enhances absorption by mixing luminal contents with digestive juices and by increasing the contact time between chyme and absorptive cell surfaces. Intestinal motility also decreases the thickness of the unstirred layer of water so nutrients and electrolytes can diffuse readily to mucosal cell membranes. The noninvasive techniques used to assess the integrity and function of the human GI system have been recently reviewed by Grootjans et al. [283].

Factors Affecting Distribution

When a toxicant gains entry into either the lymphatic or blood circulatory system, it can move rapidly throughout the body. Its effect at the target tissue is based on the affinity of the toxicant to the target. The routes of entry into various target tissues include either active transport or simple passive diffusion. As previously noted, small, low molecular weight, water-soluble toxicants are able to pass through pores in the membrane via filtration and diffusion, while polar toxicants with larger molecular weights must be actively transported across the membrane. Lipid-soluble toxicants are able to easily penetrate the membrane. The rate of uptake is ultimately determined by either the diffusion rate, which is governed by the specific characteristics of the toxicant, or the perfusion rate, which is governed by the physical rate of delivery to the target tissue [14].

Generally, the concentration of a toxicant is dependent on the volume of distribution through the body compartments. Some toxicants are limited in their distribution by size, charge, or shape; others can pass through the various compartments and become distributed throughout the organism. The plasma concentration of a toxicant is critical, as it reflects the available concentration of the substance at the target tissue. Generally, if the concentration in plasma is high and the interstitial and intracellular concentrations are low, the amount of unbound toxicant available at the target tissue increases. Once a toxicant has entered the blood plasma, it may be excreted or biotransformed into more or less toxic substances, or it may be stored. Because of protein binding, active transport, or some other physiological factor, some toxicants accumulate in certain areas of the body. This can be a protective measure if the site of accumulation is other than a potential target tissue. In this particular case, the compartment in which a certain toxicant is accumulated is termed a *storage depot*. Because the toxicant is in equilibrium with its counterparts in the plasma and elsewhere, it can be released from the storage depot as the plasma level decreases over time. Interestingly, the initial distribution of various toxicants can change based on flow rates and toxicant affinities. The details and chemical characteristics of the common storage depots available to many organisms are described elsewhere in this book. For our discussion here, the reader should be aware of the following storage depots and how they influence the distribution of toxicants in the organism.

Albumin is the major plasma protein that functions as a storage depot. Although there are other plasma proteins, albumin can bind a large number of toxicants; at least six binding regions have been identified on the protein [108,109]. The binding of various toxicants as well as endogenous substances to plasma proteins such as albumin inhibits that substance from crossing the capillary beds and entering into extracellular space for distribution. The binding mechanism

is reversible, so equilibrium can be maintained as various compartmental concentration levels change over time.

The liver and kidney both function as storage depots and can store a considerable number and amount of toxicants; both organs concentrate and remove toxicants as a primary function. Proteins found in these organs, such as metallothionein and ligandin, have been shown to bind organic acids, cadmium, and zinc, which are concentrated in these organs. It is estimated that more toxicants are stored in these two organs than in all of the other organs combined [14].

Body fat functions as a storage depot. Because of the lipophilic nature of many toxicants, many are stored in neutral body fat in large amounts. Bone also functions as a storage depot. The hydroxyapatite crystals of bone provide a very large surface area for toxicants such as lead, strontium, and fluoride to easily penetrate. The toxicants in bone are reversibly bound, so these materials can be released back into the plasma by ionic exchange and dissolution of bone lattice by osteoclastic activity.

Factors Affecting Elimination

Fecal elimination is another pathway for the GI tract to effectively remove toxicants. Three major components contribute to the process of toxicant excretion through the feces.

Biliary Excretion

Because the liver is first in line for the newly absorbed nutrients or toxicants, it can and does extract and biotransform toxicants of all sorts so the metabolites can be excreted into the bile. The bile is then reintroduced into the GI tract and excreted in the feces unless it is reabsorbed in the enterohepatic circulation system. An increase in the amount of biliary excretion can lessen the ultimate toxicity of a toxicant unless some biological effect such as hydrolysis occurs that increases the lipophilic nature and absorbability of the toxicant. Bile components are divided into three classes of compounds based on their bile/plasma concentration ratios. Class A compounds have a bile/plasma ratio of ~1 and include substances such as sodium, potassium, glucose, mercury, and thallium. Class B compounds have a bile/plasma ratio of >1 but usually between 10 and 1000. Some class B compounds would include bile acids, lead, arsenic, and many more common toxicants. Class C compounds have a bile/plasma ratio of <1 and include materials such as inulin, albumin, zinc, and chromium. Class B compounds are usually rapidly excreted by active transport directly into the bile. In general, conjugates with molecular weights greater than 325 and conjugates of both glutathione and glucuronide appear to have a higher concentration in the bile. Numerous transport systems in the hepatocytes have been identified whose function is to move these various substances to and from the liver and back into the GI tract [4,10,277,286].

Table 31.25 provides a summary of the better-known transport systems in the liver hepatocytes.

Intestinal Excretion

Studies have been performed in bile-duct-ligated animals in which the presence of a number of substances in the feces could only be attributed to direct transfer from the circulating blood to lumen of the small intestine [138–141,279]. Hence, direct excretion into the small intestine from the mucosal epithelial cells constitutes a portion of the source of toxicants excreted in the feces. In addition to the secretion of various chemicals

TABLE 31.25
Transport Systems in the Liver Hepatocyte

Transport System	Abbreviation	Function	Location	Refs.
Na^+-taurocholate cotransporting polypeptide or liver bile acid transporter	NTCP or LBAT	Transports bile acid taurocholate into the liver—part of the enterohepatic circulation of bile acids	Sinusoidal side	[110–113]
Organic-anion polypeptides	OATP1A2	Transport organic polypeptides into the liver—hepatic uptake	Sinusoidal side	[111,114–119]
	OATP1B1			
	OATP1B3			
	OATP2B1			
	OATP2A1			
	OATP3A1			
	OATP4A1			
	OATP5A1			
	OAT7			
Liver-specific transporter	LST1, LST2	Transports toxicants into the liver	Sinusoidal side	[120–122]
Organic-cation transporter	OCT1, OCT2, OCT3, OCT6	Transports toxicants into the liver	Sinusoidal side	[123–126]
Bile salt excretory protein	BSEP	Transports bile acids out of the liver	Bile canaliculi side	[127]
Multidrug-resistant protein 1	MDR1	Transports toxicants into the bile	Bile canaliculi side	[128–130]
Multiresistant drug protein 2	MRP2	Transports toxicants into the bile	Bile canaliculi side	[131–133]
Multiresistant drug proteins 3 and 6	MRP3, MRP6	Transport toxicants back into blood circulation	Blood capillaries	[134–137]

directly into the GI tract, the microflora have been shown to biotransform some toxicants to forms that become eliminated through the feces rather than reabsorbed. Because toxicants in the lumen of the intestine are also ingested by the resident microflora, it is estimated that some portion of the toxicant has been biotransformed by these intestinal microflora.

Nonabsorbed Excretion

Some ingested nutrients and toxicants simply do not get absorbed during their transit through the GI tract; for example, because of their chemical characteristics, ingested sucrose polyester, cholestyramine, and ionized compounds such as quaternary ammonium are not well absorbed and are found in fecal excretions.

TESTING THE GASTROINTESTINAL TRACT FOR TOXICANTS

A number of tests are available to detect any effects caused by the entry of toxic agents. Among them are tests that examine the structural integrity of the luminal cell well. Table 31.26 provides a summary of the types of testing utilized to examine the structural integrity and continuity of the GI tract. Toxicants can also effect changes in the rate of cell proliferation in the intestinal mucosal walls. Antineoplastic agents tend to slow the rate of cell division as part of their therapeutic mechanism, but some effects are indirect in that the toxicant can interfere with normal cell regulatory mechanisms, such as hormones, or cause tissue injury [165,166]. Several tests have been developed to examine the proliferation of cells of the mucosa. Table 31.27 is a summary of some of the available tests for mucosal cell proliferation.

The gastric secretion of hydrogen chloride from the parietal cells is a critical factor for the initiation and optimal functioning of pepsinogen and prochymosin within the stomach; therefore, toxicants that alter the gastric secretory activity can have a profound effect on the functioning of the stomach. The rate of acid secretion in the parietal cells is under both neurological and hormonal control, so the measurement of acid secretion provides an index of measurement for the status of digestion. Table 31.28 summarizes some of the procedures available for measuring gastric secretion.

Gastric emptying is a critical process in the GI tract because it controls the rate at which properly triturated chyme is released into the small intestine for optimal absorption [188–191]. The pylorus (from the Greek word for *keeper of the gate*) determines what enters the small intestine from the stomach. Under normal circumstances, it allows only chyme of the proper texture and size to enter the small intestine. The control of gastric emptying is complex and can be altered by stress [192], meal composition and size [193], pathological processes, and pharmacological interactions [190]. Methods for the assessment of gastric emptying can be divided into three basic types: (1) tracer studies, (2) imaging studies, and (3) electrical resistance studies. Representative studies from each of these categories are summarized in Table 31.29.

TABLE 31.26
Testing for Structural Integrity

Test	Description	Examined Entity	Notes	Refs.
Direct observation of mucosal cell wall	Invasive visual inspection of GI tract for lesions; quantitation of damage	GI walls	Duodenal and gastric ulcer indices are created to quantitate findings.	[142,143]
	Invasive examination of the structure and organization of the GI tract wall	GI walls	Slides are prepared to evaluate cellular organization and structure; endoscopic examination and biopsy methods.	[144–147]
	Invasive visual inspection of GI tract pretreated with dyes for lesions; quantification of damage	GI walls	Sky Blue Dye 6, Evans blue dye, or Monastral Fast Blue B is injected intravenously.	[148–151]
Mucosal permeability	Invasive exam of blood to lumen clearance of ^{51}Cr-EDTA	Assessment of leakage into lumen from blood stream	Blood and perfusate are collected to measure EDTA clearance.	[152,153]
Fecal blood loss	Noninvasive examination of feces for occult blood	Assessment of blood leakage into the GI tract by colorimetric methods	Guaiac (hemoccult) is the least sensitive and *o*-toluidine the most sensitive. HemoQuant measures fluorescence of porphyrins. Recent studies indicate these tests are insensitive.	[154–158]
	Intravenous injection of ^{59}Fe sulfate for in vivo labeling of red blood cells	Assessment of blood leakage into the GI tract by radioisotopic methods	Procedure is more sensitive than colorimetric methods; 24 h fecal collection and assay for radioactivity.	[159,160]
Cell shedding	Monitoring rate of cell loss in the GI tract	Measure DNA content of luminal fluid from surgically implanted GI tubes	Fluid is quantified for displacement of ^{125}iododeoxyuridine DNA from DNA antibodies.	[161–164,275,276]

TABLE 31.27
Testing for Mucosal Cell Proliferation

Test	Description	Examined Entity	Notes	Refs.
Cell kinetic analysis	Invasive intravenous injection of ^{3}H-thymidine to label crypt cell DNA	Assessment of the duration of the cell cycle and cell migration progress	Pulse exposure to ^{3}H-thymidine with tissue harvest and fixed slides is a 2- to 4-week process.	[167,168]
Antibodies to cell cycle antigens	Use of proliferation cell nuclear antigen (PCNA) to label S-phase cells in crypts requiring incubation of the tissues	Assessment of proliferation of GI cells	Method can be completed more quickly than autoradiography.	[169]
Flow cytometry	Incubation of intestinal crypt and villa cells and separation by DNA content	Assessment of proliferation of GI cells	Method can be completed more quickly than autoradiography.	[170,171]

TABLE 31.28
Testing for Gastric Secretory Activity

Test	Description	Examined Entity	Notes	Refs.
In vivo direct gastric acid collection	In this invasive procedure, the pylorus is tied off in an anesthetized animal; the gastric content is sampled periodically via syringe.	The sample is titrated to pH 7.0 for hydrogen ions associated with specific endogenous compounds (i.e., mucoproteins).	Titration to pH 3.5 provides an estimate of total HCl present.	[172–174]
In vivo Azure A measurement	In this noninvasive procedure, Azure A–resin complex (azuresin, Diagnex Blue) is administered by gavage, and the animal is housed to collect a 24 h urine.	Allows the quantification of the breakdown of Azure A from azuresin, which is pH dependent.	The 24 h urine is measured for the concentration of Azure A by spectrophotometry.	[175,176]
In vivo fundic pouch	In this invasive model, a dog is surgically fitted with a Heidenhain pouch to permit controlled exposures.	Permits repeated measurement of volume changes, electrolyte concentrations, and mucosal blood flow.	The denervated pouch decreases the basal acid secretory rate, which must be factored in to the findings.	[177–179]
In vitro tissue in a Ussing chamber	Segment of the stomach is surgically removed and used to separate two solutions.	Acid secretion and electrolyte flux can be quantified.	The muscle layer is removed prior to use in the Ussing chamber.	[50,180–182,287]
In vitro culturing of gastric gland cell types	Gastric tissue is prepared with pronase and collagenase, and the cell types can be isolated and examined with no anatomical barriers between the mucosal and serosal surfaces.	With appropriate corrected measures, oxygen consumption can be measured with a polarographic electrode or a Gilson respirometer as an index of secretory activity.	A variation of this method is monitoring the uptake of aminopyrine; this substance moves into the intracellular space of the parietal cell and becomes trapped in the secretory vesicles.	[50,183—188]

Understanding the factors affecting absorption of the GI tract is important in order to characterize the absorption, metabolism, and excretion of toxicants and what effect toxicants may have on the absorption and metabolism of necessary nutrients or pharmacological substances being used for therapeutic purposes. In vitro models have been developed that mimic the human GI tract in an attempt to predict reactions of food and bioactive components in physiological conditions. These simulated digestion studies have been used for a number of years to investigate the digestion, behavior, and ultimate bioaccessibility (amount of a contaminant that reaches the systemic circulation and exerts a toxic effect) of animal proteins [212], plant proteins [213], and food additives [214]; to study the effects of the foodstuff matrix on the bioavailability and bioaccessibility of ingested contaminants [36]; to examine carotenoids in biological emulsions [215,216]; and to determine the effects of ingesting contaminants from soil [217]. In vitro digestion models reflecting the conditions of the GI tract in the presence or absence of food have been used to study the bioaccessibility of compounds from soil. It is known that fasting can have a significant impact on the oral bioavailability of compounds, as the presence of food alters conditions in the GI tract. To mimic intestinal absorption, the Caco-2 transport model was used [218].

The general procedure for studies such as these is that the digestion process of the GI tract is simplified by applying physiologically based conditions such as synthetic saliva, gastric juice, duodenal juice, and bile to samples with controlled

TABLE 31.29
Testing for Gastric Emptying Activity

Test	Description	Examined Entity	Notes	Refs.
Tracer studies	*Breath tracers* are a noninvasive procedure involving ingestion of a ^{13}C-labeled substrate that produces a detectable increase in $^{13}CO_2$.	After gastric emptying, the ^{13}C-labeled substrate can be measured in the exhaled breath as $^{13}CO_2$.	Exhaled breath samples are examined by isotope-ratio mass spectrometry for the $^{13}C/^{12}C$ ratio over time.	[194–196]
	Gastric tracers are an invasive procedure involving gastric gavage of a known amount of nonabsorbable marker.	Serial aspiration samples are taken through a nasogastric tube or a catheter in the gastric wall.	The serial gastric samples are assumed to be homogeneous and are examined over time.	[190,197,198]
	Plasma tracers are an invasive procedure involving ingestion of paracetamol, which is slowly absorbed into the blood in the stomach and rapidly absorbed in the small intestine.	Serial blood samples are taken via an intravenous catheter.	The paracetamol concentration is measured in the blood L\plasma levels over time and is an indirect measure of gastric emptying.	[190,199]
Imaging studies	*Radiography* is a noninvasive procedure involving ingestion of radiopaque solids and liquids.	After ingestion, serial lateral and ventral radiographic images are taken over 4–12 h.	The movement of the radiopaque material is monitored qualitatively via the radiographic images.	[190,200,201]
	Radioscintigraphy is a noninvasive procedure involving ingestion of food containing radioisotopes.	Radiographic images are integrated with an integrated nuclear medicine computer system, and the radioactive counts are recorded over time.	With the application of various correction factors to allow for radioactive decay, the movement of gastric contents can be recorded over time.	[202]
	Ultrasonography is a noninvasive procedure used to measure flow through areas of the antral portion of the stomach.	Ultrasonographic images are taken as materials move through the stomach in real time.	Movement is measured in real time; however, further validation of this method is necessary at this time.	[203]
	Magnetic resonance imaging is a noninvasive procedure used to examine liquid- and solid-phase gastric emptying and motility.	Magnetic resonance images provide a 3D image of the stomach emptying.	Emptying and motility are measured simultaneously.	[204–206]
Electrical resistance studies	*Impedance epigastrography* is a noninvasive procedure that uses two pairs of electrodes to monitor electric current after the ingestion of food.	An electric current is applied to the epigastric region with a pair of electrodes, while another pair of electrodes monitors the changes in the current.	Ingestion of food causes changes in the pattern of electrical resistance, which is an indirect measure of gastric emptying.	[190,207,208]
	Applied potential tomography is a noninvasive procedure that uses multiple pairs of electrodes to monitor electric current after the ingestion of food.	An electric current is applied to the epigastric region with multiple pairs of electrodes that monitor the changes in electric current over the entire upper abdomen.	Ingestion of food causes changes in the pattern of electrical resistance, which is an indirect measure of gastric emptying.	[209–211]

pH and residence time periods that mimic each compartment of the GI tract. Two-stage digestion systems represent the stomach and the small intestine, and three-stage digestion systems represent either (1) the mouth, stomach, and small intestine or (2) the stomach, small intestine, and large intestine [36]. Because practically no absorption occurs in the mouth or the stomach, bioaccessibility is usually determined from the chyme resulting from the small intestine compartment. Figure 31.11 is a flow diagram of a typical in vitro digestion model.

The Bioavailability Research Group Europe (BARGE; http://www.bgs.ac.uk/barge/home.html, accessed June, 2012.) was established in 1999 to promote cooperation among European countries interested in developing and comparing data and protocols for in vitro digestion systems designed to analyze data on oral bioavailability of soil contaminants. BARGE has published comparison studies of the findings of various European laboratories [219,220], and data collected by BARGE are summarized in Table 31.30. Table 31.31 summarizes tests that can be used to help characterize and quantify absorption within the GI tract.

Normal peristalsis is necessary for digestion, the delivery of chyme to various areas for absorption, and waste elimination. Factors that control peristalsis are numerous and complex. The motility characteristics change in pattern and periodicity from one segment of the GI tract to the next.

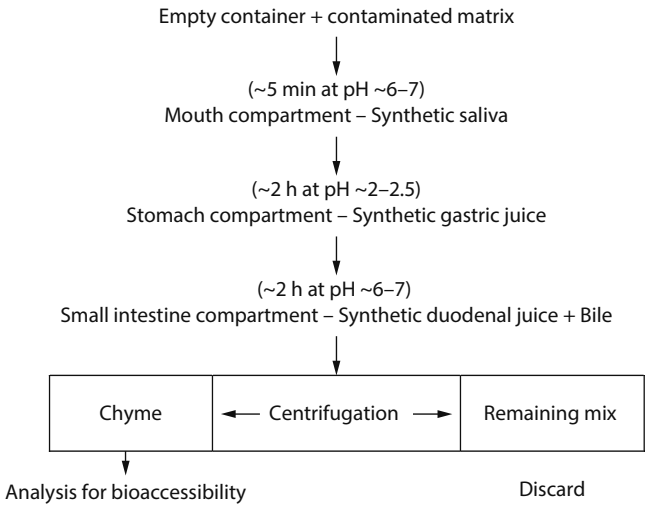

FIGURE 31.11 Depiction of a typical in vitro digestion study. (Adapted from Versantvoort, C.H.M. et al., Development and applicability of an *in vitro* digestion model in assessing the bioaccessibility of contaminants in food, RIVM Rep. No. 320102002/2004, National Institute for Public Health and the Environment, Bilthoven, the Netherlands, 2004. Available online: http://rivm.openrepository.com/rivm/bitstream/10029/8885/1/320102002.pdf)

The activity is mediated by a complex system of neurons in the wall of the GI tract that coordinate contractions between the longitudinal and circular musculature. The contractions are both neurologically and hormonally controlled. The neurological control is dependent on the following enteric (intrinsic) neurotransmitters: vasoactive intestinal peptide, enkephalin, substance P, gastrin-releasing peptide, NPY, and somatotropin. Extrinsic neurotransmitters from the vagus and pelvic nerves include choline, acetylcholine, and norepinephrine. The hormonal control is mediated by gastrin, secretin, and cholescystokinin, all of which are secreted by the walls of the GI tract in response to certain dietary components. Table 31.32 summarizes the more common methods used to analyze motility in the GI tract. Further details on common techniques used to assess gastric motility were recently reviewed by Szarka and Camilleri [258].

As noted previously, the entire length of the GI tract contains microflora, many of which are located in the large intestine [85,266]. Pathogenic strains of some bacteria such as *Shigella* and *Helicobacter pylori* can mediate some toxic effects because they invade the GI mucosa and produce physical changes in the cell wall that can lead to gastritis and peptic ulcers. Intestinal bacteria, such as *Vibrio cholerae*, *Clostridium difficile*, and *Escherichia coli*, are also capable of generating toxins that change water and electrolyte flux rates in the cell wall and ultimately produce diarrhea [285]. Other bacteria are able to activate certain foreign chemicals, such as cycasin, which is found in the nuts of cycad plants, to a form that is carcinogenic and can cause tumors in the liver, kidneys, and colon [267,268]. Table 31.33 provides a summary of some methods used to test for the potential toxic effects of bacteria in the GI tract.

CONCLUSION

The effect of a toxicant on an organism depends primarily on the relative effective concentration and the duration or persistence of the ultimate toxicant at a specific site of action. Numerous portals of entry exist for toxicants. This chapter has focused on the normal structure and function of the GI tract and how the processes of digestion and absorption influence and are influenced by toxicants that enter the GI tract. The intensity of effect of the toxicant is determined by a number of factors that are part of the normal functioning of the GI tract, such as digestion, absorption, biotransformation, distribution, and elimination. Finally, the last part of this chapter briefly describes some of the types of testing that have been developed to examine the effects of toxicants on various aspects of the normal functioning of the GI tract.

TABLE 31.30
Various In Vitro Digestion Methods Assessed by BARGE

Test	Description	Stomach pH	Stomach Incubation Interval	Intestine pH	Intestine Incubation Time	Bile Concentration	Separation Method for Chyme	Refs.
SBET[a]	Static gastric model	1.5	1 h	NA	NA	NA	0.45 μm filter	[221]
RIVM[b]	Static GI model	1.1	2 h	5.5	2 h	0.9 g/L	Centrifugation at 3000 g	[217,222]
SHIME[c]	Static GI model	4.0	3 h	6.5	5 h	1.5 g/L	Centrifugation at 7000 g	[223–225]
DIN[d]	Static GI model	2.0	2 h	7.5	6 h	4.5 g/L	Centrifugation at 7000 g	[226,227]
TIM[e]	Dynamic GI model	5.0 → 2.0 over 90 min	Gradual secretion at 0.5 mL/min	6.5–7.2	Gradual secretion at 1 mL/min	Variable	Hollow-fiber membrane	[228–230]

[a] SBET, Simplified Bioaccessibility Extraction Test (used in lead analysis in the United Kingdom).
[b] RIVM, Rijks Instituut voor Volksgezondeidand Milieu (National Institute for Public Health and the Environment) method (the Netherlands).
[c] SHIME, Simulator of Human Intestinal Microbial Ecosystems method (Belgium).
[d] DIN, Deutsches Institut fur Normung method (Germany).
[e] TIM–TNO Nutrition and Food Research computer-controlled dynamic GI model (the Netherlands).

TABLE 31.31
Testing of GI Tract Absorptive Function

Test	Description	Examined Entity	Notes	Refs.
In vivo determination of GI absorption	Follows a predetermined amount of a substance administered orally over time by determination of its concentration in systemic fluids	Test measures the bioavailability of a xenobiotic and the absorption kinetics on systemic exposure via a metabolic cage.	This is the least precise method to quantify GI tract absorption kinetics due to the many uncontrollable variables.	[231]
	Quantification of absorption	Depending on the substance, measurements can be made in plasma, urine, feces, saliva, and breath.	After oral dietary or gavage administration, samples must be collected over adequate time and with adequate volume but should not affect total blood volume.	[232,233]
	Malabsorption test (D-xylose test)	Test measures the functional integrity of the proximal small intestine.	D-xylose is given by oral gavage, and blood samples are taken periodically for 3 h; D-Xylose concentration is evaluated spectrophotometrically.	[174,234]
In vivo determination of overall GI absorption	Malabsorption test (disaccharides)	Test measures the functional integrity of the disaccharidases ability to cleave disaccharides into monosaccharides.	Disaccharide substrate is given by oral gavage and the changes in blood glucose or the by-product of glucose metabolism (hydrogen gas) measured against known blood and breath values.	[234,235]
	Malabsorption test (fats)	The focus of fat test is to distinguish between defects in the digestion of fats to fatty acids from fatty acid resorption.	An examination of feces for increased fat content (steatorrhea) is followed by oral dosing with radiolabeled triglyceride assay or ^{33}P; the reduction of triglycerides indicates digestive defect, and a reduction in fatty acid absorption suggests bile salt defect or other mucosal defects.	[82,84,236,278,290]
In vivo closed intestinal segment	Invasive procedure where an intact intestinal segment is evaluated for absorption kinetics	An intact segment of the intestine is closed off and injected with the test substance; after a period of time, the intestinal segment is removed and the rate of substance reduction is quantified.	If metabolism of the test substance occurs in the lumen, for the test results to be valid, the fate of all metabolites must be known.	[237,238]
In vivo intestinal perfusion	Invasive procedure for quantifying intestinal transport by measuring the amount of substance in the effluent against the amount infused into GI tract over time	The net flux of water transport into the GI tract can be evaluated by monitoring changes in a nonabsorbable marker.	The proximal and distal ends of the intestine to be examined are cannulated, and the test substance is administered through the proximal cannula; after a period of equilibration, samples are drawn from the distal cannula and the volumes of the test substance are compared.	[239,240]
In vitro inverted sac technique	Characterizes the carrier-mediated processes by quantification of the test substance inside an inverted length of intestine	A small segment of intestine is everted and filled with a fluid and tied at both ends; absorption is quantified by monitoring the appearance of the test substance in the fluid on the inside of the everted sac.	Because of inadequate oxygen diffusion and tissue distention, the incubations must be no longer than 5 min with rat intestine.	[241–243]

(*continued*)

TABLE 31.31 (continued)
Testing of GI Tract Absorptive Function

Test	Description	Examined Entity	Notes	Refs.
In vitro isolated GI tract mucosal cells	Isolation of various GI tract mucosal cells	GI tract mucosal cells are isolated in cultures that permit analysis of membrane transport kinetics without contamination from extraneous substance metabolism.	Isolation of these cells has facilitated more detailed understanding of the biochemical and structural characteristics of cellular membranes.	[244,245,281]
In vitro electrical potential with a Ussing chamber	Short-term exposure of a segment of GI tract suspended in a Ussing chamber to defined solutions with a voltmeter to measure electric current	In addition to voltage, the flux of electrolytes can be measured by a pulse of isotope to one side of the chamber with subsequent quantification on the other side of the membrane.	In a variation of this technique, a unidirectional flux chamber is used to assess mucosal cell transport mechanisms.	[246–248]
In vitro organ cultures	Examination of mucosal explants for studies of cell proliferation and differentiation over longer periods of time	Test permits the study of colonic carcinogenesis and chemotherapy with measurement of toxicants and their effects.	Some colonic cultures have been extended to up to 28 days with normal anatomical cell arrangement.	[249–252,280]
In vitro $CaCO_2$, T^{84}, and HT^{29} cultures	Utilizes cultures of colonic carcinoma T^{84} and subclone HT^{29}, which exhibit normal colon cell and goblet cell characteristics, respectively, in cell cultures	These cultures can be examined for electrolyte transport of nutrients and toxicants.	These cell lines exhibit normal cell characteristics, including tight junctions, apical microvilli, and vectorial electrolyte transport.	[253–256]

TABLE 31.32
Testing of GI Tract Motility

Test	Description	Examined Entity	Notes	Refs.
In vivo measurement of transit time	Gastric or duodenal administration of a suitable marker such as a ^{51}Cr-labeled sodium chromate or ^{125}I- or ^{131}I-labeled polyvinyl pyrrolidine or ^{99m}technetium and subsequent recovery of sample	Using gamma-emitting radioisotopes allows for direct GI segment assay without any processing. Small intestine movements must be administered to the duodenum via an indwelling catheter.	A rapid euthanization procedure is used to quickly extract the GI tract; to stop movement within the tract, ligatures are placed every 3–5 cm. Counts are recorded within each segment.	[257,258]
In vitro rabbit jejunum	Maintenance of rhythmic contractions in vitro	This technique is used to analyze substances that manifest inhibitory effects on GI smooth muscle.	—	[259–262]
In vitro guinea pig ileum	Excision of a guinea pig ileum with the mesenteric plexus	This technique is used to examine substances that influence neuronal conduction and neurotransmitter release and to evaluate GI smooth muscle contractions.	This method has been used extensively to assess changes in contractility in smooth muscle.	[260,263–265]

TABLE 31.33
Testing of GI Tract Microflora

Test	Description	Examined Entity	Notes	Refs.
Toxin study: rabbit ileum	Bacterial toxins are injected into closed segments of surgically ligated 3 in. sections of rabbit ileum; data have revealed surface receptors for enterotoxins.	The toxins cause fluid accumulation when injected into the intestine; quantification of the fluid can provide information about intestinal antiabsorptive or secretory activity.	Rabbit ileum provides more consistent results; the ligated segments are placed at 6 in. intervals, and samples are taken at 4–6 h intervals for examination.	[269,270]
In vivo bacterial metabolism study in germfree animals	Aseptically delivered animals maintained in germfree conditions are treated against normal rats.	Various toxicants are administered to both types of animals, which are housed in metabolism cages where their urine and feces can be quantitated.	Differences in metabolites in urine and feces can indicate changes mediated by the intestinal microflora.	[271,272]
In vitro bacterial metabolism study	Defined bacterial cultures of luminal contents are cultured with various substances.	Demonstration of enzymatic activity in a bacterial strain in vitro can provide evidence of possible bacterial role in metabolite production.	Data can be flawed by synergistic action between an extract of *Bacteroides fragilis* and the microsomal enzyme activity of the mucosa of the large intestine.	[273,274]

QUESTIONS

31.1 Describe the functions of each of the various parts of the digestive system and accessory organs.
31.2 Describe the various cellular absorption processes.
31.3 Describe the excretions of and digestive functions of the accessory organs.
31.4 Explain how substances are chemically altered when they are ingested.
31.5 Describe the intestinal first-pass effect and how it influences toxicity.
31.6 What is enterohepatic circulation?
31.7 Describe the critical factors influencing toxicity of ingested substances.
31.8 Describe the critical factors influencing distribution of ingested substances.
31.9 Describe the use of SHIME, SBET, and RIVM tests in food and soil contamination studies.
31.10 How do storage depots influence gastric toxicity?

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32 Pathophysiology and Toxicology of the Heart

Khalid Almuti, Shahid Rahman, and Daniel Acosta, Jr.

CONTENTS

OVERVIEW/INTRODUCTION

The discipline of cardiac toxicology is concerned with the adverse effects of drugs, chemicals, and xenobiotics on the heart and vascular system. These chemicals may directly and adversely cause changes in the structure and function of the myocardium. In addition, there may be indirect effects secondary to changes in other organ systems, such as the nervous systems (both central and autonomic) and the endocrine system.

Examples of functional abnormalities caused by chemicals include alterations in the heart rhythm potentially leading to lethal arrhythmias even in the absence of any preexisting cardiovascular structural abnormalities. Structural myocardial alterations may also result from the direct actions of certain chemicals including degenerative necrosis and inflammatory reactions.

This chapter presents an overview of normal and abnormal cardiac structure and physiology with selected examples of cardiotoxicants and their major mechanisms of cardiotoxicity. It also discusses the topic of myocardial cell death including the processes of apoptosis and necrosis. The reader may also wish to consult other monographs on the toxicology of the heart, found in general toxicology texts [1] or specific references on cardiovascular toxicology [2].

NORMAL CARDIAC PHYSIOLOGY

Understanding normal cardiac structure and function facilitates a better appreciation of the toxic mechanisms of different substances on the heart and vasculature. The purpose of the cardiovascular system is to provide oxygen delivery to meet the metabolic demands of the body. This is achieved by the heart acting as a pump to drive oxygenated red blood cells to the various tissue beds. The stimulus for regular cardiac contraction is the electrical action potential, which in turn leads to intracellular calcium release and subsequent force generation via actin and myosin cross-bridge formation [3]. The ultrastructure of cardiac myocytes facilitates force generation by both mechanical and electrical cell-to-cell coupling and an abundance of ATP-producing mitochondria, which fuel the metabolic demands of the myocyte.

With a thorough understanding of the normal physiology of the cardiovascular system, the major pathophysiologic mechanisms of cardiovascular disease will be addressed. We will also explore the roles by which some toxicants may precipitate or exacerbate cardiac dysfunction.

Cardiac Function and Metabolic Homeostasis

The cardiac chambers act as reservoirs that fill with and eject blood. There are multiple metabolic and neurohormonal systems that influence cardiac function. These varied inputs modulating cardiac function alter cardiac output to meet the physiologic demand for oxygen. Oxygen demand is the amount of oxygen needed to satisfy the metabolic demand of all body tissues. Oxygen delivery from the heart to distal tissues is the product of cardiac output and the oxygen content of arterial blood:

$$\text{Oxygen delivery} = \text{Cardiac output} \times \text{Oxygen content}$$

The oxygen content of arterial blood is influenced by multiple factors including the amount of oxygen delivered to the blood stream via respiration and the oxygen-carrying capacity of the blood. Hemoglobin is a major determinant of blood oxygen content and normally carries 98% of dissolved oxygen. Under a steady state of blood oxygen content, cardiac output becomes the primary determinant of changes in oxygen delivery.

Cardiac output is the product of stroke volume and the heart rate (cardiac output = stroke volume × heart rate). The stroke volume is the difference between the blood volume in the ventricle before and after contraction. The capacity of the heart to contract independently of changes in pressure conditions (i.e., preload and afterload) is defined as the contractility of the ventricle. These variables are modulated to achieve a desired stroke volume from each ventricular beat [4].

The ventricular pressure and volume are not linearly related. This is due to a property termed compliance of the heart. Unlike a rigid box, the ventricle is able to stretch as it fills with blood. Due to its compliance ability, abrupt volume changes do not cause large swings in intraventricular pressure readings. In the absence of compliance, changes in ventricular volume would otherwise cause abrupt increases in venous pressure leading to possible consequences such as pulmonary edema. The relationship of the ventricular volume and pressure may be graphically depicted as the pressure–volume curve (Figure 32.1).

Understanding the relationship between ventricular pressure and volume helps to explain how toxicants causing left ventricular fibrosis and stiffening (e.g., anthracyclines) may increase ventricular pressure at a given preload volume potentially leading to clinical disease manifestations.

Afterload is often thought of as the pressure against which the heart must work to eject blood out of the ventricle. One of the main determinants of afterload is the systemic blood pressure and arterial stiffness [5]. This pressure must be overcome to propel blood from the heart to the aorta.

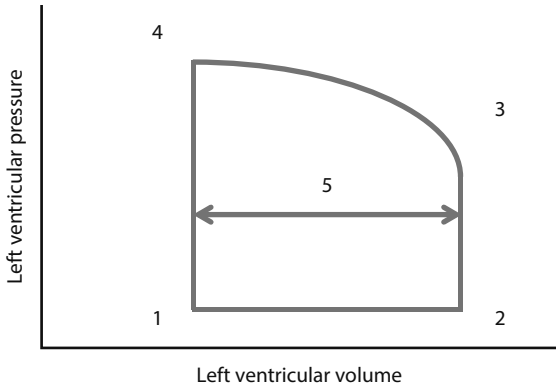

FIGURE 32.1 Pressure–volume loop. (1) End of isovolumetric relaxation, (2) end diastolic pressure, (3) end of isovolumetric contraction, (4) end systolic pressure, and (5) stroke volume.

Toxicants that cause abrupt increases in systemic blood pressure will increase the afterload and consequently the amount of work the heart must perform to overcome this pressure and to eject blood. Hence, severe hypertension, caused by drugs such as cocaine or amphetamines, may cause myocardial ischemia even without significant obstructive coronary artery disease [6].

Multiple systems modulate the contractile state of the heart to change the stroke volume to meet the demands of the body. At the level of the myocyte, increased contractility is often achieved by increasing intracellular calcium. The calcium ions interact with troponin leading to a conformational change in the inhibitory troponin molecule [7]. Subsequently, this change allows for cross-bridge formation between actin and myosin, which are the contractile components in the myocyte. Increased calcium thus leads to increased interaction between actin and myosin and increased force of contraction.

One of the primary determinants of contractility is the autonomic nervous system via the beta-adrenergic system. The beta-adrenergic receptor in the heart is a G-coupled protein receptor. When this receptor is stimulated, it leads to a downstream cascade of signals resulting in phosphorylation of the calcium channel and eventually increased calcium release [8].

Electrophysiological Basis of Contraction

The normal pacemaker cells in the heart have the capacity to modulate their function in response to extrinsic inputs from the autonomic nervous system. An increase in heart rate will lead to an increase in cardiac output via the formula stated earlier (cardiac output = heart rate × stroke volume). An appropriate increase in heart rate in response to a stimulus (i.e., exercise, pain, fever) can reach levels that depend, in part, on the person's age (younger persons can usually achieve faster heart rates). The cardiac electrical system is both sophisticated and multilayered. It is composed of cardiac myocytes that have the capacity to both generate and conduct electrical impulses.

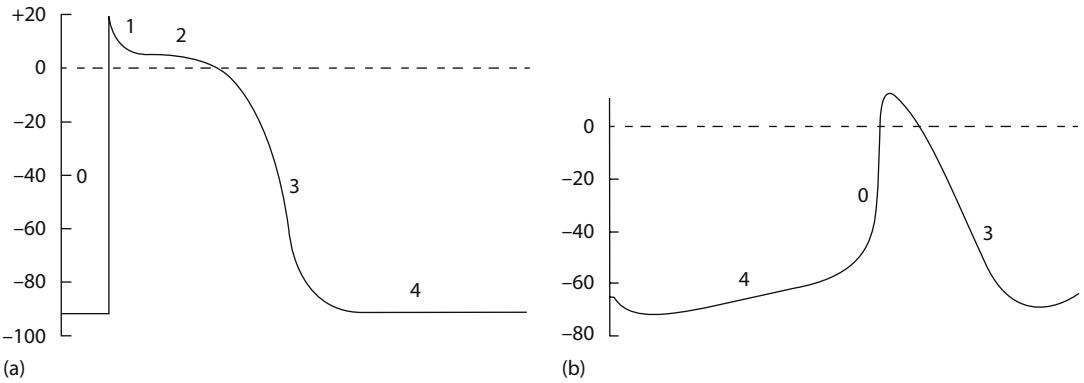

FIGURE 32.2 Typical action potentials (in mVs) recorded from cells in the (a) ventricle and (b) SA node. (Adapted from Hoffman, B.F. and Cranefield, P.E., *Electrophysiology of the Heart*, McGraw-Hill, New York, 1960.)

Cardiac myocytes are organized in a hierarchical system with one group of the cells, the sinoatrial (SA) node, being the principal pacemaker of the heart. Other cell types (i.e., atrioventricular [AV] node, His–Purkinje system, and ventricular myocytes) function as conductors of the electrical signal while retaining the capacity to generate electrical impulses themselves if called upon to do so with failure of the SA node. Under normal circumstances, the pacemaker cells of the heart produce myocardial action potentials with a great deal of regularity and dependability. This property is termed normal automaticity. Automaticity is highly regulated and depends on the interplay between multiple intracellular and extracellular (including extracardiac) factors. Such interplay allows the heart to beat with variable rates based on the various inputs and circumstances. Assuming a resting adult heart rate of 60–80 beats/min, the typical heart beats approximately 100,000 times daily. Over an average lifespan of 75 years, the heart may beat close to 2.75 billion times. This demonstrates the robustness and reliability of the system.

The SA node is a spindle-like structure that resides just below the epicardial surface of the right atrium near its junction with the superior vena cava (SVC). It receives, interprets, and acts upon signals received from its own myocyte membrane ion channels and other intracellular mechanisms. All of these data have to be consolidated in a very rapid and efficient manner with inputs from various neurotransmitters, hormones, and stretch receptors. These mechanisms collectively function as an internal clock that triggers the next myocardial action potential.

Myocardial Action Potential

The depolarization of the SA node subsequently triggers electrical impulses through the myocardium. Under normal circumstances, the resting potential of a myocyte is about −60 to −90 mV relative to the extracellular fluid potential. A slow potential change occurs just prior to initiation of a subsequent action potential. A sudden depolarization then dramatically changes the membrane potential from negative inside to positive inside. Depolarization is ultimately followed by repolarization, which resets the cell's resting potential. The process of an action potential from depolarization to the completion of repolarization is divided into five phases in cardiac Purkinje fibers, as shown in Figure 32.2. Phase 0 represents a rapid depolarization due to the inward current of Na^+. Phase 1 is associated with an immediate rapid repolarization, during which Na^+ inward current is inactivated and a transient K^+ outward current is activated, which is followed by an action potential plateau, or phase 2, which is characterized by a slowly decreasing inward Ca^{2+} current and slow activation of an outward K^+ current. Phase 3 reflects a fast K^+ outward current and inactivation of the plateau Ca^{2+} inward current, and phase 4 is the diastolic interval for the resetting of the resting potential.

The cells of the SA node are unique in that they are not dependent on the inward Na^+ current (I_{Na}) for their upstroke. Instead, the action potential upstroke is predominantly due to action of the L-type calcium current (I_{CaL}). It is thought that local oscillation in Ca^{2+} channels contributes to the depolarization of the cell membrane. The resultant Ca^{2+} influx itself results in the release of calcium stores from the sarcoplasmic reticulum (SR). This phenomenon is known as calcium-induced calcium release and plays an important role in the process of muscle contraction. Eventually, outward potassium currents repolarize the cell membrane by reestablishing a negative potential.

Electrical Mechanical Coupling

Myocyte contraction occurs when an action potential depolarizes the cardiac membrane. The depolarization causes a conformational change in L-type calcium channels, which allows a small amount of calcium to enter the myocyte. These L-type calcium channels are located in close proximity to the SR, an intramyocyte calcium storage structure. The calcium then binds to the ryanodine receptor on the SR, which subsequently leads to a conformational change in its structure resulting in the release of large amounts of stored calcium. The increase in calcium concentration in the cell allows calcium to bind to troponin, causing a conformational change in this inhibitory molecule. This conformational change permits interaction between the actin and myosin filaments

through cross-bridge formation. Adenosine triphosphate (ATP) is hydrolyzed by ATPase, present in the cross bridges, to release energy so the cross bridges can move in a ratchet-like fashion. This action increases the overlap of the actin and myosin filaments, resulting in shortening of the sarcomeres and contraction of the myocardium. This action-potential-triggered calcium increase in the plasma and myocyte contraction is referred to as excitation–contraction coupling [9].

The heart muscle as a whole has to synchronize the contraction and relaxation of individual myocytes to perform its pump function. This is achieved by gap junctions, which are special structural features of cell-to-cell interaction. Through the gap junction, major ionic fluxes between adjacent cardiomyocytes are spread, thus allowing electrical synchronization of contraction. Each single gap junction is composed of 12 connexin 43 (Cx43) units assembled in two hexametric connexons (hemichannels), which are contributed, one each, by the two participating cells. On average, a given cardiomyocyte is electrically coupled via gap junctions to 11 adjacent cells [10].

CARDIAC PATHOPHYSIOLOGY AND TOXICANTS

Arrhythmias

Tachyarrhythmias may result from one of three main mechanisms:

1. Reentry
2. Abnormal automaticity
3. Triggered activity

1. Reentry: This is the most common mechanism of arrhythmias (both atrial and ventricular) [11]. It requires a specific setup that is created by a variety of anatomic and physiologic factors. In essence, two pathways are needed, each with distinct electrophysiological properties [12,13]. An arrhythmia is initiated when an electrical impulse (usually a premature electrical impulse) reaches these two pathways, and the electrical signal travels down one of the two pathways and back up the other (Figure 32.3.). This circuit continues up and then down the two pathways in a continuous manner until interrupted [12,13]. Certain pharmacologic agents can alter some of the electrical properties of the myocytes, which are crucial for initiation and maintenance of such reentrant arrhythmias. These include the antiarrhythmic drugs amiodarone, sotalol, and lidocaine among others. They also include drugs such as beta-blockers and calcium channel blockers. In this manner, antiarrhythmic medications "break" the circuit and prevent the arrhythmia from occurring. Paradoxically, sometimes the use of antiarrhythmic drugs may promote the induction and maintenance of these circuits [14].
2. Abnormal automaticity: This phenomenon occurs when a group of myocytes develops altered electrical activity (e.g., enhanced phase 4 depolarization of the myocyte action potential). Such an alteration of the action potential properties allows myocytes to fire rapidly in the absence of an appropriate extrinsic stimulus [15]. These rapidly firing cells

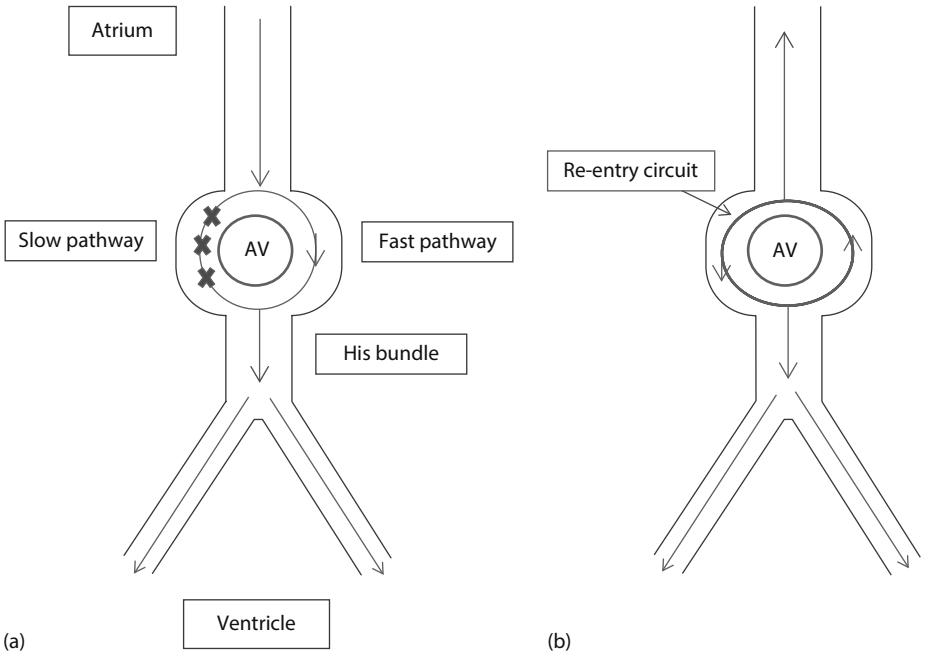

FIGURE 32.3 Schematic representation of a reentry circuit (in this case, the circuit for atrioventricular nodal reentrant tachycardia [AVNRT]). (a) Sinus rhythm is depicted; the electrical impulse enters the AV node and uses both the fast and slow pathways antegrade continuing down to the ventricles. (b) A reentrant tachycardia circuit is observed with the impulse going down one limb of the circuit, turning around, and going up the other limb (activating the atria and ventricles simultaneously).

suppress the SA node and can continue with their abnormal function for prolonged periods of time. The nature of such arrhythmias may be incessant in some cases and episodic in others. Increased automaticity may be caused by increased sympathetic tone or stimulants such as caffeine, nicotine, or amphetamines [16].

3. Triggered activity: These arrhythmias result from abnormal interruptions of the normal repolarization portion of the myocyte action potential. Such interruptions are called afterdepolarization and are further classified as either early or delayed [17]. Arrhythmias result when afterdepolarizations gain enough amplitude that a premature action potential is generated. Certain arrhythmias caused by drug toxicity (e.g., digoxin or theophylline) are related to this mechanism [18]. Such tachyarrhythmias can lead to mechanical cardiac abnormalities (e.g., tachycardia-mediated cardiomyopathy).

QT interval prolongation and related tachyarrhythmias (i.e., torsades de pointes [TdP]) may result from intrinsic or extrinsic factors. Certain inherited channelopathies may result in QT interval prolongation [19].

Alternatively, such QT interval prolongation may result from the toxic effects of certain drugs and substances.

A host of cardiac and noncardiac drugs account for most cases of acquired QT interval prolongation (Table 32.1) [20,21]. When TdP results, it may be of limited duration or it may be persistent. It may also degenerate into life-threatening ventricular fibrillation. Patients experiencing drug-induced QT interval prolongation may already have subclinical abnormalities in repolarization and the addition of the offending drug results in clinical manifestations of these abnormalities as the QT interval prolongs further. These repolarization abnormalities specifically affect phase 3 of the myocyte action potential. Such prolongation of repolarization provides more time for ectopy (i.e., early afterdepolarizations) to trigger episodes of TdP [22]. Some well-known drugs associated with TdP are methadone, phenothiazines, Haldol, and sotalol.

TABLE 32.1
QT Interval–Prolonging Substances

1. Antiarrhythmics
   A. Sotalol
   B. Dofetilide
   C. Ibutilide
2. Antimicrobials
   A. Ciprofloxacin
   B. Erythromycin
   C. Ketoconazole
3. Antipsychotics
   A. Haloperidol
   B. Phenothiazines
4. Anesthetics and opiates
   A. Sevofluran
   B. Methadone

It has been proposed that drug-related QT interval prolongation results from blockade of the outward delayed rectifier potassium (I_{kr}) channels [23]. The D,L-isomer of sotalol, an I_{kr} blocker, is estimated to have a 0.3%–7% risk of TdP. The risk increases with higher doses and the presence of conditions such as hypokalemia, renal failure, and other QT-prolonging agents among others [21,24].

Women appear to be at a higher risk of developing acquired QT interval prolongation with its clinical manifestations [25].

Bradyarrhythmias may result from failure of impulse generation and/or impulse conduction. Such failure may result from intrinsic factors related to either aging or certain disease states. In some cases, however, bradyarrhythmias can result from the toxic effects of various pharmacologic agents (i.e., beta-blockers, digoxin, antiarrhythmic agents, and calcium channel blockers). In some cases, the use of implantable pacemakers is necessary to treat marked symptomatic bradyarrhythmias.

Infarction

Myocardial infarction (known colloquially as a heart attack) occurs when the blood supply to the heart is inadequate to meet the metabolic demands of the tissue. When the myocyte does not receive adequate oxygen delivery, aerobic metabolic activity is unable to proceed and tissue ATP stores become depleted. The myocyte requires ATP for force generation and to maintain electrochemical gradients between the intracellular and extracellular space. If blood flow is stopped completely, irreversible myocardial injury begins after approximately 20 min of ischemia, and within 4–6 h, the affected myocardial segment may become completely infarcted.

The clinical syndrome of myocardial infarction is diagnosed by the occurrence of typical chest pain, electrocardiographic (ECG) findings indicative of ischemia, and the rise of cardiac biomarkers. Ischemia may lead to myocyte cell death and the release of intracellular proteins into the systemic circulation. Multiple serum assays are used to detect myocardial infarction. The primary assay in clinical use today is the troponin (either troponin I or troponin T). Troponin is the intracellular protein that inhibits actin and myosin binding. When myocyte cell injury occurs, intracellular troponin begins to leak out of the cell membrane. Troponin may be detected in the serum within 2–4 h after injury to the myocyte.

There are multiple toxic substances that may lead to myocardial infarction. One such substance is cocaine, which may cause an oxygen supply demand mismatch by several mechanisms. Cocaine exerts sympathomimetic effects on the cardiovascular system causing increased blood pressure, heart rate, and contractility. All of these sympathomimetic factors increase myocardial oxygen demand. Concurrent with the increase oxygen demand, cocaine may also cause

vasoconstriction of coronary arteries via alpha-adrenergic stimulation. During acute intoxication, the sum of these mechanisms may lead to myocardial infarction, arrhythmias, and death.

Cardiomyopathy

Cardiomyopathy is a pathologic disease process that affects cardiac myocytes. When this pathologic process impairs the ability of the ventricle to fill with or eject blood, then the clinical syndrome of heart failure may develop. When the heart's ability to maintain an adequate stroke volume is jeopardized, a cascade of events occurs to maintain cardiac output. After the acute injury to the myocardial contractile function, the body sets in motion a complex set of neurohormonal signals, which may initially stabilize cardiac output but which eventually lead to the deterioration in cardiac function. One of the earliest adaptations of the cardiovascular system to a decrease in stroke volume, as sensed by a decrease in pressure by receptors in the aorta and carotid bodies, is the stimulation of the sympathetic nervous system. This leads to an increase in heart rate to maintain cardiac output (cardiac output = HR × SV). Thus, sinus tachycardia may be a clue to depressed cardiac function. Decreased blood flow to the kidneys leads to the activation of the renin–angiotensin–aldosterone (RAAS) system. This causes sodium and water retention, peripheral vasoconstriction, and myocardial hypertrophy and fibrosis. The eventual outcome is worsened cardiac function, and clinical signs and symptoms of fluid overload, such as shortness of breath or lower extremity edema. The ventricle attempts to maintain stroke volume via increasing preload. This may lead to elevated intraventricular pressure, increased wall stress, and subsequent ischemia. Ventricular dilation and further deterioration of cardiac function may ensue [26]. Multiple toxic substances have been implicated in the development of cardiomyopathy and subsequent heart failure. Anthracyclines cause cardiac toxicity in a dose-dependent manner. Along with cumulative doses of anthracycline, risk factors have been recognized to place patients at risk of cardiac toxicity from these medications including age (both old and young), mediastinal radiation, poor cardiac function at baseline, or coadministration of other agents such as trastuzumab. The suspected mechanism of toxicity from anthracyclines is due to oxidative stress. Some studies have noted up to a 40% mortality with anthracycline-associated toxicity.

As opposed to direct myocyte toxicity posed by anthracyclines, other modes of cardiac toxicity leading to cardiomyopathy include intracellular accumulation of toxic substances. Deterioration in cardiac function is seen in hemochromatosis due to excessive iron deposition in the cardiomyocyte. This disease process has been linked to a mutation in the HFE gene, which codes for a transmembrane protein responsible for iron regulation. Regardless of the mechanism of toxicity, the overt clinical findings of heart failure are manifested when the mechanical dysfunction of the heart due to the toxic substance leads to hemodynamic compromise.

MYOCARDIAL CELL DEATH: APOPTOSIS AND NECROSIS

Toxic insults trigger a series of reactions in cardiac cells leading to measurable changes. Mild injuries can be repaired; however, severe injuries will lead to cell death via apoptosis and necrosis. If the cell survives the insults, structural and functional adaptations will take place. Apoptosis was found to be involved in cardiomyopathy in 1994 [27]. The loss of cardiac myocytes is a fundamental part of myocardial injury that initiates or aggravates cardiomyopathy. Apoptosis is an important mode of myocardial cell loss, as has been demonstrated in heart failure patients [28]. Myocardial apoptosis has been shown to play an important role in the cardiac toxic effects induced by Adriamycin® [29,30], an important anticancer agent whose clinical application is limited by its major side effect, cardiotoxicity. Exposure of primary cultures of cardiomyocytes to cadmium also induces apoptosis [31].

Many in vivo studies have shown that only a very small percentage of myocardial cell population undergo apoptosis under pathological conditions; for example, less than 0.5% of cells appeared apoptotic in myocardial tissue under the stress of dietary copper deficiency in mice [32]. At first glance, this number seems to be too insignificant to account for myocardial pathogenesis; however, this would be a false statement. In a carefully designed time course study [33], it has been estimated that cardiomyocyte apoptosis may be completed in less than 20 h in rats. Because the heart is a terminally differentiated organ, myocytes undergoing apoptosis will be lost; thus, the total cell loss can simply be accounted for by the rate of apoptosis plus necrosis. If apoptosis occurs at a constant rate of about 0.5% myocytes a day [32], the potential contribution of apoptosis to the overall loss of myocytes over a long period of time is significant.

Necrosis is a term that was widely used to describe myocardial cell death in the past. Myocardial infarction, in particular, had been considered as a consequence of necrosis [34]. Although it is now recognized that apoptosis contributes significantly to myocardial infarction [35], the significance of necrosis in myocardial pathogenesis cannot be underestimated. The contribution of necrosis to cardiomyopathy induced by environmental toxicants and pollutants is particularly important. A critical issue is how to distinguish apoptosis from necrosis.

Apoptosis and necrosis were originally described as two distinct forms of cell death that can be clearly distinguished [36]; however, these two modes of cell death can occur simultaneously in tissues and cultured cells. The intensity and duration of insults may decide the outcome; thus, triggering events can be common for both types of cell death. A downstream controller, however, may direct cells toward a programmed execution of apoptosis. If the apoptotic program is aborted before this control point and the initiating stimulus is severe, cell death may occur by necrosis [37]. Alternatively, in acute injury, apoptotic cells can progress along a continuum to eventual necrosis. To distinguish

apoptosis from necrosis, more specific oligonucleotide probes have been developed to allow the recognition of different aspects of DNA damage [38]. They have been successfully applied, in combination with confocal microscopy, to identify apoptotic and necrotic cell death in the heart with different pathogenic challenges.

Selected Cardiotoxicants

This section will focus on representative chemicals and drugs, which have been reported to have major cardiotoxic effects, and will not describe the adverse effects of cardiovascular drugs used therapeutically in the treatment of heart disease and disorders. For example, these drugs, such as beta-blockers, nitrates, calcium channel blockers, antihypertensive drugs, antiarrhythmic drugs, and digitalis and other inotropic drugs used for heart failure, may be cited for possible adverse cardiac effects in the other sections of the chapter. For the sake of completeness, some of the drugs described in the previous sections will be discussed in this section to stress their unique cardiotoxicities.

Alcoholic Cardiomyopathy

For over 150 years, the abuse of alcohol in the form of ethanol has been associated with a variety of cardiovascular diseases. Chronic, heavy use of alcohol may lead to nonischemic dilated cardiomyopathy or alcoholic cardiomyopathy, which results in decreased cardiac function and structural disease [39]. Clinically, a patient with alcoholic cardiomyopathy will demonstrate low cardiac output, four chamber dilatation, wall thinning, ventricular dysfunction, and heart failure. Alcohol abuse may also be associated with arrhythmias, hypertension, stroke, and sudden death.

The pathogenesis of alcoholic cardiomyopathy is not clearly understood, but several sources of research suggest that alterations in membrane stability of cardiac cells, decreased excitation/contraction coupling, and formation of reactive oxidative metabolites may contribute to its pathogenesis.

For example, the hepatic biotransformation of ethanol by alcohol dehydrogenase and cytochrome P45O II EI to the toxic metabolite acetaldehyde, may damage the membranes of cardiomyocytes [40]. This reactive metabolite and other oxidative by-products may cause lipid peroxidation of the cardiac cell membranes or may damage cytosolic and membrane protein thiols.

As suggested by Kang [1], the use of rats as an experimental model to investigate the chronic effects of ethanol on cardiac structure and production of myocardial fibrosis is difficult to reproduce the effects of alcohol on the human heart because of the shorter life span of the animals. However, a more recent study with a mouse model of alcoholic cardiomyopathy produced heart hypertrophy and fibrosis by genetically deleting the zinc—regulatory protein, metallothionein (MT) [41]. This study and others, as summarized by Kang [1], suggest a role for zinc deficiency in the induction of alcohol-induced myocardial fibrosis and the possible involvement of oxidative stress in the fibrogenesis because of the antioxidant properties of MT.

Other possible hypotheses of alcoholic cardiomyopathy include inflammatory and immunologic changes, disruption of calcium-mediated contractile elements, inhibition of ATP formation, apoptosis, inhibition of protein synthesis, and receptor alterations.

Cocaine

Pharmacologically, cocaine is a local anesthetic, which blocks nerve impulses by reversible inhibition of Na^+ channels and subsequent decrease in transitory Na^+ conductance in nerve fibers. In addition, cocaine produces local vasoconstriction as the result of the inhibition of norepinephrine (NE) reuptake. Cocaine may increase heart rate and blood pressure and stimulate the CNS, resulting in enhanced alertness and self-confidence. Because of its euphoric-producing effects, cocaine may be abused chronically, resulting in altered behavior and addiction.

Chronic use of cocaine may have adverse effects on the heart because of its direct and indirect actions on myocardial contractility, increase in cytosolic calcium and disruption of NE reuptake, and subsequent stimulation of beta- and alpha-adrenergic receptors. The net effect of the chronic use of cocaine is the production of cardiac arrhythmias, myocardial ischemia, myocarditis, aortic dissection, cerebral vasoconstriction, and seizures [42]. As pointed out by Kloner et al. [43], in their review on cocaine effects on the heart, cocaine abuse is a major risk factor for heart disease. It has been suggested that cocaine may directly injure cardiomyocytes, cause cell death, and result in myocardial infarction. The mechanism of cell death is not clearly understood, although it is postulated that coronary artery vasoconstriction, accelerated atherosclerosis, and thrombus formation may be factors in the production of myocardial infarction.

Antidepressants and Antipsychotics

Tricyclic antidepressants (TCAs) have been used since the 1960s for the treatment of major depressive disorder. It is now well known that TCAs can have major cardiotoxic effects in overdose situations and cardiovascular side effects are well documented [44]. These adverse effects are linked to their inhibitory actions on cardiac and vascular ion channels, anticholinergic- and quinidine-like actions, inhibition of reuptake of adrenergic amines, disruption of membrane permeability, and direct myocardial depression. Clinical signs of TCA cardiotoxicity may include sinus tachycardia, postural hypotension, QT prolongation and possible TdP arrhythmias, congestive heart failure, and AV block, leading to asystole and sudden death. Although TCAs are no longer considered the major therapeutic course of treatment for depressive disorders, TCAs are still reported as one of the most commonly prescribed drugs implicated in overdose fatalities [44].

Several major antipsychotics have been associated with sudden cardiac death, QTc prolongation, and TdP. These effects by antipsychotics on the QT interval are thought to be mediated by the blockade of the delayed rectifier K^+ current [44]. Other adverse side effects of the antipsychotics include tachycardia, orthostatic hypertension, and certain metabolic disorders, such as increased risk for weight gain, insulin resistance, and type 2 diabetes.

Androgenic–Anabolic Steroids

These agents are divided into structural classes: nonalkylated testosterone derivatives, such as testosterone, ester testosterone derivatives, nandrolone, and esters of nandrolone, and 17-alpha-alkylated androgenic–anabolic steroids (AASs), such as fluoxymesterone, methandrostenolone, stanozolol, and other related compounds. There is growing evidence for adverse cardiac effects of AASs on the heart [45], resulting in pathological changes such as hypertrophy, impaired diastolic function, myocardial necrosis and/or apoptosis, and possible ventricular fibrillation. Thus, the illegal use of exogenous androgens, especially in high doses, may induce cardiac hypertrophy and other cardiovascular problems. These actions on the heart are most likely associated with receptor and genomic mechanisms, but because a variety of acute effects may be produced in a short period of time by androgens on the heart suggest that nongenomic mechanisms may be involved in their cardiotoxicity.

Antineoplastic Agents

Anthracyclines are natural products derived from *Streptomyces peucetius* var. caesius, with doxorubicin and daunorubicin as the major ones initially isolated from the fungus. Idarubicin and epirubicin are analogs of the latter two, differing only slightly in structure. Daunorubicin and idarubicin are used extensively in the treatment of acute leukemias, whereas doxorubicin and epirubicin are more effective against solid tumors. Because of the quinone groups found as part of their chemical structures, anthracyclines can generate free radicals or semiquinone radical intermediates in malignant and normal tissues that can react with O_2 to produce superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals [46]. Because these deleterious free radicals have a prominent action on cardiac tissue, cardiotoxicity is a major result of treatment of patients with these analogs [47]. A rare form of cardiotoxicity is acute or subacute injury, resulting in transient arrhythmias, a pericarditis–myocarditis syndrome, or acute failure of the left ventricle. A more serious adverse effect is the induction of cardiomyopathy (Figure 32.4), which is the more common form of cardiotoxicity. Thirdly, a type of toxicity may be shown years after treatment with the anthracyclines: arrhythmias and late-onset ventricular dysfunction.

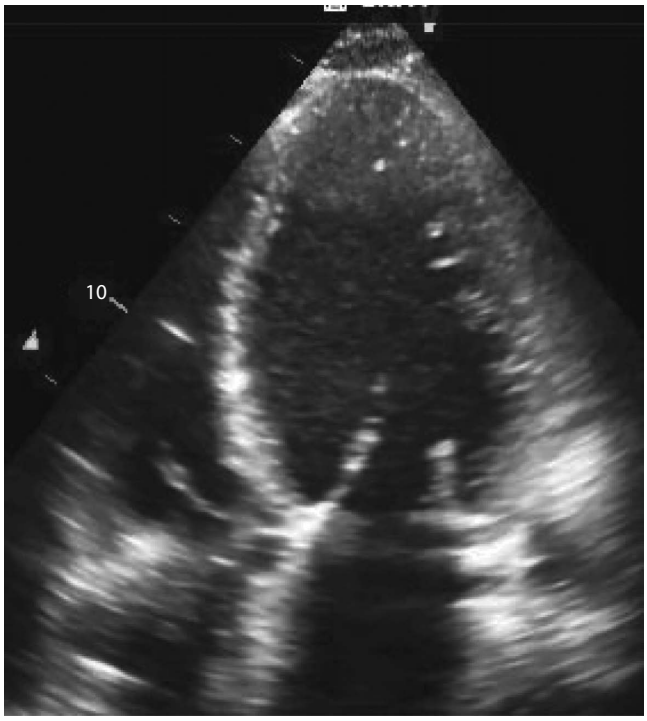

FIGURE 32.4 Transthoracic echocardiogram of a cardiomyopathy caused by the anthracycline doxorubicin in a 65-year-old patient.

The precise mechanism(s) of the cardiotoxicity of anthracyclines may be related to several biochemical and molecular events [47]:

1. The already-mentioned formation of quinone free radicals and the generation of reactive oxygen species with resulting DNA and membrane damage
2. Formation of a toxic metabolite
3. Alteration of calcium metabolism
4. Involvement of histamine and other catecholamines

Other antineoplastic agents shown to have cardiotoxic actions include cyclophosphamide, fluorouracil, bleomycin, cisplatin, arsenic trioxide, and interferons. The cardiotoxicity may be manifested clinically as arrhythmias, cardiomyopathies, myocardial ischemia, and other toxicities.

Nonsteroidal Anti-Inflammatory Agents

The major NSAIDs are aspirin, propionic acid derivatives (ibuprofen, naproxen), acetic acid derivatives (indomethacin), selective inhibitors of Cox-2 (celecoxib, etoricoxib, lumiracoxib), and enolic acids (piroxicam). Many of these agents have a variety of common and shared side effects, including GI effects, platelet actions, renal toxicity, CNS actions, uterine effects, hypersensitivity reactions, and cardiovascular toxicities [48]. The main cardiotoxic effects may be myocardial infarction, stroke, thrombosis, and closure of ductus arteriosus. It has only been most recently that the cardiovascular toxicity of NSAIDs has gained much prominence with the

TABLE 32.2
Selected Cardiotoxicants

Drug/Chemical	Proposed Mechanism(s) of Cardiotoxicity
Alcohol	Formation of reactive oxidative metabolites, zinc deficiency, induction of ATP/protein synthesis
Cocaine	Coronary artery vasoconstriction, thrombus formation
Antidepressants	Inhibitory actions on cardiac ion channels, inhibition of reuptake of adrenergic amines, disruption of membrane permeability
Antipsychotics	QT prolongation by blockade of the delayed rectifier potassium current
AASs	Unclear but may be related to receptor, genomic and nongenomic mechanisms
Anthracyclines	Formation of quinone free radicals; alteration of calcium metabolism
NSAIDs	Inhibition of COX-2, leading to thrombus formation and increased blood pressure
Erythromycin, clarithromycin, ketoconazole, terfenadine, etc.	QT prolongation, leading to TdP

withdrawal from the market of rofecoxib and valdecoxib due to increased risk for serious cardiovascular adverse events. The other NSAIDs on the market have also been suggested to have the potential to increase the risk of heart attack and stroke. The most probable explanation for the cardiotoxicity of the NSAIDs is that the inhibition of COX-2 increases the formation of thrombi and elevates blood pressure, which would them enhance the potential for cardiovascular risk in humans.

Miscellaneous Therapeutic Agents

Antimicrobial Agents [49]

Of the macrolide antibiotics, erythromycin and clarithromycin are associated with an increased risk of TdP as a result of QT prolongation. Telithromycin and azithromycin are classified as possible risks of TdP.

Fluoroquinolones were the most widely prescribed class of antibiotics for many years but are now used less frequently (ciprofloxacin, levofloxacin, and moxifloxacin). Sparfloxacin and grepafloxacin were removed from the market because of nonfatal and fatal arrhythmias secondary to QT prolongation with TdP.

The azole antifungals, such as ketoconazole and itraconazole, have been reported to cause QT prolongation and TdP, and more attention should be paid to possible adverse cardiac events in patients taking these antibiotics.

The antimicrobial agent, pentamidine, used to treat protozoal infections, has been reported to be associated with cases of QT prolongation and TdP. Similarly, certain antimalarial agents, chloroquine and halofantrine, have been associated with significant QT prolongation.

Other Drugs

Cisapride, once used as a prokinetic drug for gastrointestinal hypomotility, was removed from the market because of enhanced risk of arrhythmias associated with TdP. Sildenafil, used for the treatment of erectile dysfunction, may have some possible cardiotoxic effects by increasing cAMP in cardiac tissue. Certain antihistamines, notably terfenadine and astemizole, were removed from the market because of life-threatening ventricular arrhythmias, most likely caused by prolongation of the action potential and by blocking the delayed rectifier K^+ channel.

To mechanistically summarize the cardiotoxicants, Table 32.2 provides a brief overview of their cardiotoxicities.

QUESTIONS

32.1 Toxicants that increase the systemic blood pressure, such as cocaine, increase which hemodynamic variable leading to increase oxygen demand?
- Afterload—acute increases in afterload increase the pressure that the heart must pump against, thus increasing oxygen demand.
- Contractility and heart rate are also increased by many stimulants, which will increase myocardial oxygen demand.

32.2 What is the mechanism by which the autonomic nervous system increases contractility?
- By the stimulation of the beta-adrenergic receptor that activates G-coupled protein receptors leading to the phosphorylation of calcium channels and subsequent increased calcium release

32.3 What is the resting potential of a myocyte under normal conditions?
- 60 to −90 mVs.
- Sodium ion entry in to the cell thus leads to depolarization of the myocyte membrane.

32.4 How does the action potential cause myocardial contraction?
- Via calcium-mediated calcium release. Calcium enters the myocyte when the action potential causes depolarization of L-type Ca channels, which leads to calcium entering the cell and subsequently stimulating larger calcium release from intercellular stores.

32.5 What is the most common mechanism of action for arrhythmias to occur?
- Reentry

32.6 How do many antiarrhythmic medications suppress reentrant arrhythmias?
- By changing the electrical properties of the two separate limbs of the reentrant circuit, the circuit may be broken.

32.7 Stimulants such as caffeine, nicotine, and amphetamines may cause sinus tachycardia via which mechanism?
- Increased automaticity

32.8 TdP is precipitated by which type of arrhythmic mechanism?
- Triggered activity—afterdepolarization during the recovery phase of the action potential may lead to TdP.

32.9 When does myocardial ischemia occur?
- When the myocardial oxygen supply is not sufficient to meet the myocardial oxygen demand

32.10 Why would a cardiomyopathy lead to sympathetic nervous system stimulation?
- When the cardiac pumping function becomes jeopardized, stroke volume decreases. This decreased forward blood flow leads to stimulation of the baroreceptors in the carotid body and aorta, with subsequent stimulation of the sympathetic nervous system to cause reflex tachycardia.

32.11 How does decreased blood flow to the kidney lead to sodium and water retention in heart failure?
- Via activation of the renin–angiotensin–aldosterone system. This system is also implicated in peripheral vasoconstriction and myocardial hypertrophy and fibrosis.

KEYWORDS

Arrhythmia, Cardiomyopathy, Torsades de Pointes (TdP), Ischemia, Ethanol, Cocaine, Anthracyclines, Myocardial infarction.

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33 Neurotoxicology

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CONTENTS

OVERVIEW

Assessment of neurotoxicity encompasses a broad range of chemical, structural, and behavioral outcomes that can be affected by numerous types of toxicants. Most narrowly defined, a neurotoxin is a toxicant that directly affects the function of the nervous system. Due to the complex nature of the nervous system and a vast array of chemical, environmental, and endogenous neurotoxins, neurotoxicity takes many forms including acute distress and neuropathies, developmental abnormalities, and *silent* or latent symptoms, which become apparent only with aging and/or additional insults. Furthermore, these effects often extend beyond the nervous system and can cause systemic disturbances. This chapter aims to provide an overview of the anatomy and physiology of the nervous system including factors unique to the nervous system that increase susceptibility to neurotoxins.

* These authors contributed equally to the preparation of this chapter.

INTRODUCTION TO THE NERVOUS SYSTEM

The nervous system has numerous functions including coordination and regulation of homeostasis of systems such as the respiratory, digestive, circulatory, and endocrine systems to name a few. The nervous system also mediates movement and provides mechanisms for sensory perception. In addition, innate and acquired behaviors originate within the nervous system as well as higher cognitive functions, which define personality and enable learning, memory, and emotion. As a result, toxins that affect the nervous system can have broad and devastating effects across many physiological and psychological domains.

Anatomical Organization

The nervous system is anatomically divided into the central and peripheral nervous systems (CNS and PNS, respectively). The CNS is comprised of the spinal cord, brain stem, and forebrain and the PNS is comprised of the nerves and ganglia outside of the brain and spinal cord (Table 33.1). Structurally, the spinal cord is a bundle of nervous tissue including neuronal cell bodies, axons, glia, and structural proteins within the vertebral column stretching from the base of the skull to approximately the first lumbar vertebrae. Functionally, the spinal cord receives, sends, and processes sensory and motor information between the PNS and the brain. The brain stem is made up of the medulla, pons, and midbrain, which are critical for the regulation of vital blood pressure and respiration functions and the origination of cranial nerves that involved special senses such as taste and hearing and conveying sensory and motor information relating to the head and neck. The cerebellum is also considered part of the midbrain and receives many sensory and motor inputs and functions in part to regulate balance, posture, and fine motor skills. The forebrain consists of the diencephalon (thalamus and hypothalamus) and the cerebral hemispheres including cerebral cortex, white matter, and subcortical structures such as the basal ganglia and hippocampus. The thalamus relays sensory and motor signals to and from the cortex and acts to regulate sleep and wakefulness. Underneath the thalamus is a small set of distinct nuclei called the hypothalamus, which links the nervous system with the endocrine system to regulate sex hormones, hunger and satiety signals, and circadian rhythms. The remaining cortical and subcortical structures of the forebrain function as higher processing systems, including sensory and motor cortices, the limbic system, the basal ganglia, and the hippocampus.

The PNS can also be divided into several distinct anatomical and physiological components (Table 33.1). The somatic nervous system is composed of sensory neurons in the skin, muscles, and joints and motor neuron axons that control skeletal muscles, while the autonomic nervous system contains neurons and axons involved in visceral sensation and control of smooth muscles and exocrine glands. The autonomic system is further divided into the sympathetic nervous system that underlies the *fight or flight* response, the parasympathetic nervous system that restores homeostasis, and the enteric nervous system that is relatively autonomous and regulates the smooth muscle in the gut.

TABLE 33.1
Anatomical Organization of the Nervous System

	Major Divisions	Components	Primary Functions
CNS	Spinal cord	Cell bodies, projections, and structural matrix proteins	Receives, sends, and processes signals between the CNS and PNS
	Brain stem	Medulla	Origination of cranial nerves
		Pons	Regulation of vital functions
		Midbrain	Motor and sensory information for head and neck
		Cerebellum	Balance, posture, fine motor skills
	Forebrain	Hypothalamus	Relay and process information between the cortex and other brain regions
		Thalamus	
		Cortex	Circadian rhythm
		Subcortical ganglia	Hormonal release
			Higher-order cognitive and emotional processing
PNS	Somatic nervous system	Sensory and motor neurons innervating skin and skeletal muscle	Reception of sensory stimulation
			Innervation and stimulation of skeletal muscle
	Autonomic nervous system	Sensory and motor neurons and ganglia innervating smooth muscle and exocrine glands	Sympathetic: fight or flight response
			Parasympathetic: restoration of homeostasis
			Enteric: relatively autonomic regulation of digestion
Anatomical barriers	BBB	Capillary endothelial cells	Generate CSF
		Astrocytic feet	Isolate CNS from foreign substances and immune proteins found in systemic circulation
	Blood–CSF barrier	Ependymal cells	
	Blood–retina barrier	Retinal epithelial cells	

Notes: CSF, cerebral spinal fluid; CNS, central nervous system; PNS, peripheral nervous system.

The CNS is uniquely sequestered from the PNS and the rest of the body by several anatomical barriers—the blood–brain barrier (BBB), the blood–cerebrospinal fluid (CSF) barrier, and the blood–retina barrier (Table 33.1). The function of these barriers is to isolate the CNS, including the retina, from toxins and other damaging substances in the blood such as bacteria, viruses, and inflammatory agents and to regulate the nutritional and ionic composition of the CSF.

Development

Formation of the nervous system begins early in development when the three embryonic layers (endoderm, ectoderm, mesoderm) are formed during gastrulation. The nervous system originates primarily from ectodermal cells, which also give rise to integumentary tissue. During the third week of human gestation, the ectoderm undergoes neurulation to form the neural tube and neural crest. The neural tube and neural crest proliferate rapidly to produce neural progenitor cells, which become the CNS and PNS, respectively. These neural precursors migrate and continue to differentiate into neurons and glia; form axons, dendrites, and synapses; and establish functional circuitry including myelination of gross anatomical structures. The vast majority of nervous system development occurs during the first 2 years of life; however, several processes such as myelination, synaptogenesis, and the development of higher-order cognitive systems continue into early adulthood (Figure 33.1). Many of these processes are restricted to distinct windows in development, creating critical periods during which toxicant exposure is especially devastating for specific brain regions, and sensory, motor, and cognitive domains.

Cells of the Nervous System

There are three basic cell types found in the nervous system—neurons, glia, and microglia. Each cell type is characterized by distinct structural and physiological characteristics that determine its role within the nervous system.

Neurons

There are many types of neurons in the nervous system; however, they all share basic structural and functional features that enable formation of simple and complex networks through which sensory and motor information is processed and transmitted. All neurons are electrically excitable, postmitotic cells consisting of a cell body, dendrites, and an axon (Table 33.2). As in other cells, the neuronal cell body contains the nucleus, endoplasmic reticulum, Golgi, mitochondria, and other membranous organelles. Dendrites are specialized protrusions of the cell body that contain membrane neurotransmitter receptors or specialized sensory receptors that respond to receptor activation and sensory cues through changes in membrane potential and intracellular signaling pathways. Most neurons have a single axon extending millimeters or meters from the cell body to form synapses with target neurons or organs. Neurons can be divided into several distinct classes based on shape, location, excitability, and the expression of neurotransmitters and receptors, all of which affect neuronal signaling and processing.

Macroglia

Macroglia, commonly called glia or neuroglia, are a class of nonneuronal mitotic cells that provide energy and nutrients to neurons, maintain the extracellular environment through ion and neurotransmitter clearance, form myelin, respond to injury, and modulate neuronal signaling and plasticity. As with neurons, there are several distinct types of glia including astrocytes, oligodendrocytes, Schwann cells, ependymal cells, and radial glia (Table 33.2). Astrocytes are the most abundant glial cell and contribute to BBB formation, neurovascular coupling, regulation of extracellular ionic concentration, and neurotransmitter clearance. They have an important role in the modulation of neuronal activity through

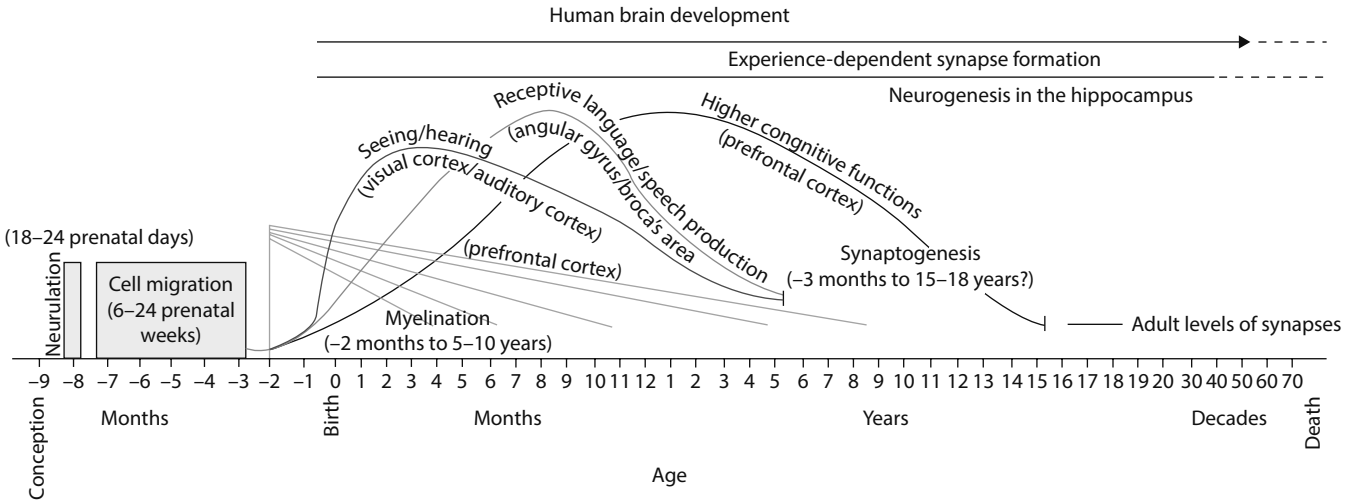
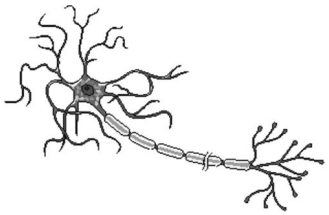
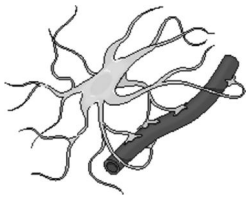
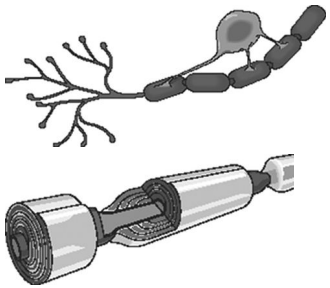
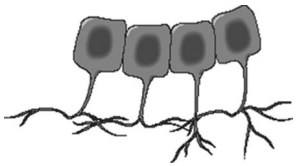
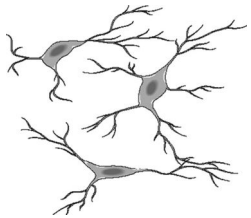


FIGURE 33.1 Human neurodevelopment. Progression of normative human neurodevelopment beginning at conception, including the developmental windows for the establishment of sensory and cognitive functions. (Reproduced from Thompson, R.A. and Nelson, C.A., *Am. Psychol*., 56(1), 5, 2001.)

TABLE 33.2
Cells of the Nervous System

	Neuron	Macroglia			Microglia
		Astrocyte	Schwann Cell Oligodendrocyte	Ependymal Cell	
			 *Myelin sheath*		
Structural and physiological features	• Dendrites, cell body, and axon • Postmitotic • Electrical excitability • Release neurotransmitters	• Structure • Contain numerous ion and neurotransmitter transporters • Release ATP and glutamate • Generate Ca^{2+} waves	• Lipid-rich myelin sheath • Schwann cells found in PNS • Oligodendrocytes found in CNS	• Line the ventricles • Form tight junctions	• Small, spaced throughout the CNS • Originate from systemic macrophages
Function	• Chemoelectric signal transmission • Network formation	• Maintain extracellular environment • BBB formation and regulation • Neurovascular coupling	• Myelinate axons • Injury response	• Blood–CSF barrier	• CNS immune response

Notes: ATP, adenosine triphosphate; CNS, central nervous system.

the release of glutamate and adenosine triphosphate (ATP). Astrocytes signal within networks formed by astrocyte–astrocyte gap junctions that allow diffusion of Ca^{2+}-induced second messengers such as inositol triphosphate (IP_3), which stimulate Ca^{2+} wave propagation across a relatively large cortical area. Elevated Ca^{2+} can stimulate glutamate release from some astrocytes. Myelin is formed by membrane projections from specialized glia cells called oligodendrocytes in the CNS and Schwann cells that wrap around axons to provide electric insulation and concentrate ion channels at the nodes of Ranvier. Myelin increases the speed and energy efficiency of action potential propagation by up to tenfold. Other types of glial cells include ependymal cells, which line the ventricles to produce CSF and form the blood–CSF barrier, and radial glia, which are neuroprogenitors and scaffolding for migrating cells during development. In adults, radial glia persist in the cerebellum and retina where they facilitate plasticity and neuronal transmission.

Microglia

Microglia differ from macroglia in that they do not originate from neural ectoderm but instead from mesodermal monocytes that have entered the brain and further differentiated into microglia (Table 33.2). Due to the BBB, the CNS is immune privileged and most systemic infections do not enter the brain; however, in cases where bacteria, viruses, and other harmful substances do cross the BBB, microglia provide an immune defense system. Microglia are distributed throughout the brain and rapidly respond to foreign substances and infections by reducing inflammation, phagocytizing foreign substances and signaling to neighboring microglia, glia, and neurons through the release of cytokines. Microglia can also kill infected cells through release of large amounts of NO and H_2O_2 and glutamate. In addition, microglia phagocytize dead cells and other naturally occurring debris.

Chemoelectric Transmission

Neurons transmit information throughout the nervous system by changes in membrane potential that are propagated along axons (electric transmission) and cause release of neurotransmitters at synapses (chemical transmission). Synapses are the junctions formed between axon terminals of a presynaptic neuron and a postsynaptic cell (Figure 33.2). Postsynaptic cells can include neurons, muscles, and endocrine tissue (such as the adrenal or pituitary glands) and may contain synapses from a single presynaptic cell or from several presynaptic cells. Astrocytes have a functional role in many synapses, forming a three-part, or tripartite, synapse with the pre- and postsynaptic cell. Astrocyte processes can surround the synapse, tightly regulating the extracellular environment through ion and neurotransmitter transport and also the release of glutamate and propagation of Ca^{2+} signaling.

Action Potential

Membrane ion channels and transporters expressed by neurons create a resting membrane potential near –70 mV. These channels establish both a chemical and electrical gradient with high intracellular K^+ and low intracellular Na^+, Cl^-, and Ca^{2+} relative to the extracellular environment. Dendrites and synapses contain a number of ligand-gated and G protein–coupled ion channels that can be activated by sensory stimuli or neurotransmitters and cause local alterations in the membrane potential. Membrane depolarization caused by Na^+ or Ca^{2+} influx is considered excitatory, while hyperpolarization due to K^+ efflux or Cl^- influx is considered inhibitory. In the dendrites, these local changes in membrane potential can spread through the cell body, and with sufficient excitatory stimulation, the depolarization can reach the axon hillock, the junction between the cell body and axon, and activate voltage-gated (v-gated) Na^+ channels. Once activated, Na^+ rapidly enters the cell driven by both chemical and electric gradients where it diffuses into the cell body and along the axon, depolarizing the membrane and activating neighboring v-gated Na^+ channels (Figure 33.2). The depolarization is propagated in an all-or-none manner along the entire length of the axon to the axon terminal where v-gated Ca^{2+} channels are activated. Ca^{2+} entry through these channels stimulates fusion of synaptic vesicles with the membrane, releasing neurotransmitters and neuromodulators.

Several events occur during action potential propagation that rapidly restore resting membrane potential (Figure 33.2). The axon contains v-gated K^+ channels that open more slowly than v-gated Na^+ channels, and in depolarized cells, K^+ flows rapidly out of the cell through these channels along the chemical and electrical gradient (recall that neurons have high intracellular K^+ levels). At the same time, v-gated Na^+ channels are inactivated by the sustained membrane depolarization, preventing further Na^+ entry. These events combined with the ongoing action of Na^+/K^+ ATPase pumps, which export three Na^+ ions from the cytoplasm and import two K^+ ions using an ATP molecule, quickly restore the membrane potential and enable the neuron to fire another action potential within milliseconds.

Several well-known toxins act by disrupting the ion channels and transporters involved in maintaining resting membrane potential and action potential generation. For example, v-gated Na^+ channels are blocked by tetrodotoxin, a toxin found in puffer fish; in low doses, it temporarily inhibits neural transmission but can cause death at high doses. Tetraethylammonium inhibits K^+ channels necessary for restoration of membrane polarization following action potentials.

Neurotransmitter Signaling

Synaptic transmission, also known as chemical transmission, involves the synthesis and release of neurotransmitters by the presynaptic cell, the interaction of the neurotransmitter with receptors on postsynaptic cells, and the subsequent neurotransmitter removal from the synapse through reuptake or degradation. Neurotransmitters can be categorized as amino acids, biogenic amines, acetylcholine (ACh), or ATP. For each neurotransmitter, there are several distinct receptors, including both ionotropic (ligand-gated ion channels) and metabotropic

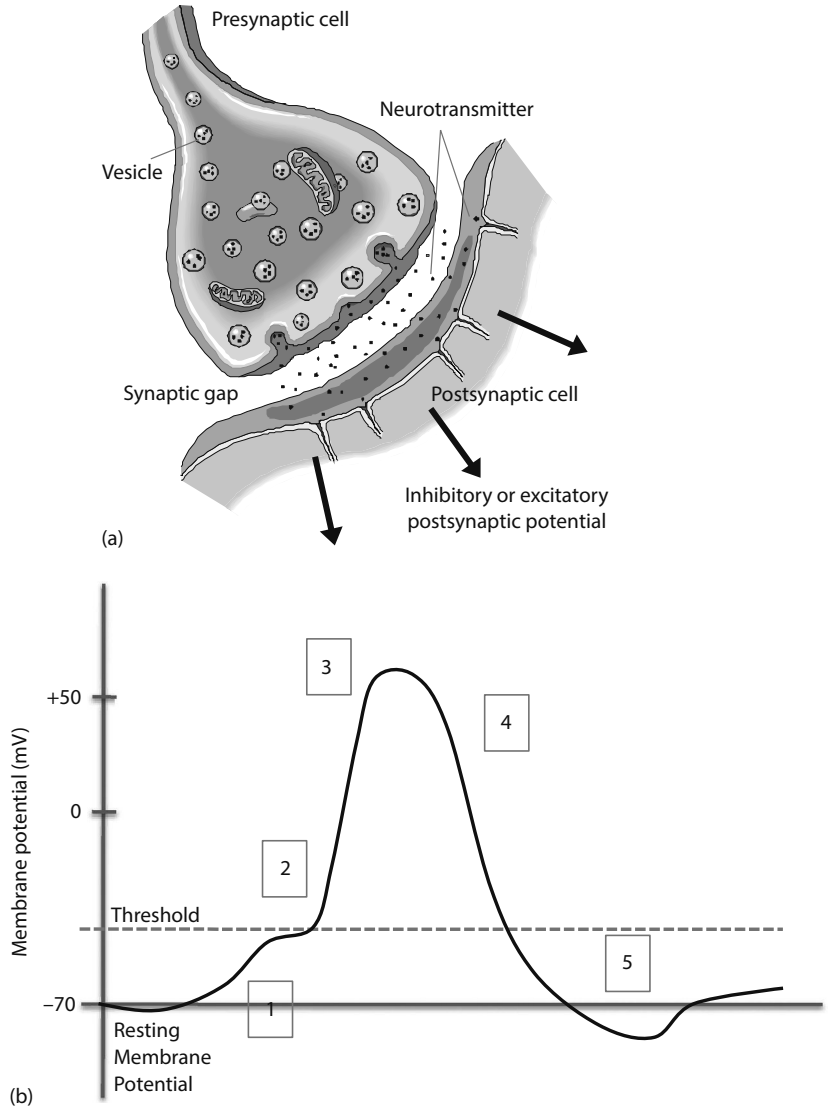


FIGURE 33.2 Chemoelectric transmission. (a) At the synapse, neurotransmitters are released from axon terminals of presynaptic neurons where they cross the synaptic cleft and bind to and activate membrane receptors on the postsynaptic cell (neurons, glia, muscles, exocrine glands). Once activated, the receptors change the postsynaptic membrane potential through local depolarization or hyperpolarization (excitatory or inhibitory postsynaptic currents, respectively). (b) Illustration of the membrane potential changes that occur during action potential propagation: (1) ion channels on dendrites cause excitatory postsynaptic currents; (2) the membrane reaches threshold depolarization at axon hillock, opening v-gated Na^+ channels, and Na^+ enters cell, further depolarizing the membrane; (3) v-gated K^+ channels open and v-gated Na channels close; (4) K^+ flows out of cell; and (5) v-gated K^+ channels close and Na^+/K^+ ATPase pumps restore resting electrochemical gradients. (LifeART collection images Copyright 1989–2001 by Lippincott Williams & Wilkins, Baltimore, MD.)

receptors (G protein–coupled receptors) (Table 33.3). The number and type of postsynaptic receptors activated as well as the length of time the neurotransmitter remains in the synapse determine the effect it has on the postsynaptic cell.

Glutamate, γ-aminobutyric acid (GABA), and glycine comprise the amino acid neurotransmitters. Glutamate is the most common excitatory neurotransmitter. The most common glutamate receptors are the AMPA and NMDA ligand-gated Na^+ and Ca^{2+} channels, which cause excitatory depolarization of the postsynaptic cell. There are also several types of metabotropic glutamate receptors, that can be excitatory or inhibitory depending on the associated G-protein. Glutamate is rapidly cleared from the synapse by glutamate transporters on both neurons and surrounding astrocytes. GABA is the most common inhibitory neurotransmitter and is synthesized from glutamate within the nervous system. $GABA_A$ receptors are ionotropic Cl^- channels, which cause hyperpolarization and inhibit action potential generation, and $GABA_B$ receptors are metabotropic receptors, which activate G protein–linked K^+ channels, also causing hyperpolarization and decreasing neuronal excitability. Glycine is the primary inhibitory transmitter used by spinal cord interneurons. Similar to $GABA_A$ receptors, glycine receptors are ionotropic Cl^- channels and decrease postsynaptic excitability.

TABLE 33.3
Major Neurotransmitters, Receptors, and Toxicants

Class	Neurotransmitter	Receptor	Postsynaptic Actions	Synaptic Clearance	Toxins
Amino acid	Glutamate	NMDAR AMPAR	Ionotropic Na^+ and Ca^{2+} channels (excitatory)	Reuptake by neuronal and astrocytic glutamate transporters	PCP
		mGluRs	Metabotropic effect dependent on associated G-protein		
	GABA	$GABA_A$	Ionotropic Cl^- channels (inhibitory)	Reuptake by GABA transporter	Hemlock Alcohol
		$GABA_B$	Metabotropic stimulation of G protein–activated K^+ channels (inhibitory)	Degradation by GABA transaminase	Picrotoxin
	Glycine	Glycine receptor	Ionotropic Cl^- channels (inhibitory)	Reuptake by transporters on neurons and astrocytes	Strychnine Bicuculline
Biogenic amine	Dopamine	Dopamine receptors	Metabotropic effect dependent on associated G-protein	Reuptake by dopamine transporter Degradation by MAO or COMT	Amphetamines Heavy metals Oxidative substances
	Norepinephrine Epinephrine	α- and β-adrenergic receptors	Metabotropic effect dependent on associated G-protein	Reuptake by norepinephrine transporter Degradation by MAO and COMT	
	Serotonin	$5\text{-}HT_3$	Ionotropic Na^+/K^+ channel (excitatory)	Reuptake by serotonin transporter	
		$5\text{-}HT_{1,2,4–7}$	Metabotropic effect dependent on associated G-protein	Degradation by MAO or COMT	
	Histamine	Histamine receptors	Metabotropic effect dependent on associated G-protein	Degradation by histamine-*N*-methyltransferase	
Other	ACh	nAChR	Ionotropic nonselective cation channel (excitatory)	Degradation by acetycholinesterase	Bungarotoxin Anatoxin Sarin Methylmercury
		mAChR	Metabotropic effect dependent on associated G-protein		Curare
	ATP and adenosine	Purinergic receptors	Metabotropic effect dependent on associated G-protein		

Notes: PCP, phencyclidine; GABA, γ-aminobutyric acid; MAO, monoamine oxidase; COMT, catechol-*O*-methyltransferase; nAChR, nicotinic acetylcholine receptor; mAChR, muscarinic acetylcholine receptor; ACh, acetylcholine; 5-HT, 5-hydroxytryptamine; NMDAR, *N*-methyl D-aspartate receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; mGluR, metabotropic glutamate receptor.

Biogenic amines are synthesized from amino acids and include the catecholamines, dopamine, norepinephrine, and epinephrine, derived from tyrosine through a common synthetic pathway. Dopamine is primarily released by midbrain neurons and has an important role in movement, affect, emotion, reward, addiction, motivation, and hormone secretion by the pituitary gland. There are at least five subtypes of metabotropic dopamine receptors, which either stimulate or inhibit cyclic adenosine monophosphate (cAMP) production through G-protein signaling. The relative expression level of receptor subtypes determines the effect in the postsynaptic cells. Norepinephrine is found in neurons of the locus ceruleus. These neurons are few in number; however, they project throughout the cortex, cerebellum, and spinal cord as well as the sympathetic PNS. Epinephrine acts as a hormone when released into the blood stream but is also found in a small number of neurons in the nervous system. The catecholamines are cleared from the synapse by specific transport proteins such as dopamine transporter and norepinephrine transporter or are degraded by monoamine oxidase (MAO) or catechol-*o*-methyltransferase (COMT) enzymes.

Serotonin (5-hydroxytryptamine [5-HT]) is another biogenic amine synthesized from tryptophan. The vast majority of systemic serotonin is found in the digestive system, although serotonergic neurons in the brain stem project throughout the brain and spinal cord and are involved in mood, appetite, and sleep regulation. There are many types of serotonergic receptors, and with the exception of the ionotropic $5\text{-}HT_3$ receptor, the receptors are metabotropic and exert excitatory or inhibitory effects depending on the subtype. Serotonin is cleared

from the synaptic cleft by the serotonin transporter or can be degraded by MAO and COMT. Histamine is also a biogenic amine derived from amino acid histidine. Histamine is best known as a proinflammatory substance released from mast cells in the immune system. In the CNS and PNS, however, it is produced and released by some hypothalamic neurons and acts as a neurotransmitter to contribute to sleep–wake cycle regulation. There are several subtypes of neuronal metabotropic histamine receptors with varying excitatory and inhibitory effects. Histamine is degraded in the synapse by histamine-*N*-methyltransferase.

ACh is the only neurotransmitter not derived from an amino acid. ACh is a polyatomic ion synthesized from choline and acetyl coenzyme A by choline acetyltransferase. ACh is used by motor neurons in the spinal cord and extensively in the autonomic nervous system. It is also synthesized in nucleus basalis neurons with widespread cortical projections. In the periphery, it is released by motor neurons and activates ionotropic nicotinic ACh receptors (nAChR) to stimulate skeletal muscle contraction. In the CNS, ACh acts largely to modulate plasticity, attention, reward, and arousal through metabotropic G protein–coupled muscarinic ACh receptors (mAChR). In the synaptic cleft, ACh is degraded by acetylcholinesterase.

ATP, ADP, AMP, and adenosine are most often considered in the context of cellular energy; however, these compounds are released in an activity-dependent manner from neurons and glia. These transmitters act on both ionotropic and metabotropic purinergic receptors to modulate autonomic function, immune response, neuronal development, and chronic pain.

In addition to these classical neurotransmitters, there are also many neuroactive substances that can affect transmission and plasticity, which are not defined as neurotransmitters. Most of these neuromodulators are small neuroactive peptides such as angiotensin-, oxytocin-, somatostatin-, and thyrotropin-releasing hormones with well-identified hormonal and endocrine functions. In addition, within the nervous system, these peptides can modulate synaptic transmission and are often found in the same vesicles as neurotransmitters. Gasses such as nitric oxide (NO) and carbon monoxide (CO) also have important signaling roles in the nervous system. They are naturally produced by nitric oxide synthases (NOSs) and by heme oxygenase, respectively, and can easily diffuse across membranes to signal to surrounding cells and have a role in inflammation signaling, redox signaling, and plasticity.

Neurotoxins can affect many aspects of neurotransmitter signaling, any of which can significantly disrupt neuronal signaling and cause physiological impairments and even death. ACh is affected by many neurotoxins and is used here as an example of the diversity of toxins that can significantly disrupt neuronal signaling. Methylmercury (MeHg), discussed in more detail in the following, disrupts ACh synthesis by inhibiting choline acetyltransferase and ACh release is impaired in the PNS by the botulinum toxin, which degrades proteins necessary for synaptic vesicle release. Botulinum does not cross the BBB so the effects are evident at neuromuscular junctions where ACh release is reduced, preventing diaphragm contraction and causing death by asphyxiation. ACh receptors are targeted by several toxins, including bungarotoxin, which blocks Ca^{2+} entry through nAChRs, thus preventing signal transmission. Anatoxin, produced by cyanobacteria, is a nondegradable nACh agonist that causes persistent muscle contraction and subsequent asphyxiation. Sarin, organophosphates, and carbamates inhibit acetylcholinesterase, prolonging the duration of signaling by preventing degradation of ACh in the synapse.

ASSESSING NEUROTOXICITY

There are a variety of markers of neurotoxicity and experimental model systems available to examine them. Histology on fixed neural tissues, magnetic resonance imaging (MRI) in living subjects, and immunocytochemistry of cell culture systems can be used to address changes in neuronal structure and physiology (macro/micro), gross deformities, and more subtle structural and even microstructural disturbances induced by a neurotoxin. Deleterious compounds can also affect neuronal metabolism, plasticity, and neurochemistry, which can likewise be measured with biochemical and electrophysiological assays. The ultimate function of the nervous system is to produce behavior and enable learning, memory, and cognition. Behavioral and cognitive disruptions are often the first indicators of the neurotoxic potential of a substance. There are a number of behavioral and cognitive assays ranging from simple associative learning to complex higher-order processing that are discussed elsewhere in this book. These behavioral assays can be helpful in assessing overt aspects of neurotoxicity in humans, rodents, and even invertebrate model systems.

Mammalian Model Systems

The use of human subjects and postmortem brains has been invaluable in the history of neuroscience. Histology on postmortem brains is useful in examining pathological changes in brain structure and composition as a result of a given disease process, such as amyloid plaques in Alzheimer's disease and Lewy bodies in Parkinson's disease (PD). Additionally, any protein, DNA, or chemical analysis can be performed on individual dissected tissues. Research using living subjects is also possible using imaging techniques, such as assessment of motor, sensory, and cognitive function, structural and metabolic measurements using MRI, and biochemical analysis of CSF. Not only do imaging technologies provide tools for research in brain function and structure in living humans, but they can be used repeatedly in the same subject to provide insight on disease progression.

Rodents, such as mice and rats, have been and continue to be widely used to study structural, biochemical, electrophysiological, developmental, epigenetic, and behavioral effects of toxicant exposure on nervous system function. As mammals, rodents have very similar genomes as humans and have all of the major brain structures, organizational patterns, and

neurotransmitters present in the human brain. In addition, neurodevelopment in rodents follows a similar progression as in humans. These features enable the assessment of nearly any neurological outcome in rodent systems.

Nonmammalian Vertebrate and Invertebrate Model Systems

Nonmammalian vertebrates, such as zebra fish (*Danio rerio*), and invertebrates are growing in popularity as a model for neurotoxicity. Zebra fish and invertebrate systems such as *Drosophila melanogaster* (fruit fly) and the nematode *Caenorhabditis elegans* are classic genetic models for neurodevelopmental research, and their small, albeit complex, nervous systems and genomes have been extensively characterized. Although invertebrates lack brains, they utilize many of the same, or very similar, neurotransmitters, receptors, and synthesis mechanisms as mammals and can exhibit neuropathologies consistent with observations in rodents and humans. In addition, mutagenesis and transgene manipulation has generated an impressive library of mutations, fluorescent reporters, and inducible expression systems in zebra fish, flies, and worms that facilitate neuronal imaging and screens for genes that modify response to toxicant exposure.[1–3] These systems are usually less expensive than mammalian models and allow for quick genetic screens, which are good for identifying genes involved in toxicity and stress responses.

In Vitro Model Systems

There are a variety of in vitro model systems that can be used to look at molecular and biochemical aspects of neurotoxicity. One of the most common in vitro models is primary culture of neurons, astrocytes, or microglia from rodent brains. There are also immortalized neuronal cell lines that can be either rodent or human in origin. One of the biggest advantages of cell culture systems is that biochemical and molecular genetic assays, such as gene silencing and florescent reporter assays, can be performed in mammalian cells that may be difficult or too costly in live animals. An emerging in vitro system for examining cellular and biochemical mechanisms of human neurotoxicity is human-induced pluripotent stem cells generated from skin fibroblasts of normal individuals as well as individuals with genetic risk factors or prior toxicant exposure. While cell culture systems provide many advantages over in vivo systems, caution in interpreting results should be used, as a single cell type rarely functions in isolation from the other surrounding cell types. Cell-based systems combined with in vivo experiments can provide complimentary data.

NERVOUS SYSTEM SUSCEPTIBILITY

The nervous system is highly susceptible to xenobiotics due to its complex and specialized components as well as its physiological functions. There are several characteristics that make the nervous system vulnerable to toxins and toxicants, which include complex structure and long period of development, interconnectivity to other organ systems, composition of differentiated postmitotic cells, selective transport into the CNS, high rate of metabolism, and myelination. In the following sections, these susceptibility factors will be discussed. It is important to note that xenobiotics can affect the nervous system through various pathways and take advantage of multiple susceptibility factors simultaneously.

Interconnectivity among Organ Systems

The nervous system is unique in that it connects to every tissue and organ system in the body, from the internal organs to the skin. Nervous system functions can be altered by inflammatory mediators, such as cytokines and chemokines, as well as hormones produced in peripheral glands, such as insulin, cortisol, and epinephrine. Indeed, many neurodegenerative diseases have been shown to have an inflammatory component to their pathology. Since the nervous system is highly interconnected to the other organ systems, it is vulnerable to not only neurospecific toxins but also toxins with broad general effects. For this reason, it is often necessary to determine whether the observed pathology is due to direct damage to the nervous system or an indirect effect of damage elsewhere. Both the liver and the kidney are involved in excretion of xenobiotics from the body. For example, diseases that alter liver and kidney function, as well as exposure to substances that reduce organ function, will cause a buildup of toxic substances or metabolites that enter the circulatory system and affect the nervous system. Additionally, if a toxin affects the nervous system, there may be pathological sequelae in other tissues. One classic example being cocaine, which affects both the release and reuptake of dopamine and norepinephrine in the brain, which is known to cause addiction, paranoia, hyperactivity, and aggression, but also increases blood pressure and causes abnormal heart rhythms leading to myocardial infarction and congestive heart failure.[4]

Differentiated Postmitotic Cells

Neurons, which are the functional cells of the nervous system required for the transmission of action potentials, are considered terminally differentiated postmitotic cells. The majority of neurons that are present in an adult individual are formed during development and differentiate into mature neurons during critical developmental windows. This is in contrast to other organ systems, such as the liver and immune systems, with regular turnover of the cells that comprise the tissues. For this reason, toxic substances that cause damage to cellular structure or cause neuronal death are especially harmful.

The loss of a neuron in the majority of the nervous system is irreversible. Neuronal loss, termed neuropathy, includes the degeneration of the cell body, axon, dendrites, and myelin sheath surrounding the axon. Cell loss may be due to either apoptosis or necrosis depending on the type of insult that led to death. Loss of neurons after xenobiotic exposure can

either be general or specific, with the loss of specific neurons resulting from a susceptibility of a particular subpopulation of neurons to the toxic substance that is not present in every neuron. Neurotoxins that cause neuropathies can either affect multiple cell types or be specific for neurons, taking advantage of vulnerabilities not present in other cell types, such as high metabolic rate or excitability. Due to the large variety of functions performed by the nervous system, neuropathies can have various functional consequences. Toxicants that produce overall damage to the nervous system, such as thallium, can produce diffuse encephalopathy and brain swelling.[5] In contrast, xenobiotics that affect subpopulations of neurons have specific functional effects. For example, quinine targets retinal ganglion cells, causing vacuolization and vision loss, while methanol causes degeneration of the putamen and retinal ganglion cells resulting in headaches, vision loss, and comas.[6,7] Death by either apoptosis or necrosis is usually cleared by phagocytosis performed by microglia. Phagocytosis by cell bodies usually occurs faster than for the axons and myelin, and for this reason, if there is extensive damage to axons and myelin in the CNS or PNS after toxicant exposure, it is prudent to investigate whether the cell bodies are affected.

Oftentimes, neurotoxins can cause axonal degeneration without affecting the cell body; this is called an axonopathy. Following an injury to the axon, the distal portion of the axon proceeds through a process of degeneration, which includes proteolysis and axonal swelling, followed by fragmentation of the axon and clearance of debris by microglia. In the PNS system, the Schwann cells proximal to the surviving portion of the axon release growth factors, which can allow for regeneration of the axon; however, if the axon is not restored, Schwann cells will decrease in number and regeneration will cease. In the CNS, oligodendrocytes secrete inhibitory factors that impede axonal regeneration while promoting astrocyte proliferation and the formation of a glial scar, making an axonopathy in the CNS permanent.

Neuropathy, axonopathy, and degeneration can be studied using a variety of histological stains in fixed tissues. One of the most thorough ways to detect degenerated axons and neurons is the amino cupric silver stain.[8] Briefly, brain tissue is imbedded in paraffin blocks and serial sectioned on a microtome. These sections are soaked in a preimpregnation solution containing cupric nitrate, silver nitrate, cadmium nitrate, lanthanum nitrate, neutral red dye, α-amino butyric acid, alanine, pyridine, triethanolamine, isopropanol, and deionized water (dH_2O). Sections are then heated in the microwave and cooled overnight before being rinsed in dH_2O, then acetone, and placed in the impregnation solution containing silver nitrate, 100% ethanol, acetone, lithium hydroxide, and ammonium hydroxide. After impregnation, the sections are transferred to a reducer solution (100% ethanol, 10% formalin, 1% citric acid, and dH_2O) while in a water bath. The sections are then rinsed in dH_2O and rapidly placed in a bleaching solution (potassium ferricyanide, potassium chlorate, lactic acid, potassium permanganate, and sulfuric acid) before being fixed in sodium thiosulfate and a rapid fixer solution and mounted on glass slides. Any degenerated axons or neurons will appear black in color as compared to normal tissues, which can be counterstained with neutral red.

While the majority of neurons are postmitotic cells, which once lost cannot be replaced, limited neurogenesis does occur during adulthood particularly in the hippocampus. Hippocampal neurogenesis results in the formation of new granule cells that incorporate into the existing cortical circuitry and is important for structural plasticity and network maintenance necessary for learning and memory. New neurons originate from adult neural stem cells of the subgranular zone of the dentate gyrus. These quiescent self-replicating type 1 cells have radial processes spanning the entire granule cell layer of the dentate gyrus and are identified by glial fibrillary acidic protein (GFAP), Sox2, and Nestin molecular markers. These cells are thought to give rise to the type 2 neural stem cells, which are intermediate cells identified as expressing Sox2 and Nestin, but not GFAP. Type 2 cells give rise to neuroblasts capable of differentiating into mature neurons.[9] Not all new neurons survive, but the ones that live are extremely sensitive to their environment. Neurotransmitters, such as GABA, as well as growth factors, neurotrophins, cytokines, hormones, and miRNAs are all known to affect the maturation of the progenitor cells. These cells can be visualized and quantified using well-defined molecular markers described earlier using immunostaining and flow cytometry protocols in order to evaluate the effects of environmental factors on adult neurogenesis. Recent evidence suggests that bisphenol A, alcohol, MeHg, cadmium, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin disrupt neurogenesis[10–14]; however, molecular mechanisms that lead to altered neurogenesis are unknown.

Anatomic Barriers

The BBB is comprised of an extensive network of specialized endothelial cells of the microvasculature of the brain, spinal cord, retina, and PNS as well as glial cells. Endothelial cells of the BBB form tight junctions around the vascular lumen of the capillaries creating a tighter seal between the blood and surrounding nervous tissue than the fenestrae present in systemic capillaries. The glial cells that comprise the BBB are primarily astrocytes, which project *astrocytic feet*, also called *glia limitans*, to surround the endothelial cells to provide structure and regulation of the tightness of the barrier. There are a small number of sites in the brain, which line the ventricles and are not protected by the BBB; these are termed the circumventricular organs and include structures such as the pineal gland, posterior pituitary, and area postrema. The lack of complete BBB in these regions allows circumventricular organs to sense and respond to signals in the bloodstream such as osmolality, toxins, and peptide hormones.

Under normal circumstances, small lipophilic substances and water can passively diffuse across the BBB; however, most other molecules must be actively transported in and out of the nervous system across the BBB. Several transport systems are used to move essential anions, cations, and metals

from the vascular lumen to the neuronal tissues. Some of these transporter systems are known to transport xenobiotics, such as the multidrug resistant protein family of transporters. Hydrophilic or large substances often pass through the BBB by molecular mimicry, a phenomenon where a xenobiotic that resembles an endogenous substrate enters the cell by hijacking the endogenous transporter or carrier molecule. MeHg is a potent neurotoxin and enters the CNS through molecular mimicry. MeHg is very nucleophilic with high affinity for the thiol group of cysteine. This MeHg–cysteine conjugate structurally resembles methionine, which is transported across the BBB via the large neutral amino acid transporter. MeHg enters the brain by taking advantage of this structural similarity.[15]

The blood–CSF barrier is comprised of modified epithelial cells of the choroid plexus and surrounding capillaries called ependymal cells. There are four choroid plexuses in the brain located in the third and fourth ventricles. The choroid plexus is responsible for the synthesis of CSF and acts as a filtration unit removing xenobiotics, excess neurotransmitters, and metabolic waste products from the CSF. The ependymal cells of the choroid plexus contain tight junctions to create a barrier between the capillary lumen and the ventricles similar to, but not as tight as, the BBB. The ependymal cells contain numerous villi and microvilli, which increases the total surface area for filtration and transport. The choroid plexus contains transporters, which can be the target for xenobiotic entry, and many of the xenobiotics that cross the BBB also enter the CNS through the blood–CSF barrier and accumulate in the CSF.

Lead (Pb) is one such metal that enters the brain through both the blood–CSF barrier and BBB. Pb exposure is known to cause the microvasculature of the brain to become leaky, resulting in increased permeability of the BBB, brain swelling, herniation, ventricular compression, petechial and cerebral hemorrhages, thrombosis, and arteriosclerosis.[16] The endothelial cells in both the microvasculature and choroid plexus cells accumulate Pb, which activates protein kinase C (PKC) signaling due to Pb-induced disruption of intracellular Ca^{2+} levels. Activation of PKC has been shown to increase endothelial permeability, which is observed during Pb toxicity.[17] Pb also inhibits functioning of the choroid plexus epithelial cells, decreasing the amount of transthyretin produced.[18] Transthyretin is an important protein involved in thyroid hormone binding and delivery and is only produced by the choroid plexus; Pb exposure decreases transthyretin and disrupts thyroid hormone signaling.

Metabolism

Neurons are energetically costly cells that are highly dependent on aerobic respiration to sustain sufficient energy to generate action potentials and maintain electrical conductivity. Even at rest, the membrane potential must be maintained by ion channels and energy requiring ion pumps. Dysregulation of these channels and pumps alters membrane potential and the ability of a neuron to depolarize or repolarize, impeding initiation and propagation of action potentials. Indeed, in conditions like epilepsy, where there is hyperactivity, and thus more frequent depolarizations and repolarizations, there is an increased energy demand, which if not met can lead to damage. Because of this high-energy need, a continual source of energy must be available to keep the neurons healthy. The brain relies heavily on aerobic glycolysis as its primary energy source, which means that any change in delivery of glucose or oxygen can be detrimental. Hypoxic and ischemic injury to the brain can result from exposure to various toxicants that either directly inhibit components of aerobic respiration machinery or decrease oxygen delivery. The production of ATP by the mitochondria involves several proteins working collectively to transfer electrons, produce ATP, and keep reactive oxygen species (ROS) production to a minimum.

It is not surprising that some of the classic neurotoxins produce their effects by the proteins involved in cellular respiration. 3-Nitropropionic acid (3-NP) is a mycotoxin produced by certain plants and fungi that decreases neuronal ATP production by specifically inhibiting succinate dehydrogenase (mitochondrial respiratory complex II). 3-NP is a suicide inhibitor of succinate dehydrogenase, meaning that 3-NP forms an irreversible complex with the enzyme, effectively making the enzyme unusable.[19] Ingestion of 3-NP by humans or livestock causes motor dysfunctions similar to Huntington's disease, and it is often used as an agent to study Huntington's in animal models. Direct injection into the brain of rats has shown that 3-NP can cause neurodegeneration through an excitotoxic mechanism.[20] Another classic neurotoxicant often used in animal models to study neurodegeneration is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). In the 1970s, MPTP was synthesized as an impurity in the synthesis of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), a synthetic opioid, and was eventually identified as the source of Parkinsonian symptoms displayed by drug abusers. MPTP itself is nontoxic; however, metabolism by MAO B in glial cells forms the toxic cation 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ is a potent reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase (mitochondrial complex I) inhibitor and leads to the disruption of the mitochondrial electron transport chain involved in respiration, free radical formation, and apoptosis. MPP^+ is thought to be selective to dopaminergic neurons by entering the cells via the synaptic dopamine reuptake transporter found primarily on dopamine neurons. Heavy metals are well-known neurotoxicants that decrease ATP production in the brain by inhibiting cellular respiration components. Rats exposed to low levels of Pb, below the threshold of safe for humans, had decreased ATP levels and reduced Na^+/K^+ ATPase activity. Rats exposed to arsenic inhibited complex I, complex II, and complex IV.[21,22]

Decreased oxygen delivery to the brain after exposure to a toxicant usually results either from a global phenomenon affecting all organs in the body or from alterations in the circulatory system. In the brain, the most metabolically active regions are severely affected by reduced oxygen including the basal ganglia, hippocampus, cerebral cortex,

and cerebellar Purkinje cells. Carbon monoxide poisoning produces hypoxia by competitively binding to hemoglobin in place of oxygen, which has effect on the entire body. Acute CO exposure severely damages these metabolic brain regions and may also cause white matter damage and cortical atrophy.[23] Decreased oxygen delivery to the brain may also be caused by toxicants that cause hypoperfusion, a condition where there is decreased blood flow to an organ or tissue, which if prolonged can result in tissue damage or death. Research performed in rats and monkeys suggests that global hypoperfusion caused by cyanide (CN^-) is responsible for the brain lesions characteristic of cyanide poisoning rather than other direct forms of CN^- damage to the CNS.

Although a xenobiotic may affect metabolic pathways, its neurotoxicity does not have to depend on its alterations in metabolism. Acrylonitrile is a widely used vinyl monomer used in the manufacturing of acrylic fibers, plastics, and synthetic rubber with known neurotoxic effects. In humans, acute acrylonitrile exposure causes limb weakness, labored or irregular breathing, dizziness, impaired judgment, hallucinations, cyanosis, nausea, collapse, loss of consciousness, convulsions, and death. Chronic exposure to acrylonitrile causes depression and lability of autonomic functions such as lowered arterial pressure, labile pulse, diffuse dermographia, increased sweating, and change in orthostatic reflex. Similar effects, including agitation, seizure, and death, have been observed in rats exposed to acrylonitrile. The precise mechanism for acrylonitrile's neurotoxicity is unknown. Acrylonitrile has been shown to undergo Michael addition reactions with nucleophiles including glutathione (GSH), the cell's must abundant thiol responsible for maintaining cellular redox status. Exposure to high levels of acrylonitrile can deplete GSH. This depletion of GSH and subsequent change in redox status results in acrylonitrile metabolism by cytochrome P450 and formation of CYP2E1 and CN^-, the initially proposed mechanism of toxicity. However, inhibition of CYP2E1 with 1-benzylimidazole reduced blood levels of CN^- in rats exposed to acrylonitrile, but did not protect the animals from acrylonitrile-induced mortality.[24] In addition to being able to bind to GSH, acrylonitrile can covalently bind to proteins, including the catalytically active cysteine of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme involved in glycolysis and ATP production.[25] Acrylonitrile decreases some glycolytic intermediates, but in spite of this, there is no evidence for ATP depletion in the brains of rats exposed to acrylonitrile.[25] This illustrates that although there may be the potential to alter the metabolism critical for neuronal functioning, xenobiotic neurotoxicity may involve several other mechanisms.

Myelination

Myelination is a unique characteristic only found in the nervous system. Myelin consists of lipid-rich sheath that encases neurons of both the CNS and PNS. Its primary function is to insulate the neuron and allow for rapid conductance of the action potential along the neuron. The presence of myelin decreases membrane capacitance and increases the electrical resistance of the neuron, allowing for significantly faster rates of action potential propagation as compared to unmyelinated neurons. In the CNS, myelin is formed by oligodendrocytes, while in the PNS, it is formed by Schwann cells. Both Schwann cells and oligodendrocytes form myelin by wrapping concentric layers of membrane and cytoplasm around the axons, excluding cytoplasm from the inner surface of their membranes and reducing the extracellular surfaces of the bilayers allowing for a compact stack of lipid- and protein-rich myelin. The lipids that comprise myelin are predominantly galactocerebroside and sphingomyelin. Structural proteins are required to form and maintain proper myelin structure. Myelin basic protein (MBP), myelin oligodendrocyte glycoprotein, and proteolipid protein are adhesion proteins associated with the intracellular spaces of wrapped myelin sheath in the CNS, which when mutated can cause genetic diseases of poorly formed or maintained myelin. Demyelination, the loss of myelin while the axon is preserved, occurs through a variety of pathological mechanisms.

Loss of myelin results in slowing or aberrant action potential conductance. Demyelination by toxins can result from intramyelinic edema or direct damage to the myelinating cell. Intramyelinic edema occurs when the myelin sheets separate, which can be reversible or can progress to demyelination of the axon. A variety of toxicological mechanisms can lead to intramyelinic edema, including disruption of MBP transcription by triethyltin, disruption of ion gradients by hexachlorophene, and vacuole formation resulting in the splitting of myelin sheets by closantel.[26–28] Intramyelinic edema can lead to swelling and spongiosis of the brain, which is often not reversible. Direct damage to the myelinating cells has been observed following exposure to dideoxycytidine, cuprizone, and dithiocarbamate pesticides.[29–31] It was once thought that oligodendrocytes were incapable of reforming myelin after demyelination in the CNS; however, it is now known that oligodendrocyte precursor cells can be stimulated to remyelinate damaged axons in mouse models of demyelination/remyelination.[32] The functional consequences of demyelination depend on the extent of demyelination that occurs after exposure to a toxic substance. Diffuse demyelination may result in global nonspecific effects including headache and photophobia, whereas demyelination confined to the PNS can present as a peripheral neuropathy such as limb paralysis.

RECEPTORS AND SIGNALING

The mechanisms by which extracellular signals are transferred to the cell cytosol and nucleus, commonly referred to as *cell signaling* or *signal transduction*, are receiving much attention in all areas of biology and medicine. Neurotransmitters, hormones, and growth factors serve as first messengers to transfer information from one cell to another by binding to specific cell membrane receptors. This interaction results in activation or inhibition of specific enzymes and/or opening of ion channels, which lead to changes in intracellular metabolism and, in turn, to a variety of effects, including activation

of protein kinases and transcription factors. These intracellular pathways can be activated by totally different receptors and are very interactive or *cross talking*, so they can control and modulate each other. As the area of signal transduction is very broad, a review of all its aspects is well beyond the scope of this chapter, and only a few examples are discussed in the following for illustrative purposes.

Some of the most studied signaling pathways are those activated by membrane receptors. These include, for example, enzyme-linked receptors and G protein–linked receptors.[33,34] Enzyme-linked receptors (such as those of insulin or of epidermal growth factor) are receptors in which the ligand-binding domain, the membrane-spanning region, and the effector enzyme are usually separate domains of the same protein. Binding of the ligand to the receptor activates the effector enzyme. The most widely studied of these receptors are those with tyrosine kinase activity. Tyrosine kinase autophosphorylates the receptor and also phosphorylates other substrate proteins. This initiates a cascade of processes whereby a large number of proteins are activated, including Ras, Raf-1, JAK-STAT, as well as mitogen-activated protein kinases (MAPKs) and p70S6 kinase.[33]

G Protein–Mediated Signaling

G Protein–linked receptors are activated by a wide variety of ligands, including neurotransmitters and hormones. These receptors have a seven-transmembrane domain, extracellular loops with the ligand-binding domain, and intracellular loops, which interact with G-proteins. The G (for guanosine diphosphate (GDP)/guanosine triphosphate (GTP)-binding)-proteins consist of three subunits, α, β, and γ. Upon interaction with the ligand, the GDP bound to the α-subunit is replaced by GTP, and Gα dissociates from Gβγ. Gα-GTP then activates an effector enzyme, which starts a signal transduction cascade. Depending on the nature of the G-protein, a receptor can activate or inhibit the same enzyme (e.g., adenylate cyclase), or activate a totally different enzyme. Furthermore, in addition to Gα, Gβγ has also been shown to activate an array of effector enzymes, including those also activated by Gα.[35] This model of G protein–linked receptor is utilized, for example, by the adenylate cyclase system. Upon activation by Gα-GTP, adenylate cyclase catalyzes the conversion of ATP to cAMP; the latter acts as a second messenger by activating protein kinase A (PKA). This occurs via the binding of two cAMP molecules to each of the regulatory subunits of PKA, thus releasing the catalytic subunit. In turn, PKA phosphorylates, and thus changes the activity of substrate proteins in the cytoplasm, in the membrane, as well as in the nucleus (e.g. the transcription factor cAMP response element-binding protein (CREB)). If the Gα-subunit is of the inhibitory type (Gi), then inhibition of adenylate cyclase and a decrease of cAMP levels will ensue.

Another pathway that utilizes this system regulates the metabolism of membrane phosphoinositides. In this case, Gα-GTP (Gq) activates the enzyme phospholipase Cb (PLCb), which catalyzes the breakdown of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG), both of which act as second messengers. It should be noted that PLCs can also be activated by other receptors (e.g., that of nerve growth factor), thus underlining the complex entangled web of interactions in signal transduction pathways.[33] $InsP_3$ binds to specific receptors in the endoplasmic reticulum and mobilizes calcium from intracellular stores, thereby allowing activation of a variety of cellular enzymes. DAG, in concert with calcium and phosphatidylserine, activates certain subtypes of PKC. In turn, PKC phosphorylates a number of cellular substrates, including transcription factors. Only classical PKCs (such as a and g) are activated as indicated; novel PKCs (e.g., PKC e) do not need calcium for activation, and atypical PKCs (e.g., PKC z) are activated by other means. In addition to DAG, other important lipid mediators can be generated in cells by G protein–linked receptors through multiple pathways.

An important pathway is represented by the hydrolysis of phosphatidylcholine, one of the major membrane phospholipids, by phospholipase D (PLD), to generate phosphatidic acid (PA), which can activate atypical PKC z. Activation of classical and novel PKCs stimulates PLD activity, thus generating PA; this can also be converted to DAG by a PA hydrolase, thus providing a mean for a prolonged stimulation of PKCs. Other important lipid mediators are arachidonic acid, the precursor of all prostanoids, which is generated by hydrolysis of membrane phospholipids by phospholipase A_2, and ceramide, which is released from sphingomyelin by sphingomyelinase.

Phosphoinositide-3 Kinase Signaling

Phosphoinositide-3 kinase (PI3K) represents another important cell-signaling pathway, which plays important roles in the control of cell growth, proliferation, and survival.[36] When activated by surface receptors or interaction with Ras, class I PI3K produce phosphatidylinositol (3,4,5)-trisphosphate [$PtdIns(3,4,5)P_3$]. PI3K regulates cellular functions by recruiting $PtdIns(3,4,5)P_3$-binding proteins to the plasma membrane. The prototype of these molecules is protein kinase B (PKB), also known as Akt, which is activated by ligation of $PtdIns(3,4,5)P_3$ and by phosphorylation by 3′-phosphoinositide-dependent kinase 1 (PDK1).[37] PDK1 also phosphorylates some PKC isoforms (e.g., PKC z) and downstream kinases (e.g., p70S6 kinase). The ability of certain receptors to increase intracellular levels of cyclic GMP (cGMP) and the fact that such effect was indirect, that is, it necessitated an additional second messenger, has been known for quite some time. Initial candidates for such role included calcium ions and arachidonic acid metabolites. However, it was later discovered that NO, synthesized from L-arginine by NOS, binds to a heme moiety attached to guanylate cyclase and stimulates the formation of cGMP.[38,39] The NOS family consists of three isoforms: neuronal NOS (nNOS) and endothelial NOS (eNOS), which are constitutively expressed and require the formation of calcium–calmodulin complexes for their activation, and inducible NOS (iNOS), which exerts its activity in a calcium-independent manner. NO is involved in

a wide array of cellular effects in the CNS, ranging from neuroprotection to neurotoxicity.[40,41] The diversity in response appears to be related to the steady-state concentration of NO. In general, low concentrations of NO (1–300 nM) would promote cell survival, whereas at higher concentrations (1 mM), nitrosative stress would prevail, leading to cell cycle arrest and apoptosis.[42]

Lead, A Case Study for Cell Signaling: Neurotoxicant Interaction

In recent years, investigation of the molecular mechanisms of neurotoxicity has started to consider a variety of signal transduction pathways as potential targets for neurotoxicants. Such studies have followed three basic experimental approaches: the study of the direct effects of a chemical on a particular step in cell signaling (e.g., a protein kinase); the study of the direct effects of a neurotoxicant on second messenger responses activated by an endogenous compound (e.g., a neurotransmitter-induced activation of phospholipid hydrolysis); and, to a limited extent, the alterations in signal transduction resulting from other toxic actions (e.g., oxidative stress).

Interactions of neurotoxicants with signal transduction pathways may occur in neuronal or in glial cells, and may lead to inhibition or stimulation of cell proliferation and/or cell differentiation, to the direct induction of cell death, to inhibition of pathways that provide protection against cell death, or to other effects on cellular function. As such, such interaction may be relevant to neurotoxicity in adults, to developmental neurotoxicity, as well as to neurooncology. The relevance of interactions with signal transduction pathways in the neurotoxicity and developmental neurotoxicity of a number of chemicals (mostly ethanol, polychlorinated biphenyls [PCBs], domoic acid, aluminum, cyanide, organotin compounds, and certain pesticides) has been discussed in a number of reviews and book chapters in the past two decades.[43–47] In this chapter, one example of such interactions is discussed, related to mechanisms possibly underlying the developmental neurotoxicity of Pb.

Pb was discovered thousands of years ago and has been used extensively ever since. In the 1800s, commercial use of Pb increased further, as it was found to be highly effective in paints. In the early 1920s, an organic form of Pb (tetraethyl lead) was found to be very effective as an antiknocking agent in gasoline, starting the long successful sale of leaded fuels. The toxic effects of Pb, however, did not go unrecognized. In recent decades, several efforts to reduce the use of Pb (e.g., banning of Pb paints in the 1970s, phasing out of leaded gasoline in the 1980s) significantly curtailed Pb contamination; yet Pb remains a major, ubiquitous, ecosystem pollutant. Pb is a neurotoxic metal both in adults and in children. In adults, the main effects of Pb poisoning are a peripheral neuropathy, precisely a myelinopathy, which is reversible upon chelation and cessation of exposure. At higher concentrations (100 μg/dL in blood), an encephalopathy can also develop. In contrast, the developing CNS is exquisitely sensitive to the effects of Pb, even at much lower blood levels. In the 1970s, the blood Pb action level in children was 60 μg/mL, the level associated with clinical signs of toxicity in adults.[48] In those years, epidemiological studies clearly showed an association between body burden of Pb in children and adverse neurobehavioral outcomes, namely, lower academic performance and shortened attention span.[49] The phasing out of leaded gasoline and the limitation on smokestack Pb emissions caused Pb blood level in children in the United States to decrease by 80% in the period 1978–1991. Over the years, the level of concern for blood Pb has decreased to 25 μg/mL, then in 1991 to 10 μg/mL, where it stands today. However, blood Pb levels as low as 2 μg/mL have been associated with declines in IQ and various adverse behavioral effects,[50] and there is widespread belief that there is no proven safe lower limit for Pb exposure.[51]

Animal studies in multiple species have confirmed that developmental Pb exposure causes similar cognitive dysfunctions, learning impairment, and distractibility.[52] Pb has been shown to exert neurotoxicity during differentiation and synaptogenesis[53]; however, the greatest adverse effects are seen during the latest stages of brain development, suggesting that Pb may interfere with the apoptotic process and the trimming/pruning of synaptic connections.[54] In vivo and in vitro studies have shown that Pb may disrupt the BBB by injuring astrocytes, with a secondary damage to the endothelial microvasculature.[55] Developmental Pb exposure has been shown to target the hippocampus, cerebral cortex, and cerebellum.[56]

Though the exact mechanisms of Pb neurotoxicity have not been fully elucidated, the prevailing hypothesis is that lead interferes with the action of calcium; indeed, in several systems, Pb can mimic calcium actions at the cellular level and/or disrupt calcium homeostasis.[57,58] Given the role of calcium as a cofactor in many cellular processes involved in signal transduction, it is not surprising that several cell-signaling pathways are affected by Pb. One of the most interesting findings is the ability of Pb to substitute for calcium in activating certain PKCs. The first observation was that Pb, in a concentration range of 0.1–10 mM, would activate PKC and cause translocation of PKC from the cytosol to the particulate fraction in isolated immature rat microvessels.[59] Pb was also shown to activate PKC, partially purified from rat brain, at concentrations as low as 10^{-15} M, several orders of magnitude lower than calcium itself.[17] A later study confirmed that Pb could activate PKC at 10^{-11} to 10^{-8} M, while calcium activates PKC in the range from 10^{-8} to 10^{-5} M.[60] Two studies, utilizing recombinant human PKCs, detailed the interactions of lead with these enzymes.[61,62] Calcium-dependent PKCs, but not all other PKCs, have a calcium-binding domain (C2) in the regulatory region. At low concentrations (10^{-12} M), Pb binds to a site (Ca1) within C2 that causes activation of PKC a; at higher concentrations, Pb binds to another site (Ca2), also in C2, which causes inhibition of enzyme activity by antagonizing calcium. In addition to the dual effects mediated through the interactions of Pb with the C2 domain, at still higher concentrations, Pb inhibits PKC activity by interacting with the catalytic domain. Indeed, Pb was found

to inhibit PKC e and PKC z, which lack the C2 domain.[62] These findings may explain the reports describing contrasting effects of lead on PKCs.[63]

Activation of PKC by Pb has been suggested to be involved in its inhibition of astroglia-induced microvessel formation[64] and to contribute to its ability to release neurotransmitters from bovine adrenal chromaffin cells, brain synaptosomes, and PC12 cells.[61,65,66] In vivo exposure to Pb during brain development has been shown to cause a decrease of PKC g in the hippocampus.[67,68] PKC inhibitors block long-term potentiation, a possible functional equivalent of memory storage, suggesting that Pb-induced decreased PKC activity (possibly resulting from down-regulation caused by sustained activation) may relate to learning and memory deficits caused by this metal. An increase in PKC e expression was also reported following in vivo developmental Pb exposure, perhaps as a compensatory mechanism due to prolonged inhibition.[69] In human astrocytoma cells, activation of PKC a, followed by the sequential activation of MAPK (Erk 1/2) and its target $p90^{RSK}$, was shown to mediate the mitogenic effect of Pb in these cells.[70,71] Such effects may be involved in the reported ability of Pb to induce CNS tumors (gliomas and astrocytomas) in both animals and humans.[72,73]

In summary, the ability of lead to substitute for calcium and/or to alter the action of calcium appears to be responsible for its effects on a number of cell-signaling systems. In particular, its interactions with PKCs may play a role in the ability of lead to cause cognitive alteration upon developmental exposure and may contribute to its mitogenic action possibly related to its carcinogenic effects.

VULNERABILITY OF THE DEVELOPING NERVOUS SYSTEM

Several lines of evidence suggest that the developing nervous system may be more susceptible, and/or differentially susceptible, to toxic insult than the adult nervous system. Different parts of the CNS develop at different stages (Figure 33.1); cell proliferation, migration, and differentiation contribute to the formation of definite brain structures, in which the correct number of cells in the proper location is necessary for proper function.[74,75] Even within a single brain region, subpopulations of neurons may have different rates of development; for example, in the cerebellum, Purkinje cells develop early (embryonic days 13–15 in the rat, corresponding to gestational weeks 5–7 in humans), while granule cells are generated much later (postnatal days 4–19 in the rat, corresponding to gestational weeks 24–36).[74] Failure in cell proliferation or cell migration because of exposure to toxic insults (e.g., irradiation or MeHg) has profound deleterious effects on the developing brain.[76] Though neurons maintain the ability to make new synapses throughout life, the period of brain development when synaptogenesis occurs is critical for the formation of the basic circuitry of the nervous system.[77] Furthermore, in the developing nervous system, neurotransmitters may have function other than neurotransmission, such as modulation of cell proliferation, survival, and differentiation.[78] Thus, any toxicant that interferes with neurotransmission during development may cause permanent defects in the CNS.

Developmental neurogenesis produces more neurons than those found in the mature nervous system, and excess neurons are pruned by finely regulated apoptotic processes at different developmental times.[79] Any chemical interfering with apoptotic processes may trigger degeneration of neurons that would not otherwise have been deleted or may promote survival of unnecessary cells.[80] In addition, pruning, defined as loss of synapses, also occurs physiologically in the developing brain, and chemicals interfering with this process (which is longer lasting than neuronal loss to apoptosis) would have most significant adverse effects on brain functions.[81]

While all these considerations relate to the development of neurons, it has become apparent that glial cells (astrocytes, oligodendrocytes, and microglia) also play a relevant role in brain development and may be the target of toxic action.[82] Indeed, several chemicals (e.g., alcohol, nicotine, and certain pesticides) exert profound neurotoxic effects when exposure occurs during the brain growth spurt, characterized by extensive glial cell proliferation and maturation.

Separate guidelines for developmental neurotoxicity testing have also been developed both in the United States and in Europe.[83,84] Maternal exposure to the test chemicals is from gestational day 6 to postnatal day 10 or 21 to ensure exposure in utero and through maternal milk. Tests involve measurements of developmental landmarks and reflexes, motor activity, auditory startle test, learning and memory tests, and neuropathology. As for neurotoxicity testing, developmental neurotoxicity testing has been proven to be useful and effective in identifying compounds with developmental neurotoxicity potential.[85] This is not to say that current developmental neurotoxicity testing guidelines cannot be improved; indeed, it has been pointed out that they may be overly sensitive and produce a high rate of false positives[86] or, in contrast, that they may be too insensitive and not comprehensive enough.[87] Furthermore, issues have been raised regarding historical control data, toxicokinetic parameters, maternally mediated toxicity versus direct effects, selection of tests, and their analysis and interpretation.[88,89]

In the past several years, the need to develop acceptable alternatives to conventional animal testing has been increasingly recognized by toxicologists in order to address the escalating costs and time required for toxicity assessments, the increasing number of chemicals being developed and commercialized, and the need to respond to recent legislation (e.g., Registration, Evaluation, Authorisation, and Restriction of Chemicals [REACH] and the Cosmetics Directive [76/768/EEC] in the EU). In addition, efforts have been directed toward the development of alternative models to reduce the number of animals used for toxicity testing, utilizing either mammalian cells in vitro or nonmammalian model systems (e.g., zebra fish or *C. elegans*), which could serve as tools for neurotoxicity and developmental neurotoxicity testing, particularly for screening purposes.[90] These alternative tests should serve as Tier 1 tests to allow the screening of

compounds whose potential developmental neurotoxicity is unknown. Given the complexity of the nervous system and the multiple facets of possible neurotoxic effects, it is highly unlikely that a single test (as the Ames test for mutagenicity) will cover the spectrum of neurotoxicity. Rather, a battery of tests should be considered, which may include some in vitro tests with mammalian cells and one or two tests with nonmammalian models. This may be complemented by quantitative structure–activity relationship-based computational approaches. Novel approaches, part of the *omics* technologies, may also find a role in such endeavors. Alternative models for developmental neurotoxicity should attempt to mimic several processes that may occur in vivo, and given the complexity of the CNS, the scenario for developmental neurotoxicity is much more complex than that for other target organs of toxicity.[90]

Examples of Developmental Neurotoxicants

There are approximately 200 chemicals that have been found to be neurotoxic in humans,[91] and for many more, there is at least some evidence of neurotoxicity deriving from animal studies. Of these, several are developmental neurotoxicants. However, of over 80,000 chemicals on the market, only a handful (about 200) have undergone developmental neurotoxicity testing according to the established guidelines.[85] As said, the developing brain is often more sensitive than the adult brain to toxic insult; thus, neurotoxicity is observed at much lower exposure levels. This is the case, for example, of lead or MeHg. In several cases, the effects of developmental chemical exposure are different from those observed in adults upon similar exposure, for example, ethanol or valproate exposure. In some instances, developmental exposure to neurotoxicants results in morphological alteration of the CNS, with accompanying changes in functions.[76] However, functional changes may result from more subtle biochemical/molecular alterations without major structural abnormalities. Exposure to chemicals that may adversely affect the nervous system has been suggested to be associated with a number of developmental disabilities that are diagnosed in children at an alarmingly increasing rate including learning disabilities, attention-deficit hyperactivity disorder, dyslexia, sensory deficits, mental retardation, and autism spectrum disorders.[48,91]

One example of a developmental neurotoxicant, MeHg, is presented in the following. Lead, discussed earlier as an example of neurotoxicant interfering with signal transduction, is another example. MeHg is probably one of the most studied developmental neurotoxicants due to several episodes of human poisoning over the years, and continued low-level exposure in many populations worldwide, mainly through the consumption of contaminated fish.[92–94] With the possible exception of a case in Sweden in the early 1950s, due to consumption of MeHg-contaminated seed grain, the first evidence of the deleterious effects of MeHg exposure on brain development emerged from Japan in the mid-1950s.[95] Many children born to mothers who consumed MeHg-contaminated fish around Minamata Bay presented with severe neurological deficits, while their mothers appeared unaffected or suffered only mild symptoms.[96] About a decade later, another extensive episode of poisoning occurred in Iraq, due to consumption of MeHg-contaminated grain.[97]

In both cases, notable differences were found in the distribution of pathological changes in the young, exposed in utero or as children, and in adults.[98] In particular, while damage in adults is restricted to the cerebellum and the visual cortex, diffuse damage is seen in the developing brain.[99] It was estimated that the in utero developing nervous system has a fivefold greater vulnerability to MeHg.[100] Signs and symptoms in MeHg-poisoned children included spastic paresis, mental retardation, movement disorders, seizures, primitive reflexes, and speech difficulty.[93,96] The mechanisms of MeHg developmental neurotoxicity have been studied extensively, and a very complex picture has emerged. MeHg binds with high affinity to sulfhydryl groups, which are relevant for the proper functioning of a large number of proteins. As such, several key cellular processes are affected by MeHg.[76,92,94] For example, MeHg has been reported to cause apoptotic cell death, to cause retraction of growth cones and extension, to impair the cytoskeleton, to affect cellular metabolism, and to reduce cell proliferation and neuronal migration.

The ban of alkylmercury compounds for use as fungicides and stricter controls on fish contamination have avoided further catastrophic events as those of Japan and Iraq. However, low-level contamination of fish is persistent and may be responsible for developmental neurotoxic effects, particularly in populations with high seafood consumption. Three major longitudinal studies have examined the potential effects of low-level MeHg exposure in New Zealand, the Seychelles, and the Faroe Islands.[100–102] In all locations, MeHg exposure is entirely due to diet, which consists mainly of marine animals. Two of these studies (in New Zealand and the Faroe Islands) reported a correlation between maternal levels of MeHg and subtle neurobehavioral deficits in the offspring. In particular, a small decrease in IQ points and deficits in memory attention and visuospatial perception were noted in both studies.[100,102] In the Seychelles study, such relation between MeHg exposure and neurodevelopmental effects was not apparent.[103] In the Faroe Islands population, concomitant exposure to PCBs present in whale meat and whale blubber may be an important confounder, as both MeHg and PCBs may have independent neurological effects in this population.[104,105] Another important confounder in these and more recent studies[95] is the beneficial effect of fish consumption ascribed to omega-3 fatty acids.[106,107] Thus, a balance exists between adverse (MeHg) and beneficial (omega-3) effects, which may dampen toxicity or obscure benefits. As MeHg contamination of fish is difficult to avoid, the best choice for the pregnant consumer is to choose fish species with minimal MeHg burden and relatively high omega-3 content (e.g., sardine, salmon), in order to minimize risk and maximize benefits.[107] Exposure limits for MeHg have been set, with provisional tolerable weekly intake values ranging from 0.7 to 1.6 μg/kg, depending on the regulatory agency.[107]

SILENT NEUROTOXICITY, LONG-TERM EFFECTS, AGING, AND RELATIONSHIP TO NEURODEGENERATIVE DISEASES

Manifestations of neurotoxic effects upon adult and/or developmental exposure are usually seen shortly after exposure. Yet, evidence is emerging that deleterious effects of toxicants may not become clinically evident for some months, or even several years after exposure. This period during which the individual may manifest no evidence of toxicity is referred to as the *silent* or latent period.[108] Silent toxicity is defined as persistent biochemical or morphological injury, which remains clinically unapparent unless unmasked by experimental or natural processes.[108,109] Silent toxicity may be compared to the process of carcinogenesis, in which molecular and cellular damages occurs years if not decades before any clinical manifestation of the disease.[110]

In case of neurotoxicity, an example of silent toxicity is represented by the Parkinsonism dementia known as Guam's disease, in which latencies of decades have been reported before clinical signs and symptoms from an undefined exposure.[111] Similarly, bovine spongiform encephalopathy (mad cow disease), a variant of Creutzfeldt–Jakob disease, can have a latency period of decades.[112] The delay between exposure and clinical expression of neurotoxic injury may be ascribed to various causes. For example, a selected population of neurons may be affected, but the known plasticity of the brain would compensate for such loss. However, further exposure to exogenous influences (e.g., stress, disease, additional chemical exposure) and/or the natural aging process unmasks the existing deficit. Alternatively, the organism may be initially able to compensate for a certain deficit, but progressive loss of function eventually overcomes the functional reserve and plasticity of the brain.[108]

The possibility that such latent period between exposure and clinical manifestation would occur in the context of development is even more probable. The concept that adult disease may have a fetal origin has been introduced by David Barker and is known as the *Barker hypothesis*.[112] Exposure to chemicals may cause direct damage or alter developmental programming, whose resulting functional deficits become apparent later in life. A famous example is that of diethylstilbestrol, in which in utero exposure leads to an increase in vaginal adenocarcinoma around the time of puberty.[114]

In the context of developmental neurotoxicity, prenatal infection has lasting effects on the dopaminergic system. Perinatal exposure of rats to the Gram (–) bacteriotoxin lipopolysaccharide causes a 30% loss in dopaminergic neurons in the substantia nigra and persistent injury to the dopaminergic system.[115,116] This study suggests that depending on the gestational time of infection, human infants with prenatal infections would be born with fewer dopaminergic neurons than nonexposed infants. This would be initially inconsequential, as clinical signs of PD are not apparent until about 80% of dopaminergic neurons are lost. However, this early-life damage would predispose an individual to develop PD as the aging process brings along a normal progressive loss of dopaminergic neurons.[117] Developmental exposures to certain pesticides, such as the herbicide paraquat and the fungicide maneb, which also target dopaminergic neurons, have also been implicated in the later development of PD.[118] Similarly, developmental exposure to the now banned organochlorine insecticide dieldrin has been shown to cause long-lasting alterations of the dopaminergic system, typical of a silent dopaminergic dysfunction.[119]

In some occasions, early mild damage may worsen as the individual matures and ages. For instance, neurotoxic effects of developmental exposure to MeHg may be unmasked only during aging.[120,121] In utero exposure to methylazoxymethanol, which causes microencephaly, caused a premature decline in cognitive functions,[122] and the neurotoxic effects of neonatal exposure of triethyltin, a glial neurotoxicant, were exacerbated by aging.[123] In other situations, this may not be the case, yet the neurotoxic effects of developmental exposure appear to be irreversible, and even if they do not worsen with age, they are certainly long lasting, as shown, for example, for PCB-126.[124] Perturbations of cognitive functions due to developmental Pb exposure remain for life,[125] and perinatal Pb exposure has been associated with the development of schizophrenia and of Alzheimer's disease.[126–128] It has also been suggested that exposure to Pb during critical periods of brain development may alter expression and regulation of amyloid precursor protein later in life, potentially altering the course of amyloidogenesis and acting as a risk factor for the onset of Alzheimer's disease–like pathology.[127,129,130]

In addition to direct effects on neurons and glia in the nervous system, chemicals that act as endocrine disruptors can also disrupt neurodevelopment. For example, polybrominated diphenyl ether (PBDE) flame retardants are thought to exert developmental neurotoxicity by two general, and not mutually exclusive, modes of action, one related to effects on thyroid hormones and the other involving direct effects of PBDEs on the developing brain.[106] Thyroid hormones are known to play a relevant role in brain development,[131] and hypothyroidism is associated with a large number of neuroanatomical and behavioral effects.[132,133] PBDEs perturb the thyroid system both during development and adulthood, leading to a reduction of circulating thyroid hormone.[134] The exact mechanism(s) underlying these effects are unclear, but reduction in thyroid hormones may contribute to developmental neurotoxicity of PBDEs although effects have also been seen in the absence of thyroid hormone changes.[135] Experimental compounds such as propylthiouracyl, which inhibits thyroid hormone synthesis and causes hypothyroidisms, are clear developmental neurotoxicants, as evidenced by behavioral and morphological alterations in the CNS.[133,136] Decreases of T_4 following developmental exposure to PBDEs or PCBs range from 10% to 60%. Such decreases may well contribute to their developmental neurotoxicity as evidenced by neurological decrements observed in children of mothers with 25% decrease in T_4.[132]

DRUGS OF ABUSE

There are many classes of *drugs* that have neuropharmacological properties. In cases where these drugs are misused or abused, they can result in severe toxicity to the system and thus can be considered neurotoxicants. While they may exert their toxicity in several organ systems, many of them have mechanisms of action that interfere with the physiology of the nervous system. Besides their primary actions, an additional concern for many of these drugs of abuse is their use in combination with other drugs or alcohol, which can often exacerbate toxicity. Other concerns include long-term abuse, which can lead to cognitive impairment, confusion, psychosis, coma, and death, and exposure during critical windows in brain development can result in altered development of brain circuitry. For many of these drugs of abuse, the full toxicological profiles are not worked out. While there are many and various neurotoxicological consequences of drug exposure, the following section details the effects on neurotransmitters.

Narcotics are usually used to treat pain, but according to the National Institute on Drug Abuse, they are some of the most highly abused prescription drugs.[137] CNS depressants, prescribed to treat anxiety and sleep disorders and stimulants are also highly abused.[137] Examples of narcotics include opium, morphine (Avinza), codeine, heroin, hydrocodone (e.g., Vicodin), and oxycodone (e.g., Percocet). These drugs bind to opioid receptors throughout the body and reduce the feeling of pain. For example, morphine and heroin are mu-opioid agonists.[138] These drugs, when used in combination with CNS depressants, increase the risk of respiratory depression.

Classical stimulants include nicotine, caffeine, cocaine (coke, blow, crack), and amphetamines. Nicotine affects the nicotinic cholinergic receptors.[139] Cocaine works on the dopaminergic system by blocking the dopamine reuptake transporter.[140] Amphetamines alter catecholamine neurotransmission both at the level of the transporter and by disrupting vesicular storage of dopamine.[141]

Depressants including alcohol, cannabinoids, barbiturates, benzodiazepines, gamma-hydroxybutyric acid (GHB), and paraldehyde act by slowing down the activity of the CNS by affecting neurotransmitters like GABA. They may also have hallucinogenic effects. For example, benzodiazepines activate a subset of $GABA_A$ receptors on ventral tegmental area interneurons, specifically the alpha-1 subtype, altering the dopamine effect.[142] Marijuana, hashish, and synthetic marijuana (known as K2 or *spice*) exert their toxicity via tetrahydrocannabinol (THC), the main psychoactive ingredient, acting through cannabinoid receptors.[137]

Hallucinogens, including D-lysergic acid diethylamide (LSD), peyote and mescaline, psilocybin (mushrooms), phencyclidine (PCP), MDMA (ecstasy), and other phenethylamines, affect the serotonergic system. LSD, peyote, and psilocybin disrupt the serotonergic system, while PCP acts primarily on the glutamatergic receptors. LSD has been shown to act as a partial agonist of $5\text{-}HT_{2A}$ and $5\text{-}HT_{1A}$ receptors, with stronger effects through $5\text{-}HT_{2A}$.[143,144] MDMA exerts its effects on the serotonergic system by binding to the serotonin transporter, resulting in increased and prolonged serotonin signaling; it can also be taken up by the transporter and cause release of serotonin.[145,146] The same effects are seen in the dopaminergic and norepinephrine systems, although to a lesser extent.

Inhalants, which can be any number of things that are delivered via the nasal passages or the mouth, are categorized into nitrites, gasses, aerosols, and volatile solvents.[137] Some classic examples include glue, lighters, and markers. They can affect many neurotransmitters through one of the primary acute effects of hypoxia and through long-term effects including myelin breakdown.

The United States Department of Justice regulates these drugs of abuse under the Controlled Substances Act, which has five schedules for regulation, based on the substance's medical use, potential for abuse, and safety or dependence liability. Drugs that fall under Schedule I categorization are those that have a high potential for abuse and have no currently accepted medical use in treatment in the United States. Examples include heroin, LSD, and marijuana. Schedule II includes those drugs that have a high potential for abuse yet have an accepted medical use in treatment, but abuse may lead to severe psychological or physical dependence; examples include morphine, PCP, cocaine, methadone, and methamphetamine. Schedule III includes those substances that have less potential for abuse than those in Schedules I and II and have accepted medical uses in treatment, and abuse may lead to moderate or low physical dependence or high psychological dependence. Examples include anabolic steroids and codeine. Schedule IV substances have low potential for abuse compared to higher schedules.

SUMMARY

In summary, the nervous system is a complex network of differentiated cells and signaling cascades, which are especially vulnerable to both neurospecific and global toxins and toxicants. Due to its complexity and unique vulnerability factors, no two exposures to the nervous system are alike, with toxic effects that may be seen immediately, only during development, or later in life. For this reason, careful analysis of neurotoxicity of a given agent should be assessed in a case-by-case basis across multiple experimental models and exposure paradigms. With such a large list of known neurotoxins and the even broader classes of potential neurotoxins, complete neurotoxic profiles rarely exist. The use of multiple cell– and invertebrate-based systems and high-throughput arrays and screens will prove indispensible in teasing out which chemicals and compounds we should be most concerned of.

QUESTIONS

33.1 What are functional consequences of altering sodium homeostasis in a neuron?

33.2 Why is it a common characteristic that mitochondrial electron transport chain inhibitors and uncouplers are neurotoxic?

33.3 A new small molecule has been synthesized that is structurally related to scopolamine, a known neurotoxin with anticholinergic activity found in the *Atropa belladonna* (deathly nightshade) plant, and you have been asked to assess its neurotoxic potential. What would be the expected sites of action? What kind of clinical symptoms in a rat dosed with a high or a low concentration?

33.4 It is observed that compound X causes abnormal myelination in the PNS. How might it be determined whether compound X causes demyelination or interferes with remyelination?

KEYWORDS

Neuron, Glia, Neurodevelopment, Behavior, Myelination, Heavy metal, Neurotransmission, Blood brain barrier

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34 Assessment of Male Reproductive Toxicity

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CONTENTS

INTRODUCTION

Reproduction is a complex process requiring exquisite integration of signals along the hypothalamic–pituitary–gonadal axis in both sexes. To be successful, functional gametes must be produced, matured, released, and transported effectively. Specific behavioral repertoires also must be executed precisely to ensure efficient mating and fertilization. Our current state of knowledge about the physiological, biochemical, and molecular orchestration of reproduction is captured in many excellent reference volumes [1–5]. Based on this understanding, male reproductive function would be expected to be impacted by chemicals that act through a variety of mechanisms, including disruption of the spermatogenic cycle in dividing cells (spermatogonia) as well as those undergoing meiotic processes (spermatocytes); damage to DNA, especially during DNA repair-deficient stages (condensed spermatids); and interference with a vast array of endocrine and paracrine signaling pathways vital to the regulation and integration of reproductive function.

Much of today's research in reproductive biology is motivated by the widely recognized need to understand both fertility and infertility in humans and wildlife so as to develop effective technologies for both contraception and treatment of infertility. Studying the extent to which industrial chemicals in the environment or therapeutic drugs may alter reproduction serves to facilitate our understanding of reproductive biology. Such understanding is essential if protective actions by regulatory agencies are to be based on sound science. The goal of this chapter is to summarize how information on the potential for chemicals to interfere with male reproductive capability is obtained and used by regulatory agencies, industry, and others.

The incidence of infertility is high in humans relative to many other species. It is estimated that 8% to 15% of couples seek medical treatment for infertility [6]. Although infertility is a function of the couple, in a sizable number of cases, the infertility can be attributable to deficiencies in the male. In fact it has been estimated that 98% of male subfertility cases result from deficits in sperm production (quantity) or sperm quality, while only 2% represent ejaculatory dysfunction [7].

In addition, male-mediated effects on offspring have been demonstrated [8,9]. The potential for the male to contribute to reproductive failure and adverse pregnancy outcomes is significant, but the causes of male infertility are only partially understood. We now know that genetic alterations (e.g., aneuploidy), chromosomal aberrations (e.g., translocations), and gene polymorphisms account for a significant proportion of male infertility cases [10–12]. It is inherently difficult to decipher the contribution of chemicals in the environment to the etiology of subfertility in men but despite difficulty, it is imperative to attempt to elucidate the contribution of environmental exposures because, unlike genetically based infertility, chemically induced infertility is presumably preventable.

An assessment of environmental risk factors is further complicated by the growing awareness that exposures during fetal and neonatal development have the potential to impact reproductive function during adulthood [13–15]. Accordingly, it is critical to consider the impact of exposures at all periods of the life cycle, not just on adults; therefore, male reproductive test protocols and epidemiological studies must be designed to evaluate critical windows of exposure and developmental bases of adult disease.

Whereas numerous environmental, occupational, and therapeutic agents have been identified as male reproductive toxicants in typical rodent models such as mice and rats, relatively few have been shown to cause similar effects in human males. This discrepancy is likely due to the reduced resolving power that is inherent in many human studies. Agents that have been shown to cause reproductive effects in men include heavy metals, chemotherapeutic agents, radiation, pesticides such as dibromochloropropane and ethylene dibromide, and other chemicals such as carbon disulfide, chloroprene, and 2-ethoxyethanol [16–18].

More is known about chemicals that are capable of causing adverse male reproductive system effects in animal models because experimental protocols are designed using controlled exposures over a range of doses [19]. The toxicology literature is rich in reviews of many studies relating male reproductive effects to exposures that target male germ cells at specific stages of development, the supporting Sertoli cells, and, importantly, the endocrine regulation of spermatogenesis and sperm maturation [3,4,20–24]. The outcomes of such exposures have included not only reduced fertility but also embryo/fetal loss, birth defects, cancer, and other postnatal structural or functional deficits.

Of particular relevance regarding animal toxicology studies is the growing awareness that exposures to chemicals that mimic or block endocrine signaling during fetal development can significantly alter male reproductive organ and tract development, sometimes with far-reaching impacts such as decreased sperm production or increased risk of testicular cancer later in life [13–15]. This literature indicates that, for certain agents, the developing male reproductive system could be the most affected or the most sensitive target.

Concerns about human male reproductive health have been elevated by reports of downward secular trends in sperm counts, as well as increases in incidences of male reproductive tract malformations (e.g., hypospadias and testicular maldescent) and testicular cancer observed during the latter half of the twentieth century [25–28]. It has been suggested that exposures during reproductive system development to environmental agents that mimic or antagonize endogenous hormones may be causally related to such effects and that low sperm counts are only part of a syndrome of testicular dysfunction in humans that also may include reproductive tract malformations such as hypospadias, cryptorchidism, and predisposition to testicular cancer [29,30]. These concerns prompted the U.S. Environmental Protection Agency (EPA) and international partners to design and launch a comprehensive, tiered testing program to screen for endocrine-active and endocrine-disrupting chemicals (EDCs) [31].

Any attempt to extrapolate effects observed in an animal model to humans must take into account that fertility of the

human male may be particularly susceptible to agents that reduce the number or quality of sperm produced. Whereas in some strains of mice and rats sperm production can be reduced by 90% before effects on fertility by natural mating are observed [32,33], the number of functionally normal sperm in men appears to be much closer to the threshold for reduced fertility. With that being the case, smaller decreases in sperm production in men could have serious consequences on their reproductive potential. If the number of normal sperm per ejaculate is sufficiently low and/or the number of qualitatively normal sperm is reduced, subfertility/infertility is likely to occur. The incidence of subfertility in men has been considered to increase at sperm concentrations below 20×10^6 sperm/mL of ejaculate [34]; nevertheless, some men with low sperm concentrations but otherwise healthy sperm are able to achieve conception, and many subfertile men have concentrations greater than 20×10^6, illustrating the importance of sperm quality. Results from a recent prospective study indicate that human conception rate may begin to decline when sperm concentrations fall below 60×10^6 sperm/mL [35]. Thus, it is reasonable to assume that reductions in sperm production and/or sperm quality by a toxic agent may further decrease the human male reproductive potential.

Ascertaining the relationships between environmental exposures and male reproductive risks for an informed risk assessment process requires not only a sufficient amount and type of experimental data but also a systematic approach to evaluating those data. The approach utilized by the U.S. EPA for male reproductive toxicity risk assessment [36] is presented in this chapter, with particular emphasis on a critical analysis of the strategies and endpoints that are available for male reproductive risk assessment. Although the focus of this chapter is on the assessment of reproductive risk in the male, it is important to recognize that studies that focus solely on the contribution of one parent, although valuable for better elucidation of path to phenotype in that parent, may either miss or substantially underestimate the true reproductive risk of a couple exposed to an environmental chemical. Thus, EPA's Guidelines for Reproductive Risk Assessment [36] is an initial study designed to thoroughly identify the potential for reproductive risk in either sex.

HAZARD CHARACTERIZATION AND DOSE–RESPONSE ASSESSMENT

A paradigm for the risk assessment process has been described in detail in two publications prepared by the National Academy of Sciences [37,38]. Although devised primarily for cancer risk assessment, many of the components also apply to the assessment of noncancer health effects such as reproductive toxicity. The major components of that paradigm are (1) hazard identification, (2) dose–response assessment, (3) exposure assessment, and (4) risk characterization. The EPA's Guidelines for Reproductive Toxicity Risk Assessment is best characterized by hazard characterization followed by quantitative dose–response analysis for risk assessments.

Protocol Selection

Testing protocols describe the procedures to be used to provide data for risk assessments. The quality and usefulness of those data are dependent on the design and conduct of the tests, including endpoint selection and resolving power. The most widely accepted comprehensive protocol is the multigeneration reproductive test [39]. This test detects reproductive toxicity in either sex and provides dose–response information but may not provide all of the information required for a comprehensive risk assessment. The protocol was revised in 1996 to add specific sperm measures (motility, morphology, and numbers) and is continually being evaluated for its effectiveness. It is noteworthy that recent recommendations would modify it further with the goal of enhancing its sensitivity to detect reproductive effects while expanding its scope to detect developmental immuno- and neurotoxicity [40]. The latter changes would also reduce the cost and number of animals necessary for noncancer health effects testing. The EPA has *harmonized* the multigenerational test protocol with those from other federal agencies such as the Food and Drug Administration (FDA) and international organizations such as the Organisation for Economic Co-operation and Development (OECD) to achieve uniformity of approaches at the international level [41]. Results of this protocol may indicate that modifications or follow-up testing should be conducted to determine the most affected sex or elucidate the path to phenotype underlying the observed toxicity [19]. Subacute or acute exposure protocols may be used to study the ontogeny of an effect or better confirm the path to phenotype.

Species Selection

It is intuitively obvious that data from human studies are desirable when estimating specific exposure levels below which there is no appreciable risk; however, human exposure data on single chemicals are rarely available, so it is necessary to extrapolate human risk from data derived from animal studies. Generally, the rat is the species of choice and the default species for reproductive toxicity testing. However, if pharmacokinetic and/or mechanistic data are available that suggest that the rat is not appropriate, a different species should be selected. In any case, confidence in the results of testing for male reproductive toxicity is increased when multiple species have been examined. Advantages of the rat as a test species include the availability of an extensive reproductive toxicity database for this species, uniformity of reproductive endpoints within strain, and consistently efficient reproductive performance. In addition, the basic mechanisms underlying male reproductive function in the rat are well researched and reasonably representative of those in human males.

For a second mammalian test species, the rabbit has specific advantages that make it a good choice. First, ejaculated semen can be collected from bucks using an artificial vagina, allowing longitudinal assessment of semen parameters. Second, by the feasibility of collecting semen ejaculates

TABLE 34.1
Duration (Days) of Spermatogenesis and Epididymal Transit to Provide Recommended Minimum Duration of Exposure in Various Test Species

Test Species	Rat	Mouse	Rabbit	Monkey	Dog	Human
One cycle of the seminiferous epithelium	12.9	8.9	10.7	9.5	13.6	16
Duration of spermatogenesis	59	41	48	43	61	72
Duration of epididymal transit	10	9	12.7	10.5	11.3	5.5
Minimum duration of exposure	69	50	61	54	72	78

(as opposed to cauda epididymal sperm only in rats), alterations in the accessory sex gland secretions can be assessed, and levels of toxicants and/or their metabolites can be measured in seminal fluid. This offers the opportunity to relate specific alterations in semen quality with levels of a given chemical in both rabbits and men. Furthermore, ejaculated semen samples can also be used for artificial insemination (AI). As discussed in detail later, the use of AI with a known number of sperm can be a highly effective strategy for detecting adverse effects on the sperm fertilizing ability in both rabbits and rats. Finally, the use of rabbits circumvent the strain differences associated with rats and has the additional advantage of detecting possible germ cell neoplasia following toxic exposures as this species, unlike rats, manifest germ cell tumors and their precursor lesion called carcinoma in situ (CIS) or intratubular germ cell neoplasia [42].

Under some circumstances, data from other mammalian or nonmammalian species may be appropriate for incorporation into human health risk assessments; for example, mice in which specific genes have been knocked out may make excellent models for elucidating the mechanisms of toxicant action. The use of invertebrate species (e.g., frogs, fish) has become increasingly important in the screening for potential EDCs; chemicals likely to affect all aspects of the ecosystem.

Dose Selection and Duration

To increase the likelihood of detecting toxicity, at least one relatively high, dose level and an array of endpoints are included in the study design. For toxicity testing, the test guidelines specify that the highest dose should produce systemic toxicity but not mortality. Dose–response assessment requires the generation of dose–response curves that adequately describe the increments in degree of effect as well as any changes in pattern of endpoints affected with changing dose level. Dose–response data should also include sufficiently low-dose levels such that a low level of response or no effect is produced. Spacing between doses is especially critical in dose–response assessment. If the gaps between dose levels are too large, the estimate of the lowest-observed adverse-effect level (LOAEL) could be too high, and the no-observed adverse-effect level (NOAEL) could be too conservative. Adequate coverage of a range of low doses is recommended for the testing of all suspected reproductive toxicants with at least one dose being less than 1000-fold the known or predicted maximum human exposure [43]. If a single chemical fails to alter a sensitive male reproductive endpoint at an exposure that is 1000-fold greater than a maximum human exposure, it is probably not a relevant reproductive health risk.

Adverse effects of a toxicant may not be observed in sperm or in fertility until at least 70 days of exposure has lapsed (Table 34.1). This is the length of time to cover the complete process of spermatogenesis (approximately 60 days) and epididymal sperm maturation (approximately 12 days) in the rat [44]. Damage that is limited to spermatogonial stem cells, for example, would not appear in cauda epididymal sperm or in ejaculates for 10 weeks in the rat. Regardless of species studied, the minimum exposure for an initial study to identify male reproductive toxicity should span the duration of spermatogenesis plus transit of sperm through the epididymis during maturation. The duration of one cycle of the seminiferous epithelium varies across species but the total duration of spermatogenesis is commonly equivalent to 4.5 cycles of the seminiferous epithelium. Duration of transit through the epididymis also varies widely among species [44–46] thereby necessitating different minimum durations of exposure for each test species.

This exposure recommendation assumes that levels and cumulative effects of the agent at the sites of attack reach steady state within one cycle of the seminiferous epithelium after initiation of treatment. If that assumption is not valid for an agent, the treatment period may need to be extended accordingly. In studies using shorter dosing periods, a prolonged follow-up may be necessary to determine the persistence of an effect, particularly effects on the earlier stages of spermatogenesis. So again, final assessments should be done on the above minimum exposure times. Knowledge of relevant pharmacokinetic and pharmacodynamic data can facilitate selection of doses, treatment duration, and interim, posttreatment assessments for shorter-duration studies.

Duration of Mating

In fertility testing, pairs of animals are cohabited for periods of time sufficient to ensure conception in healthy animals. This is generally 2 weeks in rats, which allows for at least two opportunities for normally cycling females to conceive. With natural mating, a large impact on sperm production (decreased quantity and/or quality) is necessary before an

adverse effect on fertility can be detected. Moreover, alterations in mating behavior or sperm number in a male may go undetected as a male has multiple mating opportunities during each estrus and the multiple estrous periods in a two week period. As a result, an adequate number of sperm may be ejaculated to ensure fertility even when the males have reduced sperm production as a consequence of treatment. During cohabitation, females should be examined daily for presence of seminal plugs or by vaginal lavage for evidence of mating. Females are usually separated from the male on the day following mating. This practice limits mating to one estrus but still allows numerous copulations during that estrus. Study designs that provide for only a single copulation can increase the sensitivity of fertility testing [47]. Control over the number of copulations is best done by maintaining the animals on a reverse light–dark schedule so estrus and matings occur during normal working hours. If sexually experienced rats are used, copulatory behavior can be rapidly and accurately monitored, with several males observed simultaneously [48].

Number of Animals

The number of animals per dose group in a toxicology study is determined by the number of animals expected to survive and yield data, the expected variation between animals in the endpoints to be examined, the magnitude of effect to be detected, and the level of probability selected for statistical significance. The number of animals required per treatment should be calculated by standard statistical methods as part of the study design process [49]. Estimates of the coefficients of variation for some parameters used for tests of the male rat reproductive system have been reported by several authors [50–52]. In general, when multiple endpoints are used in a study of male reproductive toxicity, 20 males per treatment are sufficient to detect effects. In tests designed to evaluate fertility via natural mating, it is often recommended to start with more males per treatment group to obtain 20 pregnancies per treatment, and some protocols specify mating two females per male. Given the inherent lack of sensitivity afforded by natural mating, it must be determined whether this increased numbers of animals is justified.

TESTING PROTOCOLS

Single- vs. Multigeneration Studies

Comprehensive reproductive toxicity studies in laboratory animals generally involve continuous exposure to a test substance and evaluation of reproductive capability for one or more generations. The objective is to detect effects on the integrated reproductive process as well as to study effects on the individual reproductive organs. The single-generation reproduction test evaluates effects of subchronic exposure of peripubertal and adult animals on reproductive organs and performance. In a multigenerational study, animals producing the first generation of offspring are considered the parental (P) generation, and the subsequent offspring generations

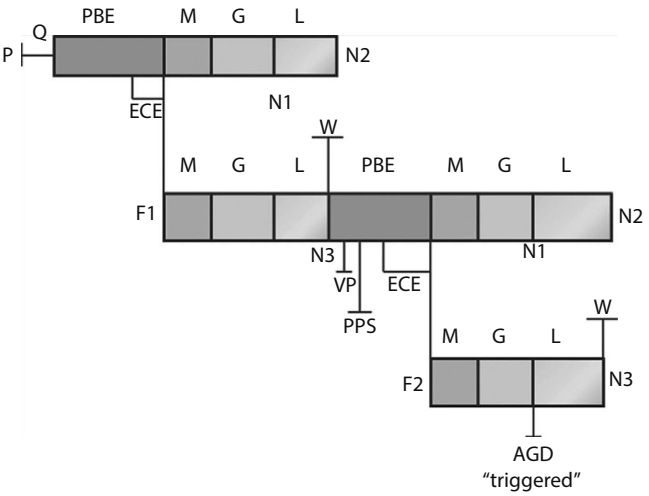

FIGURE 34.1 Schematic depicting the EPA's harmonized multigeneration reproduction test. Exposure in each generation continues until necropsy (N). Q, quarantine; PBE, prebreeding exposure (10 weeks); M, a 2-week mating; G, gestation; L, lactation; W, weaning on postnatal day 21; PPS, preputial separation monitored in F1 males from postnatal day 35; VP, vaginal patency monitored in F1 females from postnatal day 22; ECE, a 3-week evaluation of estrous cyclicity; AGD, anogenital distance measured in the F2 pups at birth if pubertal landmarks (PPS or VP) are affected in the F1; N1, necropsy of the parental males (assessments include organ weights, histology, and sperm measures); N2, necropsy of the parental females (assessments include organ weights and histology); N3, necropsy of three F1 and F2 weanlings per sex per litter (assessment is a macroscopic evaluation with organs weighed and preserved for histology).

are designated filial generations (e.g., F1, F2). Both the P and F1 generations are mated in a standard two-generation reproduction test.

According to the EPA's multigeneration protocol illustrated in Figure 34.1, both sexes of the P generation are dosed beginning at 5–9 weeks of age. This allows effects on spermatogenesis and sperm maturation in the epididymis to be expressed and increases the likelihood of detecting histologic lesions in the testis and epididymis. Both the P and F1 generations are exposed for 10 weeks (rats) prior to breeding. Dosing of the P and F1 males and females is continued until necropsy at the end of gestation and lactation, respectively. F1 and F2 offspring are exposed continuously during gestation and lactation until weaning on PND21. For F1 offspring, exposures are continued beyond weaning, through adulthood. This allows detection of effects that occur from exposures throughout reproductive development, from sexual differentiation through adulthood. Thus, males in the P, F1, and F2 generations have different exposure histories with P males exposed from puberty through adulthood, F1 males exposed throughout the differentiation and development of the male reproductive system, and F2 males exposed during sexual differentiation and early development. The study consists of three groups of increasing dose levels and a concurrent control group. Enough males and females are mated to ensure 20 pregnancies per dose group for each generation. Cohabitation

is terminated when evidence of mating is detected. Randomly selected F1 male and female offspring are dosed after weaning for at least 10 weeks. More than one litter may be produced from either P or F1 animals. Depending on the route of exposure of lactating females, it is important to consider that offspring may be exposed to a chemical by ingestion of maternal feed or water (diet or drinking water studies) as well as via the milk.

In single- and multigeneration reproduction tests, reproductive endpoints evaluated include visual examination of the reproductive organs, weights and histopathology of the pituitary (both sexes), testes, epididymides, male accessory sex glands, uterus, ovaries, and vagina. The parental and F1 animals are evaluated to obtain the number and quality of sperm produced, estrous cycle normality, number of ovarian primordial follicles, and pubertal landmarks associated with normal development of the reproductive system, including the age at vaginal opening in females and the age at preputial separation in males. In addition, anogenital distance (AGD), an endocrine-sensitive endpoint in pups, may be triggered in the F2 animals if developmental effects (e.g., delayed puberty) are observed in the F1 animals. Recent recommendations are to include AGD in an augmented test design and to increase the number of F1 and F2 pups maintained through weaning, puberty, and adulthood [52]. An evaluation of reproductive competence is standard for P and F1. Both male and female mating and fertility indices are calculated. Litters (and often individual pups) are weighed at birth and examined for the number of live and dead offspring, gender, gross abnormalities, and growth and survival to weaning.

Inclusion of testicular histopathology and sperm evaluations help risk assessors judge whether effects on fertility can be attributed specifically to the male, in which case the male alterations are used to set the NOAEL, LOAEL, and margin of exposure (MOE); however, identification of effects in one sex does not exclude the possibility that both sexes may have been affected adversely. Data from matings of treated males with untreated females and vice versa (crossover matings) are necessary to establish sex-specific dose–responses for specific effects.

An EPA workshop has considered the relative merits of one- vs. two-generation reproductive effects studies [53]. The participants concluded that a one-generation study is insufficient to identify all potential reproductive toxicants, because it would exclude detection of effects caused by prenatal exposure and unless exposure beginning prior to puberty also excludes peripubertal effects. A single-generation test would also miss effects on germ cells that could be transmitted to and expressed in the next generation, that is, epigenetic alterations. A one-generation test might also miss adverse effects with delayed or latent onset because of the shorter duration of exposure for the P generation. These limitations are shared with the shorter-term screening protocols described in the following. Because of these limitations, a comprehensive reproductive risk assessment should begin with a two-generation test or its equivalent.

In studies where parental and offspring generations are evaluated, additional risk assessment issues arise with regard to the relationships of reproductive outcomes across generations. Increasing vulnerability of subsequent generations is sometimes observed. Predictions of increased risk of subsequent generations could be strengthened by knowledge of the reproductive effects in the adult, the likelihood of bioaccumulation of the agent as duration of exposure is increased during reproductive development, and the potential for increased sensitivity resulting from exposure during critical periods of reproductive development [54], particularly exposure to endocrine-active agents [25]. An instructive example of the use and interpretation of multigenerational test data to evaluate the potential for effects to worsen across generations (or not) is provided by the Center for the Evaluation of Risks to Human Reproduction in its evaluation of bromopropane [55].

Several recent reports suggest that exposures during early phases of testis or prostate differentiation and development may alter the epigenetic programming of the organ, resulting in altered function during adulthood, including increased predisposition to cancer [56,57]. Such alterations may potentially be transmissible across subsequent generations, even in unexposed offspring. Such observations may impact both testing strategies and risk assessment approaches in the future.

On the other hand, subsequent generations may be less affected than the F1 offspring. This can occur when the F1 and F2 animals represent survivors who are (or become) more resistant to the agent than the P generation. Therefore, results between generations or between sequential litters within a generation should not necessarily be compared directly. Significant adverse effects in any generation should be considered a cause for concern unless inconsistencies in the data indicate otherwise.

A review of 20 positive multigeneration reproduction studies has provided some insights on the relationship of male toxicity to effects on offspring within a given generation and the relationships of reproductive outcomes across generations [58]:

- The presence of toxicity in the adult male (P), reproductive or otherwise, was not a prerequisite for the occurrence of effects on offspring.
- Approximately one-half of the studies were classified as *positive increasing*. In these cases, the second-generation (F1) animals exhibited effects that were more severe than those in the first generation or occurred at equivalent or lower doses.
- The increasing toxicity across generations is consistent for chemicals that bioaccumulate; however, exposure of sequential generations that involve different developmental stages (P vs. F1 adults) might also contribute to differential effects across generations.
- The multigeneration reproduction test does not address the issue of reversibility; however, inclusion of additional mated pairs in a study can provide additional animals for a reversibility test or developmental toxicity evaluation.

Subchronic Studies

Subchronic toxicity tests may have been conducted before a detailed reproduction study is initiated. In the subchronic toxicity test with rats, exposure usually begins at 6–8 weeks of age and is continued for at least 70 days. The initiation of exposure at 8 weeks of age (compared with 6) allows the animals to reach a more mature stage of sexual development and ensures an adequate length of dosing for observation of effects on the reproductive organs with most agents as discussed earlier. Dosing is often done orally (i.e., gavage, in diet, or in drinking water) but may be by inhalation or dermal application. Animals are monitored for clinical signs throughout the test and are necropsied at the end of dosing, without evaluating reproductive function. The endpoints evaluated for the male reproductive system include visual examination of the reproductive organs, plus weights and histopathology for the testes, epididymides, and accessory sex glands.

Scientists in the National Toxicology Program have examined the feasibility and value of incorporating some basic measures of male reproductive toxicity into the protocols of their standard 13-week, prechronic toxicity studies to serve as a reproductive screening battery. These include epididymal sperm morphology, quantitative evaluations of epididymal sperm motility, and enumeration of epididymal sperm reserves. Data were collected at the end of 50 13-week studies, of which 25 were conducted in mice and the remainder in rats [51]. In this evaluation, reproductive organ weights and sperm motility appeared to be the most statistically powerful endpoints. Based on the results of these studies, the authors recommended that multiple endpoints of spermatotoxicity be evaluated in screening tests. This test may be useful to identify an agent as a potential reproductive hazard but usually does not provide information about the integrated function of the reproductive systems (sexual behavior, fertility, and pregnancy outcomes) nor does it include effects of the agent on immature animals.

Short-Term Male Reproductive Studies

Although short-term tests (i.e., less than 70 days for the rat) have been proposed to screen chemicals for testicular toxicity, the risk of false negatives due to insufficient duration of exposure is high. A serious limitation, especially when using them for chemicals with unknown toxicity, is that effects of exposures during male reproductive development would not be evaluated. That said however, these tests are appropriate when prior information exists about the target organ (i.e., testis or epididymis) or if a chemical is suspected to alter spermatogenesis or epididymal sperm maturation based on a structure–function relationship with a known toxicant. Short-term tests are also of value, if not essential, in the elucidation of the path to phenotype, for example, determining which specific germ cells are compromised by exposure and how these cells might be altered to account for the observed phenotype (e.g., dysgenetic acrosomes). Because a wide variety of short-term tests have been used to assess reproductive toxicity, only a few examples are presented here.

Studies to Detect Toxicity in the Epididymis

This protocol was developed to identify toxicants that alter the structure or function of the epididymis within 5 days of toxicant exposure [59]. Based on the known transit rate of rat sperm through the epididymis, sperm that are within the proximal region of the epididymis at the onset of exposure (day 1) can be recovered from the proximal cauda epididymidis, the first site in which fertile sperm can be found, 4 days later (day 5). By limiting the exposure period in this manner, spermatids that would be within the testis at the onset of exposure are precluded from assessment. With toxicants that compromise testosterone production, it is possible to implant testosterone-filled Silastic® capsules to clamp serum testosterone at control levels. Also, the efferent ducts can be ligated to prevent toxicant-induced perturbations in testicular fluid from altering the epididymis. This protocol has been used to identify epididymal toxicity resulting from exposure to chloroethyl methanesulfonate [60], as well as epichlorohydrin and hydroxyflutamide [61]. To detect antiandrogenic effects from a chemical such as hydroxyflutamide, however, it is necessary to castrate the animals to lower endogenous androgen levels to the extent that competition of test chemical for the androgen receptor is effective. To prevent the loss of androgen-dependent function within the epididymis following castration, a testosterone-filled Silastic® capsule can be implanted to maintain androgen status at a level that maintains epididymal sperm maturation over 4 days [62]. A similar short exposure design has been used to identify sperm defects associated with cyclophosphamide-induced postimplantation loss [63], as well as infertility of epididymal sperm that results from the metabolic inhibitors ornidazole and α-chlorohydrin [64].

Importantly, one manifestation that seems to be common to the epididymal toxicants ethane dimethane sulfonate (EDS), chloroethyl methanesulfonate, hydroxyflutamide, and epichlorohydrin, as well as developmental exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and methoxychlor, is reduced epididymal sperm number without any concomitant reduction in testicular sperm number. This suggests that epididymal sperm transit is accelerated by certain chemical exposures. Indeed, when the short-term epididymal toxicity protocol was modified to test this hypothesis, accelerated transit was demonstrated [61]. The acceleration appeared to be independent of androgen status but was significantly correlated to several constitutive epididymal proteins. Accelerated sperm transit in humans, who have a much shorter period of epididymal transit, could have adverse effects on the process of sperm maturation and numbers of sperm ejaculated.

Studies to Detect Toxicity in the Testis

Linder et al. [65] originally proposed a short-duration test to screen chemicals for spermatotoxicity in structure–function studies or to set priorities for chemicals requiring further evaluation. Depending on the duration of dosing and the day on which necropsies are performed, the protocol may

cover a period of up to 2.5 weeks (e.g., dose for 5 days, necropsy 14 days later). The 14-day period allows for spermatids that are compromised at the onset of exposure to appear in the epididymis. In a validation study, groups of male rats were dosed for 1–5 days with 14 chemicals shown to produce minimal testicular effects in subchronic studies, and necropsies were performed 2–3 days and 13–14 days after dosing was terminated. Reproductive organ weights (testis, epididymis, seminal vesicle, and prostate), sperm counts (testicular and epididymal), and sperm motion parameters (computer-assisted sperm analysis [CASA]) were measured. Both the testes and epididymides were subjected to critical histopathologic evaluation. Spermatotoxicity was detected for the ten most potent testicular toxicants. Results for the other four chemicals, which were judged to be minimally toxic in a subchronic test, were essentially negative; thus, chemicals that produce moderate to severe damage to germ cells in latter stages of spermatogenesis are detectable with this short-duration test. More recently, this protocol has been abbreviated to encompass a 14-day exposure with a necropsy following administration of the last dose. With this, it has been established that the disubstituted haloacetic acid by-products of drinking water disinfection (i.e., dibromoacetic acid [66], dichloroacetic acid [67], and bromochloroacetic acid [68]) produce lesions in the latter stages of spermatogenesis. The histopathologic profile of these insults includes the formation of atypical residual bodies, fusion of sperm, and delayed spermiation.

Studies Using Other Animal Models

As discussed earlier, while testicular toxicity has been demonstrated in rat model following exposure to water disinfection by-products, comparable or even longer-term exposure to dibromoacetic acid did not produce any significant testicular toxicity in mice [69]. As species and strain differences among rodent animal models have become apparent, alternative animal models including nonrodent, amphibian, and aquatic animal models have been used to test testicular toxicity of environmental chemical pollutants. For example, when rabbits were chronically exposed to dibromoacetic acid in drinking water [70] or a mixture of common chemical contaminants in groundwater near hazardous waste sites [71], testicular toxicity with persistent sperm abnormalities such as vesiculated acrosomes and acrosomes shared between two or more sperm was observed even at relatively low levels.

Studies to Identify EDCs

In Vivo and In Vitro Screens

A battery of in vivo and in vitro tests has been proposed to fulfill congressional mandates to screen for EDCs. The Tier I in vivo tests to screen for alterations in male reproductive development in the rat are the Hershberger and pubertal assays [72,73]. The Hershberger assay attempts to identify androgenic or antiandrogen influences by a particular chemical. Young castrate male rats are used to increase the sensitivity of detecting a change in androgen status in sex accessory organs; the rationale is the same as that used earlier when testing for effects of antiandrogens in the adult epididymis, that is, lower endogenous androgen levels via castration. Androgen-dependent sex accessory organs are weighed following exposure to the putative EDC in groups of animals without or with concurrent exogenous androgen supplementation. Testosterone propionate is given daily during exposure when antiandrogenic activity is tested [74]. Castration, by removal of both the testis and the epididymis, is done at 6 weeks of age [75], around the time of preputial separation but before biological puberty, that is, the appearance of sperm in the epididymis is attained around 55 days of age (Klinefelter, unpublished). One week after castration, treatment groups are assigned on the 49th day and typically animals are necropsied between 60 and 70 days of age. Thus, there is typically a 2–3-week exposure period. The androgen-dependent organs that are evaluated include the glans penis, the levator ani, bulbocavernosus muscles, ventral prostate, and seminal vesicles with coagulating glands. It should be noted that this assay could be performed equally well if castration involved removal of only the testis. In this case, the epididymis would also be weighed.

The pubertal male assay is similar in concept except dosing begins just after weaning, on postnatal day 23, and continues until postnatal day 53 [76]. Two dose groups are required in addition to the vehicle control group. This assay is capable of detecting alterations due to wider array of toxicants including estrogenic chemicals, chemicals that affect steroid hormone biosynthesis directly, and chemicals that alter the hormonal status of the hypothalamic/pituitary axis [77–81]. In addition to weights of the sex accessory organs including the epididymis, various serum hormones (e.g., Follicle-stimulating harmone (FSH), Luteinizing harmone (LH) testosterone) are measured, and histology of the testis and epididymis is evaluated. Due to the increased exposure in this assay, subsequent testing is required to confirm target organ(s) implicated in the observed toxicity and its time course.

A slightly longer protocol, but more comprehensive protocol than either the Hershberger or pubertal assays, would initiate dosing during pregnancy and continue through puberty. This assay would have the advantage of detecting alterations in male sexual differentiation and early development of the gonad and accessory sex glands. Abnormalities such as cleft phallus, cryptorchidism, ectopic testes, epididymal agenesis, altered AGD, and retained nipples would be detected.

As indicated earlier, nonmammalian assays are now increasingly popular to screen for endocrine-active chemicals. Here again, the focus has been on agonistic/antagonistic activities of androgenic and estrogenic pathways. The fish short-term assay [82–85] measures many endpoints common to mammalian reproduction, evaluates both sexes simultaneously, and actually assesses fertility. Briefly, four females and two male fish are housed in a flow-through tank of water containing one of three test concentrations or a vehicle control. Exposures continue for 21 days at which time fish

evaluated for alterations in gonadal histology, hormones, and secondary sex characteristics; fertility is assessed throughout the exposure period.

There are five primary in vitro tests screen for chemicals that bind estrogen and androgen receptors or affect steroidogenesis by altering the steroidogenic enzymes, including aromatase. There are multiple androgen and estrogen receptor–based assays being utilized today. Competitive binding assays are used to ascertain whether a test chemical has significant affinity for either the androgen or estrogen receptor in vitro [86,87]. Certainly, before denoting any chemical as either an agonist or antagonist for androgen or estrogen receptor–mediated action, binding to the steroid receptor must be demonstrated. However, receptor binding alone does not constitute an alteration in receptor-mediated action. Once bound to the receptor, the steroid–receptor complex must undergo conformational change to interact with the hormone response element in a responsive gene to initiate transcription of that gene.

Cell-based transcriptional activation assays have evolved to evaluate the ability of a chemical to up- or down-regulate steroid receptor–mediated gene transcription. For these assays, cells are transfected with a plasmid containing a hormone response element and coupled to a reporter gene such as luciferase. Often, the cells used in these assays have no endogenous steroid receptor so a vector coding for the steroid receptor is also transfected. Both androgen [88]- and estrogen [89,90]-based assays have been developed. In general, transcriptional activation assay, both mammalian cell and yeast based, has proven less standardized than receptor binding assays [91], and neither assay is particularly well suited for testing chemicals requiring metabolic activation. One factor that is typically ignored in both of these in vitro assays is that the level of endogenous ligand used in the assay is rarely added at physiological levels.

Screening for the ability of chemicals to alter steroidogenesis has been done using minced testis cultures, as well as cultures of transformed or dedifferentiated cells. For the minced testis cultures, small aliquots of testis parenchyma are weighed and incubated for several hours with and without LH stimulation [92,93]. This method is applicable to ex vivo evaluation using parenchyma derived from testes (at any age animal) following in vivo exposure. The use of transformed cells such the mouse MA-10 Leydig cell lines is limited because these cells lack the enzymes required for conversion of progesterone to testosterone [94]. The rat R2C Leydig cell line lacks the LH receptor precluding detection of alterations in LH-stimulated testosterone production [95]. Human H295 cells have been used recently. These cells are dedifferentiated cells isolated from an adrenocortical carcinoma capable of producing cortisol, androgens, and estrogen [96]. However, these cells also do not express a functional ACTH receptor and cannot respond to LH. Most recently, mouse BLTK1 cells have been used [97]. These cells were obtained from a testis tumor that developed in transgenic mice expressing the inhibin promoter. This cell line appears to respond to LH and contains the requisite steroidogenic enzymes. The disadvantage of these cells is that the amount of testosterone they produce seems quite low compared to normal Leydig cells. However, this is true for all cell lines.

There is no reason that preparations of highly purified rat Leydig cells [98,99] discussed in the succeeding text could not be used for 96-well plate cultures to screen for the potential of chemicals to alter steroidogenesis by the Leydig cell directly. These cells respond quite well to LH, obviously have all the requisite steroidogenic molecules in play, and produce hundreds of nanograms of testosterone in only a few hours, thereby increasing sensitivity to detect subtle, chemical-induced compromise in testosterone synthesis.

Hypothesis-Based Testing

As pointed out earlier, the in vivo screens to detect EDC activity are not designed to provide data regarding target organ/cell or path to phenotype. Most of the EDC research published today represents hypothesis-based testing. For example, if a chemical is known to alter steroidogenesis in an adult animal or in an in vitro screening assay, one might consider a test to evaluate the potential for this chemical to alter testosterone throughout reproductive development. In this case, exposures would continue in utero, through puberty, and into adulthood. At the end of gestation, during the pubertal period, and as adults, the exposed males would be evaluated. This approach leads to literally dozens of studies done on phthalates. The early work established that phthalates were capable of significantly reducing testosterone production by the fetal testis [100]. This decrease in testosterone production was associated with aggregation of fetal Leydig cells and subsequently linked to postnatal phenotypic alterations such as cryptorchidism, agenesis of the epididymis, and hypospadias [101]. Gene expression studies later established that at higher exposures, key steroidogenic molecules were compromised [102]. Most recently, we have learned that Leydig cell aggregation occurs at much lower dose levels and this dysgenesis precedes the decreased ability of the fetal Leydig cell to produce testosterone [103]. This is simply one example of how sophisticated studies with more targeted approach can be utilized to gain a better understanding of the underlying toxicity resulting from a chemical exposure. And, a better understanding such as this leads to the discovery of molecular targets that might be applicable to human studies and facilitate a better human risk assessment.

Such hypothesis-based, target-oriented studies using nonrodent models are also necessary as the outcomes in other animal models might provide additional information than those observed in rodent models. For example, when exposed to dibutyl phthalate (DBP) [104], it was found that rabbits manifest germ cell atypia with implications for the development of germ cell cancer (see in the succeeding text under "Histopathological Evaluation" section) that was not reported in rats. Likewise, when *Xenopus laevis* frog embryos [105,106] were chronically exposed to DBP at very low levels, survival, development (metamorphosis), and spermatogenesis were impacted.

IN VIVO ENDPOINTS TO EVALUATE MALE REPRODUCTIVE TOXICITY

The following sections describe various endpoints that can reflect male reproductive toxicity and their use in risk assessment. A comprehensive assessment of male reproductive toxicity requires information on multiple endpoints that are capable of detecting the range of potential adverse effects. These should include measures of fertility and reproductive behavior. Because the fertility assessed by natural mating in rodents has limited sensitivity, endpoints should also be included that are capable of detecting effects on components of the male reproductive system that support those functions (e.g., production of normal spermatozoa and normal differentiation of the reproductive tract and external genitalia).

Alterations in these reproductive endpoints may be the result of direct or indirect toxicity to the male reproductive system. In either case, exposure to an agent has caused a reproductive effect and there may be cause for concern. Careful evaluation of the dose–response curves for the various target organs and effects will hopefully point to the most sensitive target organ/tissue. This organ/tissue then would be the focus for setting the NOAEL, LOAEL, or benchmark dose and estimating human health risk. Statistical analyses are important in determining the effects of a particular agent, but the biological significance of data is most important. When many endpoints are investigated, statistically significant differences may occur by chance. On the other hand, apparent trends with dose may be relevant biologically even though statistical analysis does not indicate a significant effect. In the following discussion, endpoints are identified in which significant changes may be considered adverse, but there needs to be concordance between statistically significant results and known biological relevance of these results. The following sections describe various male-specific endpoints of reproductive toxicity that are useful in assessing male reproductive risk.

Reproductive Organ Weights

Monitoring body weight during treatment provides an index of the general health status of the animals, and such information may be important for the interpretation of reproductive effects. Reduction in body weight or weight gain may reflect a variety of responses, including rejection of chemical-containing food or water because of reduced palatability, treatment-induced anorexia, or systemic toxicity. Less than severe reductions in adult body weight induced by restricted nutrition have shown little effect on the male reproductive organs or on male reproductive function [107,108]. When a relationship between a body weight decline and a significant effect on male reproductive organ weight is not apparent, it is not appropriate to dismiss the altered organ weight as secondary to systemic toxicity. So an alteration in a reproductive organ (or other reproductive measure for that matter) is still considered adverse if more modest body weight changes exist. In the presence of severe body weight depression (i.e., >10% relative to control), the altered reproductive endpoint may have resulted from a more generalized toxic effect and is not typically considered a specific, adverse reproductive outcome.

The male reproductive organs for which weights may be useful for reproductive risk assessment in adults include the testes, epididymides, pituitary gland, seminal vesicles (with coagulating glands), and prostate. Organ weight data may be presented as both absolute weights and as relative weights (i.e., organ weight to body weight ratios). Organ weight data may also be reported relative to brain weight because, subsequent to development, the weight of the brain usually remains quite stable [109]. Evaluation of data on absolute organ weights is important, because a decrease in a reproductive organ weight may be quite independent of body weight. Typically, at the lower effective dose levels of most potent reproductive toxicants, there is no change in body weight.

Normal testis weight varies only modestly within a given test species [110]. This relatively low interanimal variability suggests that absolute testis weight should be a precise indicator of gonadal injury; however, damage to the testes may be detected as a weight change only at doses higher than those required to produce significant effects in other measures of gonadal status [111–113]. This contradiction may arise from several factors, including a delay before cell deaths are reflected in a weight decrease. The simultaneous occurrence of germ cell death and Leydig cell hyperplasia may offset any observed change in testis weight, and blockage of the efferent ducts by cells sloughed from the germinal epithelium or the efferent ducts themselves can lead to an increase in testis weight due to fluid accumulation [114,115], again, an effect that could offset the effect of germ cell depletion on testis weight. Nonetheless, a significant increase or decrease in testis weight in the absence of a comparable body weight change is indicative of an adverse effect.

Pituitary gland weight can provide valuable insight into the reproductive status of the animal; however, the pituitary gland contains cell types that are responsible for the regulation of a variety of physiologic functions, including some that are separate from reproduction, so changes in pituitary weight may not necessarily reflect reproductive impairment. If weight changes are observed, gonadotropin-specific histopathologic evaluations may be useful in identifying the affected cell types. This information may be used then to judge whether the observed effect on the pituitary is related to reproductive system function and therefore an adverse reproductive effect.

The weights of the epididymis, the prostate, and the seminal vesicles are androgen dependent and may reflect changes in the animal's endocrine status or testicular function. Separation of the seminal vesicles and coagulating gland is difficult in rodents; however, the seminal vesicle and prostate can be separated and results may be reported for these glands separately or together, with or without their secretory fluids. From a developmental perspective, the epididymis and seminal vesicles are considered to be testosterone-dependent organs, while the prostate is considered to be dependent on

dihydrotestosterone [116]. Because the developing seminal vesicles and prostate respond to hormone stimulation differently, these organs should be examined separately in any developmental study.

Significant changes in absolute male reproductive organ weights constitute an adverse reproductive effect. Such changes also may provide a basis for obtaining additional information on the reproductive toxicity of that agent, but significant changes in other important endpoints that are related to reproductive function may not be reflected in organ weight data. For this reason, the lack of an organ weight effect should not be used to negate significant changes in other endpoints that may be more sensitive.

Histopathological Evaluation

Histopathologic evaluations of test animal tissues have a prominent role in male reproductive risk assessment. Organs that are often evaluated include the testes, epididymides, prostate, seminal vesicles (often including coagulating glands), and pituitary. Tissues from lower dose exposures are often not examined histologically if the high dose produced no difference from controls. Histologic evaluations can be especially useful by (1) providing a relatively sensitive indicator of damage; (2) providing information on toxicity from a variety of protocols; (3) with short-term dosing, providing information on site (including target cells) and extent of toxicity; and (4) indicating the potential for recovery.

The quality of the information presented from histologic analyses of spermatogenesis is improved by proper fixation and embedding of testicular tissue. With adequately prepared tissue, a description of the nature and background level of lesions in control tissue, whether preparation induced or otherwise, can facilitate interpreting the nature and extent of the lesions observed in tissues obtained from exposed animals [117–120].

Many methods of histopathologic evaluations of the testis only record lesions if the germinal epithelium is severely depleted or degenerating, if multinucleated giant cells are obvious, or if sloughed cells are present in the tubule lumen. More subtle lesion, such as atypical germ cells, missing types of differentiating germ cells, or retained spermatids that can significantly affect spermiation may not be detected when testicular tissue is not optimally processed for histopathology. Also, familiarity with the intricacies of morphology of the testis and the kinetics of spermatogenesis of each test species is essential for the identification of less obvious lesions that may accompany lower dose exposures or lesions that result from short-term exposure [119]. Several approaches for qualitative or quantitative assessment of testicular tissue are available that can assist in the identification of less obvious lesions that may accompany lower dose exposures, including the use of the technique of staging. A text atlas that provides extensive information on tissue preparation, examination, and interpretation of observations for normal and high-resolution histology of the germinal epithelium of rats, mice, and dogs is available [119]. Also, a decision-tree scheme for staging rat spermatogenic cycle has been published [121].

Cell staging is based on the examination of cross sections of the seminiferous tubules. The proliferation and differentiation of germ cells is a highly ordered, time-dependent process; thus, the temporal and spatial relationships of the various spermatogenic cell types can be defined for the different stages of the spermatogenic cycle (Figure 34.2) [119,121]. Based on differences in light absorption of differentiating germ cells, transillumination has also proven useful for isolating segments of rat seminiferous tubules at specific stages for biochemical analyses [122]. Knowledge of the cytoarchitecture of the testis can allow identification of specific lesions that have resulted from toxicity to the germ cell at a given stage of development.

Quantification of cell staging may include analysis of frequency distributions of the cell stages present or the proportion of tubules that have identifiable stages with all expected cell types [119,121]. The cell-staging approach is being applied more frequently in the evaluation of environmental agents [123–130]. It is not, however, required for routine toxicology testing.

Similar to rodent animal models, kinetics and staging criteria for less frequently used animal model, the rabbit, have also been well documented and have been used to delineate the nature of seminiferous epithelial lesions in toxicological studies [131,132]. As a valid alternative vertebrate reference system for comparative analysis with higher vertebrates, frogs and fishes also constitute good animal models because of the simplicity of their testicular organization and cystic pattern of spermatogenesis; in fact, a nomenclature for fish germ cells to improve the comparability with higher vertebrates has been proposed [133,134]. These animal models perhaps simulate polluted aquatic environmental condition better than mammalian models. It has been demonstrated that simple, yet thorough, qualitative as well as quantitative evaluation of spermatogenic effects can be performed following exposure to ubiquitous waterborne toxicants such as phthalates (Figure 34.3) [106].

Morphometry is a term applied to a variety of specialized techniques to obtain quantitative data on cellular or organelle characteristics [111,119,135]. The methods may be applied to measure diameters, areas, or volumes of testicular compartments (e.g., tubular vs. interstitial), specific cell types, or subcellular structures. Cell counts may also be obtained as well as ratios of one cell type to another (e.g., pachytene spermatocytes to Sertoli cell). Although not required in test protocols, these specialized methods can be used in mechanistic studies, which, in turn, can inform the risk assessment. Aside from an evaluation of the seminiferous epithelium, the interstitium of the testis also warrants a careful evaluation. Some reproductive toxicants can result in Leydig cell hyperplasia, which may or may not progress to adenoma formation. While most, if not all, of the discussion to date regarding Leydig cell hyperplasia and its association with Leydig cell adenoma formation has centered around mechanisms of toxicant-induced hyperplasia in the adult testis [136,137], it is possible that the fetal or prepubertal Leydig cell is uniquely capable of becoming hyperplastic and that the mechanisms of initiation may

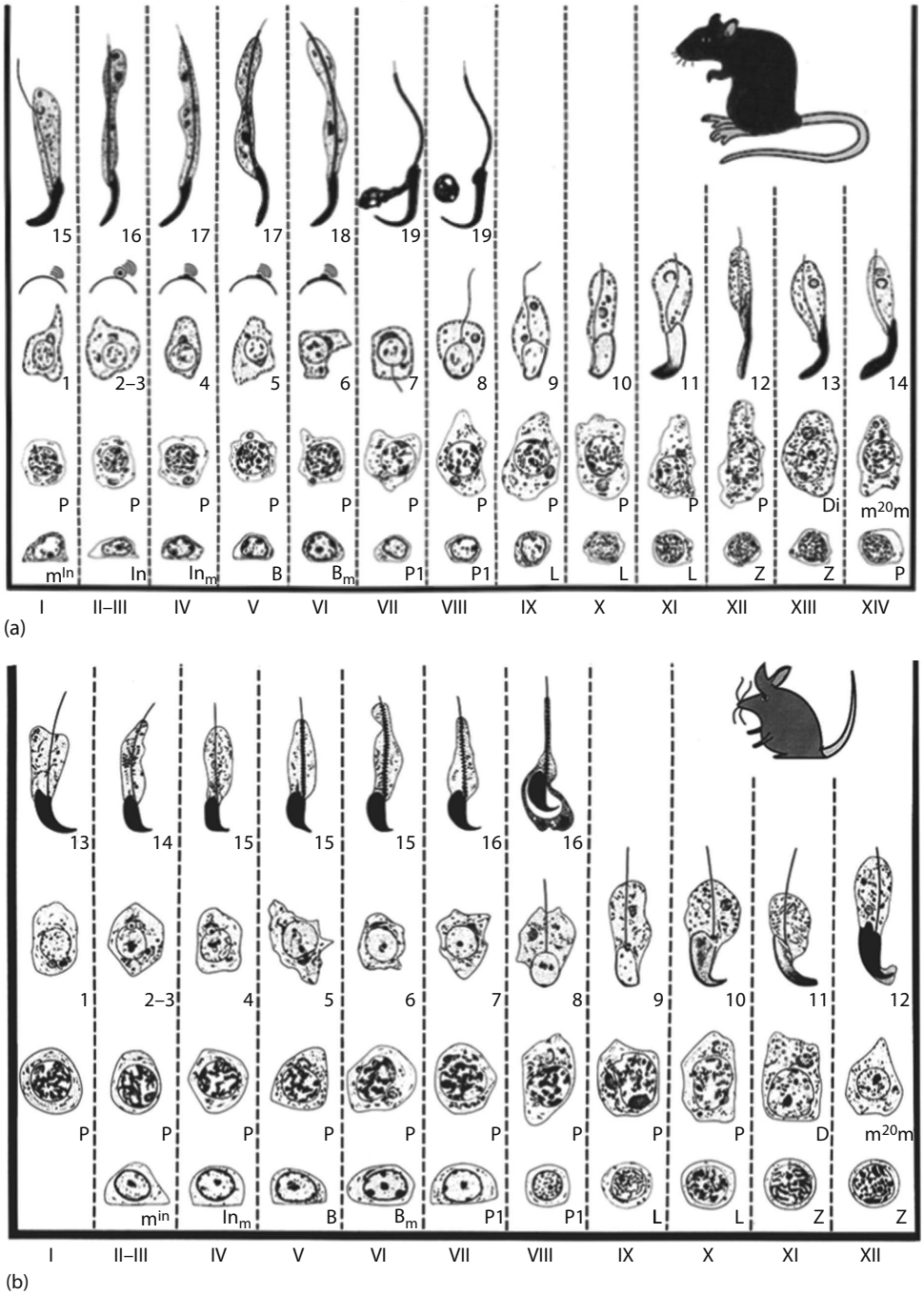

FIGURE 34.2 Cycle maps of spermatogenesis for the rat (a) and the mouse (b). The vertical columns, designated by Roman numerals, depict cell associations (stages). In the scheme provided, stages II and III are combined into a single stage called II–III. The developmental progression of a cell is followed horizontally until the right-hand border of the cycle map is reached. The cell progression continues at the left of the cycle map one row up. The cycle map ends with the completion of spermiation. The symbols used designate specific phases of cell development. (Reprinted from Russell, L.D., Ettlin, R., Sinha Hikim, A.P., and Clegg, E.D. et al., *Histological and Histopathological Evaluation of the Testes*, Cache River Press, Clearwater, FL, 1990. With permission of Cache River Press.)

differ. Transgenic mice that are deficient in anti-Mullerian hormone exhibit Leydig cell hyperplasia [138]. In this regard, gestational exposures to phthalates have been associated with an increased postnatal incidence of Leydig cell hyperplasia and adenoma formation [139,140], respectively.

Although adenoma formation does bear clinical significance, the relevance of Leydig cell hyperplasia alone, as a reproductive effect in test species, remains controversial [137]. Numerous modes of action have been postulated for induction of Leydig cell hyperplasia, including androgen receptor antagonists, testosterone biosynthesis inhibitors, aromatase inhibitors, and estrogen agonists. Each of these modes of action would result in a change in the testosterone-to-estradiol ratio within the Leydig cell. Even though the importance of

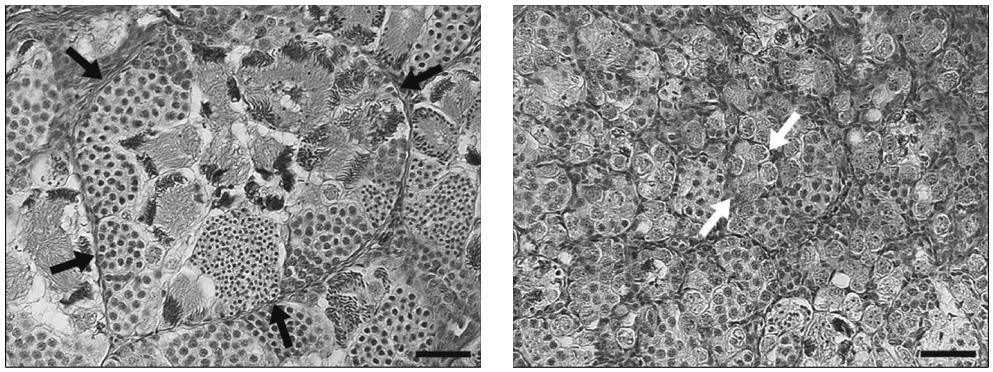

FIGURE 34.3 A typical seminiferous tubule cross section from a normal adult (33-week-old) African clawed frog, *Xenopus laevis* (a), is shown in contrast to regressed seminiferous tubules in an age-matched experimental animal (b) exposed to 10 ppm DBP beginning at sexual differentiation (Nieuwkoop and Faber stage 52; 3 weeks of age) continuing until metamorphosis (stage 66; 8 weeks of age). The number of germ cells in a nest in any stage of spermatogenesis is conspicuously less in the DBP-exposed animal compared to the control animal. Note the number and variety of cell nests that constitute a seminiferous tubule showing the pattern of organization and progression of spermatogenesis in *Xenopus laevis* in contrast to a mammal. This unique pattern of seminiferous tubular architecture in frogs makes it easier to evaluate the impact of toxicants on the process of spermatogenesis relative to commonly used laboratory animal models. Both images were photographed at the same magnification. Bar = 50 μm.

this ratio has been demonstrated in recent years [141], its direct association with Leydig cell hyperplasia has not been demonstrated. Moreover, few studies have demonstrated Leydig cell number in a definitive fashion [140,142]. There is really no way to confirm an increase in number of Leydig cells in the testis without performing a thorough morphometric analysis in which the number of Leydig cells are enumerated using Leydig cell–specific probes (i.e., antibodies that recognize steroidogenic enzymes specific to Leydig cells).

In utero exposure to the phthalates has been linked to fetal Leydig cell dysgenesis, specifically the formation of clusters of fetal Leydig cells [100,143]. Using the Leydig cell–specific marker, 3β-hydroxysteroid dehydrogenase, and the Sertoli cell marker, anti-Mullerian hormone, Mahood et al. [144] reported that fetal Leydig cells get trapped in seminiferous cords during cord formation and these Leydig cells persist in the seminiferous epithelium at adulthood. Similarly, Sertoli cells and/or germ cells were noted within the clusters of fetal Leydig cells in the interstitium. A morphometric study of Leydig cells within testes before and during pubertal, that is, progenitor and immature Leydig cells, and in the adult testis following in utero exposure to phthalates has not yet been performed. Leydig cell hyperplasia may occur after birth since prepubertal exposure to di(2-ethylhexyl)phthalate (DEHP) was shown to result in hyperplasia in adults [140]. If studied adequately, Leydig cell dysgenesis may become one of the early phenotypes associated with the testicular dysgenesis syndrome (TDS) in humans, which encompasses decreased sperm quantity/quality, hypospadias, cryptorchidism, and testicular germ cell cancer [145], assuming that Leydig cell dysgenesis occurs in humans exposed to phthalates.

Testicular dysgenesis has been linked to abnormal male reproductive development or masculinization that is now known to be dependent on testosterone as well as anti-Mullerian hormone and insulin-like factor 3 (Insl3) [15]. Indeed, Insl3 mRNA and protein have recently been shown to be decreased in cryptorchid testes of rats exposed to selected phthalate esters during gestation [146,147]. Cryptorchid testes frequently manifest testicular CIS cells prior to frank formation of germ cell tumors. Recently, light and electron microscopic evaluations have revealed that the driving force in the progression to CIS cells is the chemical nature of the toxic insult and not the abdominal location of the testis itself [148]. It is interesting that those chemicals that were shown to produce a progression from cryptorchid to CIS cell–containing testes—1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethylene (DDE) and estradiol—would each alter the normal testosterone-to-estrogen ratio during reproductive development. It should be noted that the manifestation of CIS and CIS-like lesions has been documented only in the rabbit testis either spontaneously [149] or following intentional exposures to DBP [104] or 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) [150]. The morphological features of testicular CIS in rabbits duplicate CIS lesions encountered in deer [151], horses [152], and humans [153–155]. Testicular CIS is not known to occur in rats emphasizing the importance of use of nonrodent animal models in studying testicular dysgenesis and testicular cancer (Figure 34.4) [42,156].

The basic morphology of other male reproductive organs (e.g., efferent ducts, epididymides, accessory sex glands, and pituitary gland) has been described as well as the histopathologic alterations that may accompany certain disease states [137–160]. Compared with the testes, less is known about structural changes in these tissues that are associated with exposure to toxic agents. With the excurrent ducts and accessory sex glands, histologic evaluation is usually limited to the height and possibly the integrity of the secretory epithelium. Evaluation should include information on the efferent ducts and caput, corpus, and cauda segments of the epididymis. The presence of debris and sloughed cells in

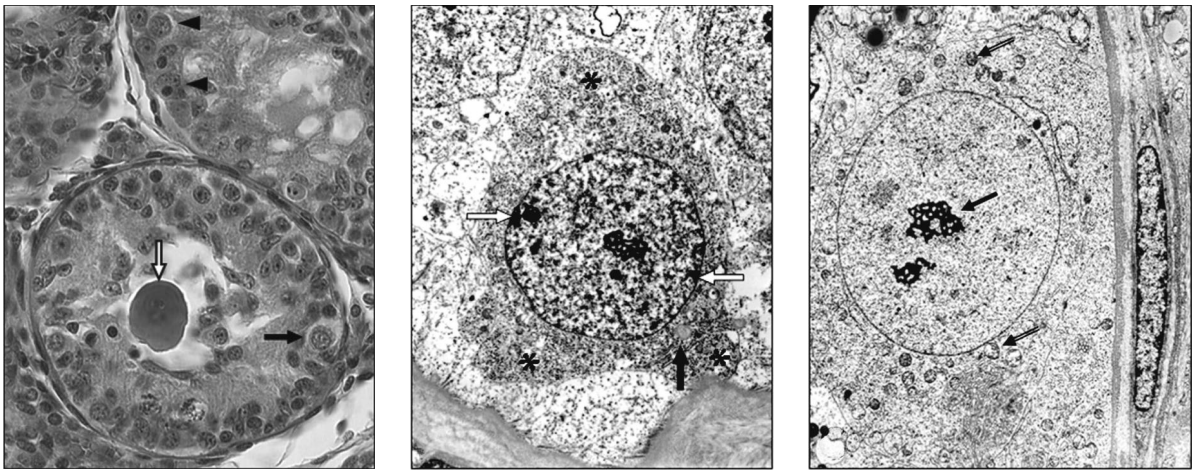

FIGURE 34.4 Light and transmission electron micrographs of rabbit testis showing germ cell atypia resembling CIS or intratubular germ cell neoplasia in humans. (a and b) Testes of 24-week-old rabbits exposed to DDT or a mixture of DDT and vinclozolin in utero and during the first 4 weeks of life. (a) Atypical germ cells resembling CIS (arrow heads and black arrow; ultrastructure of a cell similar to the one pointed by black arrow is shown in panel b). The CIS cell cluster indicated by arrow heads have irregular nuclear contours, and the large, gonocyte-like CIS cell (black arrow) has glycogen and lipid inclusions that can be discerned at ultrastructural level in panel b. A concretion or microlith (black arrow), commonly associated with neoplastic changes, is located in the lumen of the seminiferous tubule. (b) A CIS cell. Characteristic features include abundant aggregates of glycogen (asterisks) in the cytoplasm, lipid droplets (black arrow), and meandering nucleoli with fragments of nucleolonema (white arrows) adhering to nuclear membrane. (c) Atypical gonocyte resembling the precursor of CIS in the testis of a 25-week-old rabbit exposed to dibutyl phthalate. Cellular atypia ranged from alterations in the nucleolonema solid arrow to swollen mitochondria (arrowheads) in addition to accumulation of lipid and irregular membranous profiles in cytoplasm. These ultrastructural features are not typical of normally differentiating germ cells and have been attributed to neoplastic germ cells in humans (see text for references documenting identical electron microscopic profiles).

the epididymal lumen is a valuable indicator of damage to the germinal epithelium or the excurrent ducts. The presence of lesions such as sperm granulomas, leukocyte infiltration (inflammation), or the absence of clear cells in the cauda epididymal epithelium should be noted. Information from examinations of the pituitary gland should include evaluation of the morphology of the cell types that produce the gonadotropins and prolactin.

Historically, the degree to which histopathologic effects are quantified has been limited to classifying animals, within dose groups, as either affected or not affected using qualitative criteria. Little effort was made to quantify the extent of injury, and procedures for such classifications were not applied uniformly. This prompted the development of improved methods and more uniform approaches for evaluating the seminiferous epithelium [118–120]. These efforts have reinforced the importance of quantifying the extent of histopathologic damage per individual and have established high-quality histopathology as a sensitive and value-added endpoint in reproductive toxicity testing.

With proper tissue preparation and analysis, data from histopathologic evaluations provide a relatively sensitive tool that is useful for the detection of low-dose effects. Furthermore, changes in testis histology provide insights regarding the sites and mechanisms of action for the agent on that reproductive organ. When similar targets or mechanisms exist in humans, the basis for interspecies extrapolation is strengthened. Depending on the experimental design, information can also be obtained that may allow prediction of the eventual extent of injury and degree of recovery in that species and humans [161].

Significant and biologically meaningful histopathologic damage in excess of the level seen in control tissue of any of the male reproductive organs should be considered an adverse reproductive effect. Significant histopathologic damage in the pituitary gland should be considered as an adverse effect, but they should be shown to involve cells that control gonadotropin or prolactin production to be considered a reproductive effect. Although thorough histopathologic evaluations that fail to reveal any treatment-related effects may be quite convincing, consideration should be given to the possible presence of other testicular or epididymal effects that are not detected histologically (e.g., genetic damage to the germ cell, decreased sperm motility) but may affect reproductive function.

Sperm Evaluations

The EPA harmonized reproductive test guidelines [39] call for evaluation of epididymal (or vas) sperm number, sperm morphology, and sperm motility. Data on these parameters provide an estimation of both sperm quantity and quality and are therefore more informative than sperm numbers alone. Decreased sperm production will be reflected by reductions in both the number of homogenization-resistant spermatids in the testis and the number of sperm stored in the cauda

epididymidis. Likewise, alterations in sperm morphology or motility can be a reflection of a testicular insult as well as altered maturation in the epididymis. Data on sperm quantity and quality can be obtained noninvasively by collecting serially timed ejaculates from rabbits, thus enhancing the ability to confirm effects seen in rodents and establishing the feasibility to detect such effects in humans. Note that epididymal sperm measures in rodents are relatively consistent among animals of the same strain, whereas comparable measures in human semen are highly variable both between and within men [35]. Brief descriptions of these measures in rats are provided in the following, followed by a discussion of various additional sperm measures that are useful in a comprehensive male reproductive risk assessment.

Sperm Number

Sperm count has been frequently reported in the literature on humans [162]. Sperm number from test species may be derived from testicular, epididymal, or ejaculated samples [163]. Of the common test species, ejaculates can only be obtained readily from rabbits or dogs; however, ejaculates can be recovered from the reproductive tracts of mated females of other species including the rat. Indeed, by using hormonally primed, receptive females and limiting mating to only a few hours, numbers of ejaculated sperm can be recovered from the uterus of the mated females to differentiate the effects a chemical may have on mating behavior versus sperm number in the epididymis [164]. In this study, the chemical denervation induced by guanethidine exposure resulted in infertility that was linked to a deficit in mating behavior rather than to alterations in epididymal quantity or sperm quality.

Ejaculated sperm number from any species is influenced by several variables, including the length of abstinence and the ability to obtain the entire ejaculate. Intra- and interindividual variation is often high but is reduced somewhat if ejaculates are collected at regular intervals from the same male, as can be done with the rabbit [165]. Likewise, repeated measures study designs in epidemiology studies can improve detection sensitivity so fewer subjects are required [166]. When a preexposure baseline is obtained for each man, then changes during exposure or recovery can be better defined because each individual serves as his own control or baseline [167]. Unfortunately, in most epidemiology studies, abstinence is not strictly controlled and often only a single ejaculate is collected per man.

In toxicity testing using rats, cauda epididymal sperm are enumerated to determine the epididymal sperm reserves. While samples for sperm motility and morphology may be derived from the vas deferens, the cauda epididymal sperm sample can be used for all evaluations of sperm quality including fertility via in utero insemination (IUI) [168]. In this manner, various endpoints such as sperm number, sperm motility, sperm morphology, the sperm membrane proteome, and fertility can be correlated from a given population of sperm from an individual animal (Figure 34.5).

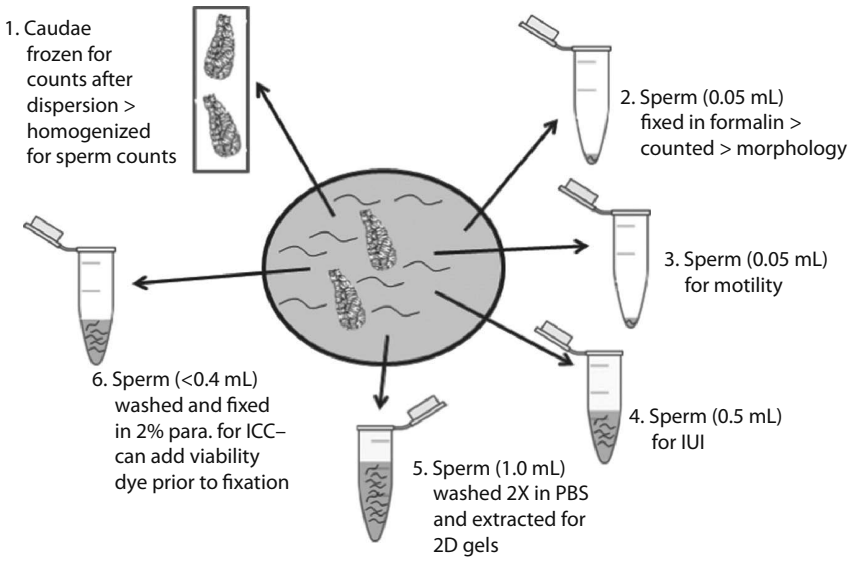

FIGURE 34.5 Diagram illustrating how multiple quantitative and qualitative endpoints can be obtained from a given population of sperm from an individual rat. Two caudae are placed in 2 mL of an isolation buffer [170], poked several times with a scalpel blade and sperm are allowed to disperse. (1) After several minutes of dispersion, the two caudae are frozen for subsequent homogenization to enumerate cauda sperm. (2) A fixed volume of the dispersed sperm is fixed in formalin, counted, and slides are prepared for an evaluation of morphology. For a total cauda sperm count, the sperm count obtained from this aliquot is corrected for 2 mL and added to the sperm number obtained from the cauda tissue homogenate. (3) Another aliquot of sperm is further diluted for an evaluation of sperm motion parameters. (4) Another, larger aliquot of sperm is used for IUI, typically 5 million cauda sperm are inseminated per horn in approximately 0.1–0.2 mL. (5) 1.0 mL is washed in buffer and the sperm extracted in detergent for subsequent analysis of the membrane proteome by 2D gel electrophoresis. (6) Remaining sperm are washed and fixed for immunocytochemical staining; these sperm also can be treated with a viability dye prior to fixation enabling dual fluorescent imaging for both viability and membrane marker of interest.

Automated counting has improved the efficiency with which this endpoint can be obtained [169]. It is not recommended to express the epididymal sperm count relative to the weight of the cauda epididymidis because sperm contribute very little to epididymal weight and ratio data may actually mask declines in sperm number. Thus, absolute sperm counts can improve resolution for detecting an effect. As is true for ejaculated sperm counts, epididymal sperm counts are influenced directly by the level of sexual activity [170].

As indicated, an estimate of sperm production in the testis may be derived from counts of the distinctive elongated spermatid nuclei that remain intact after homogenization of testes in a detergent-containing medium [170–172]. These elongated spermatid counts are a measure of sperm produced from spermatogonial stem cells [32]. If an evaluation was conducted when the effect of a lesion would be reflected adequately in the spermatid count, then spermatid count may serve as a substitute for quantitative histologic analysis of sperm production [120]. However, spermatid counts may be misleading if the duration of exposure (e.g., 14 days) is shorter than the time required for a spermatogonial stem cell to develop into an elongated spermatid (59 days in the rat). Also, spermatid counts reported from some laboratories have large coefficients of variation that may reduce the statistical power and thus the usefulness of that measure.

Sperm Morphology

Sperm morphology refers to structural aspects of sperm and can be evaluated in cauda epididymal, vas deferens, or ejaculated samples. A thorough morphologic evaluation identifies abnormalities in the sperm head and flagellum. Because of the suggested relationship between an agent's mutagenicity and its ability to induce abnormal sperm, sperm head morphology has been a frequently reported sperm variable in toxicology studies on test species [173]. The tendency has been to conclude that increased incidence of sperm head malformations reflects germ cell mutagenicity; however, not every mutagen induces sperm head abnormalities, and other nonmutagenic chemicals may alter sperm head morphology. For example, microtubule poisons may cause increases in abnormal sperm head incidence, presumably by interfering with spermiogenesis, a microtubule-dependent process [174]. Sperm morphology also may be altered due to degeneration subsequent to cell death; thus, the link between sperm morphology and mutagenicity is not consistent.

An increase in abnormal sperm morphology has been considered evidence that the agent has gained access to the germ cells [175]. There is evidence that stem germ cells (spermatogonia) are indeed targeted as occurrence of some unique sperm abnormalities, namely, acrosomal dysgenesis characterized by vesiculation and sharing between two or more spermatids was found to perpetuate long after cessation of exposure to a test chemical (lasting beyond more than at least one duration of spermatogenesis) [42]. These ramifications of experimentally induced testicular dysgenesis in rabbits arch across exposures to a variety of environmental agents including common chemical contaminants in drinking water, water disinfection by-products, and pesticides such as vinclozolin. Interestingly, similar unique sperm defects also have been observed in clinically subfertile stallions; and once observed on breeding soundness evaluation, these defects continue to occur over a lifetime indicating a possible permanent effect on stem germ cells [176]. Perhaps these domesticated animals are unwittingly exposed in feed and drinking water to the very chemical contaminants that the rabbits were exposed to in experimental paradigms (Figure 34.6).

Exposure of males to toxic agents may lead to sperm abnormalities in their progeny [177,178]; however, transmissible germ cell mutations might exist in the absence of any morphologic indicator such as abnormal sperm. The relationships between these morphologic alterations and other karyotypic changes remain uncertain [179].

The traditional approach to characterizing morphology in toxicologic testing has relied on subjective categorization of sperm head, midpiece, and tail defects in either stained preparations by bright field microscopy [180] or fixed, unstained preparations by phase contrast microscopy [163]. Such approaches may be adequate for mice and rats with their distinctly angular head shapes. Because human sperm exhibit considerable heterogeneity of structure and categorizing normal sperm involves subjectivity, the World Health Organization (WHO) has provided consensus guidance on classification of sperm morphology [34] and emphasizes that reference values relating this outcome to fertility depend largely on the strictness with which a normal sperm is defined. Data that categorize the types of abnormalities observed and quantify the frequencies of their occurrences may provide more information in a toxicology or epidemiology study than simply quantifying the percentage of normal sperm.

In comparison with other sperm measures, sperm morphology is a relatively stable endpoint in humans and test species. This feature may enhance its use in the detection of spermatotoxic events. The majority of studies in test species and humans have suggested that abnormally shaped sperm may not reach the oviduct or participate in fertilization [181,182]. The implication is that the greater the number of abnormal sperm or the smaller the number of normal sperm in the ejaculate, the greater the probability of reduced fertility. A prospective human male fertility study [35] has reported that the number of normal sperm (by strict criteria [183] in ejaculates) was highly related to the probability of couples achieving pregnancy within one year.

Sperm Motility

The biochemical environments in the testes and epididymides are highly regulated to ensure proper development and maturation of the sperm and the acquisition of critical functional characteristics such as progressive motility and the potential to fertilize [184]. With chemical exposures, perturbation of this balance may occur, producing alterations in sperm properties such as motility. Chemicals (e.g., epichlorohydrin) have been identified that selectively affect epididymal sperm motility and also reduce fertility [185]. Rat sperm motility is an established male reproductive toxicology

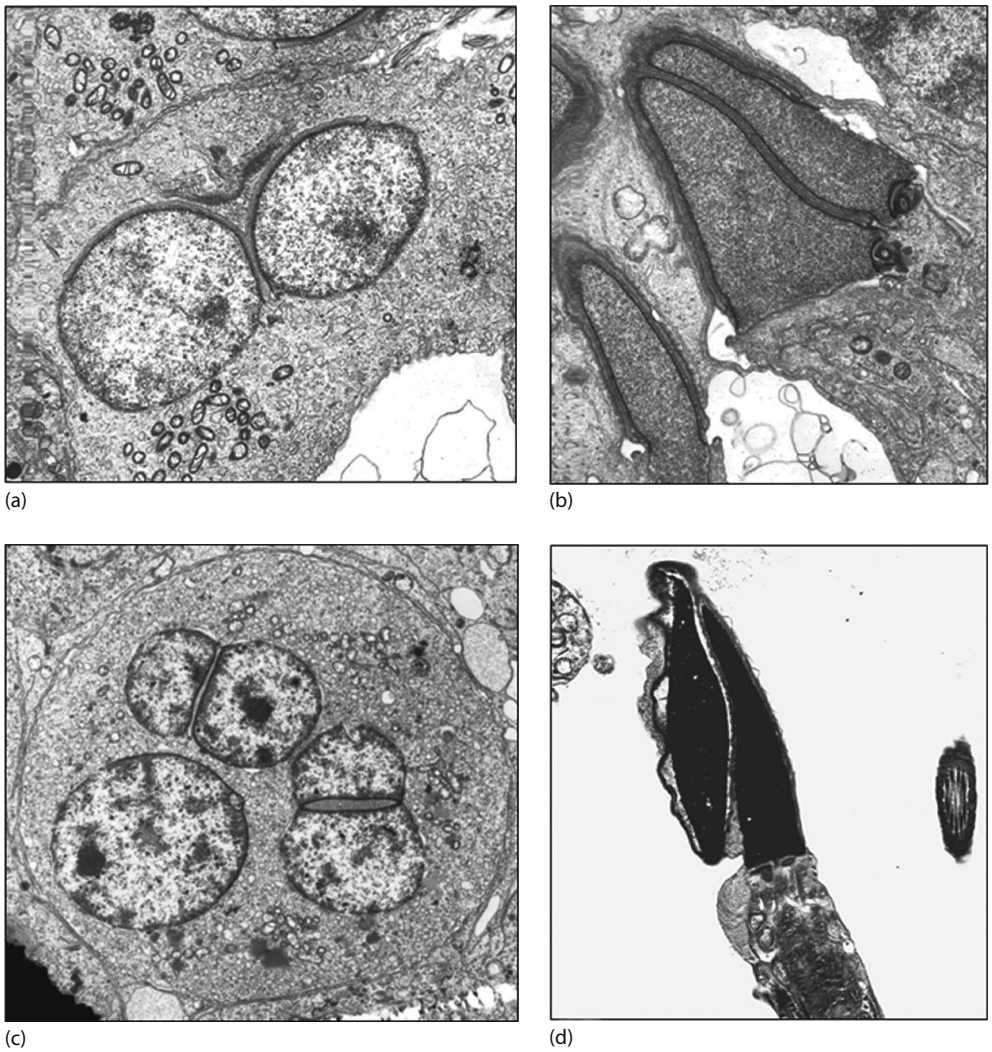

FIGURE 34.6 Transmission electron micrographs of acrosomal dysgenesis-shared acrosomes. Morphogenesis of this unique malformation as observed in rabbits exposed developmentally to common industrial chemical pollutants in drinking water (a, b) and antiandrogenic pesticide vinclozolin (c). As two adjacent round spermatids differentiate, they share a common Golgi apparatus (a) and acrosomal vesicle that spreads around both spermatid nuclei (b) as they condense and elongate resulting in conjoined sperm (d). These unique defects also have been shown to result from developmental exposure to a variety of environmental pollutants including water disinfection by-products in both rabbits and rodents. The profile shown in panel (d) is from an equine seminal ejaculate; once found in a stallion, it is not unusual to encounter such otherwise uncommon sperm morphological defects in follow-up breeding soundness evaluations. This clinical observation, together with the fact that these unique acrosomal-nuclear defects also are manifested in experimental rabbits long (5–10 months) after cessation of exposure, lends credence to our hypothesis that some stem spermatogonia and/or Sertoli cells are permanently affected perpetuating these malformations. Indeed, the multinucleated giant cell containing a cohort of postmeiotic germ cells showing syncytia of spermatid nuclei sharing acrosomes (panel c; from a rabbit exposed developmentally to an antiandrogenic pesticide, vinclozolin) indicates that this is a distinct possibility.

endpoint [186–189], and sperm motility assessments are an integral part of reproductive toxicity testing [40]. Motility measurements are typically obtained on epididymal rat spermatozoa, but sperm recovered from the vas deferens can be similarly analyzed [190].

Motility estimates may be obtained on ejaculated, vas deferens, or cauda epididymal samples. Standardized methods are necessary because motility is influenced by a number of experimental variables, including abstinence interval, method of sample collection and handling, elapsed time between sampling and observation, the temperature at which the sample is stored and analyzed, the extent of sperm dilution, the nature of the dilution medium, and the microscopic chamber employed for the observations [163,191–195].

Sperm motility can be evaluated in real time under phase contrast microscopy, or sperm images can be recorded and stored in video or digital format and analyzed later, either manually or by CASA [196–200]. For manual assessments, the percentage of motile and progressively motile sperm can be estimated and a simple scale used to describe the vigor of the sperm motion.

The application of video and digital technology to sperm analysis allows a more detailed and more objective evaluation of sperm motion, including information about the individual sperm tracks. It also provides a permanent record of the sperm tracks that can be reanalyzed as necessary (manually or computer assisted). With computer-assisted technology, information about sperm velocity (straight line and curvilinear) as well as the amplitude and frequency of the track is obtained rapidly and efficiently on large numbers of sperm [196]. Using this technology, chemically induced alterations in sperm motion have been detected [59,201,202], and such changes have been related to the fertility of the exposed animals [186,203,204]. These studies indicate that significant reductions in sperm velocity are associated with infertility, even when the percentage of motile sperm may not be affected. The ability to distinguish between the proportion of sperm only quivering in space with those that are progressively motile is important [163]. CASA parameters can be defined to provide various objective classifications for progressively motile sperm. This is done simply by setting user-defined thresholds for one or more CASA parameters (e.g., straightness) based on the motion characteristics of sperm from control animals [40,185]. The computer then calculates the percentage of motile sperm that exceed these thresholds and deems these progressive.

Sperm Membrane Integrity

Exclusion of vital dye remains a standard method for evaluating sperm viability by assessing sperm membrane integrity. A combination of the SYBR-14 dye and propidium iodide (PI) seems to be particular effective [205]. PI is impermeant and can bind to sperm DNA only if the cell membrane is compromised. SYBR-14 is a permeant dye that can bind DNA when the sperm membrane is intact. Cell permeant viability dyes can also be used to target specific organelles if the sperm membrane is damaged. For simultaneous evaluation of both viability and a specific membrane protein, a fixable impermeant live/dead stain can be used. These dyes will result in intracellular staining wherever the membrane is compromised.

Exposure of the sperm membrane to an increased level of reactive oxygen species results in lipid peroxidation of the sperm membrane, which in turn leads to loss membrane integrity. Lipid peroxidation assays can reveal whether the plasma membrane is damaged due to oxidation stress. Typically, the level of lipid peroxidation is determined by measuring malondialdehyde (MDA) [206] formed when the unstable lipid peroxides break down.

Numerous sperm membrane proteins have been identified over the years and deemed important for fertility. Most of these were considered pivotal based on their cellular localization (e.g., acrosome) and/or abundance in a sperm extract. We discovered a novel fertility protein by virtue of toxicity testing [168,207–210]. Diminutions in this protein (SP22) in extracts of cauda epididymal rat sperm were linked to toxicant-induced effects, particularly chemicals thought to alter epididymal function such as EDS, chloroethane methanesulfonate, epichlorohydrin, α-chlorohydrin, 6-chloro-6-deoxyglucose, ornidazole, and hydroxyflutamide. With each of these exposures, the decrease in SP22 was highly correlated with decreased fertility via IUI. We also demonstrated that testicular toxicants such as brominated haloacids also decrease the level of this protein on both rat cauda epididymal sperm and ejaculated rabbit sperm [70,210], and again, the decrease was correlated with fertility via IUI (rat) and intravaginal insemination (rabbit). Importantly, the toxicant-induced diminutions in the levels of SP22 proved to be more sensitive than other more typical measures of sperm quality (i.e., sperm motion and sperm morphology). Because SP22 actually originates in the testis, it is a feasible biomarker of effect following exposure to both testicular and epididymal toxicants. Antibodies to recombinant SP22, both rat and human, are now available to immunostain sperm from human and laboratory animals alike for chemical-induced alterations in fertility.

Sperm Nuclear Integrity

The ability of sperm to fertilize an egg does not guarantee fertility. The sperm genome must also be intact for embryonic/fetal development to proceed normally. A variety of assays for DNA and chromosome damage in sperm are being used in human epidemiology and rodent toxicology studies. Of these, the sperm chromatin structure assay has been most widely used [211,212]. This assay is based on the properties of the fluorescent, metachromatic dye acridine orange (AO), which fluoresces green when intercalated into normal double-stranded DNA but red when bound to denatured DNA (or RNA). In this assay, sperm are first acid denatured, then stained with AO and examined using flow cytometry. The relative abundance of red fluorescence is an indication of abnormal chromatin structure and DNA damage; increased percentages of sperm with excess red fluorescence have been correlated with infertility in humans [213]. Other tests for detecting DNA damage in human and rodent sperm, including the comet assay, the TUNEL assay, and measurement of oxidative DNA adduct sites, are being increasingly used to evaluate the effects of environmental contaminants on the genetic integrity of sperm [212]. Importantly, these assays can be measured in sperm from a single ejaculate. For example, DNA fragmentation assessed by TUNEL, oxidative DNA damage assessed by detection of 8-oxoguanine, and sperm membrane lipid peroxidation was studied recently and demonstrated that oxidative DNA damage is not necessarily related to membrane damage [214]. New assays for detecting aneuploidy and chromosome breakage in sperm are also beginning to be applied in epidemiology and toxicology studies. These are based on the use of chromosome-specific fluorescent probes detected by fluorescence in situ hybridization (FISH) methods [215]. For example, exposure to air pollution was recently reported to be associated with increased incidence of an extra Y chromosome [216], and a similar effect was found to be associated with smoking [217]. Because these methods are also being developed for use with rat sperm [218], it should be possible in the near future to design studies to compare responses in humans and rats for this endpoint.

Fertility Assessments

Animal data on reproductive success (or the lack thereof) are difficult to verify in human populations. First, humans are characterized by low rates of conception; thus, an insufficient number of pregnancies may occur in an exposed population to provide sufficient power to detect an effect. Moreover, both partners may be exposed to the toxicant, making it more difficult to ascribe reproductive failure solely to the male. Studies of gender-specific occupational work groups may provide some clarification as to the male's contribution. Such studies also provide the opportunity to study individuals with higher exposures than those encountered in the general population. By contrast, fertility measures in rodents that are mated naturally are inherently insensitive due to relative superior sperm quality compared to humans. Nonetheless, most studies rely on data obtained following natural mating. Table 34.2 illustrates some of the most important indices to evaluate. To distinguish between fertilization failure and early pregnancy loss, additional experimental approaches are needed [219]; for example, oocytes can be recovered from the oviduct the day after mating and examined to evaluate fertilization directly, and the zygotes can be cultured in vitro to the blastocyst stage to evaluate preimplantation developmental potential [220].

Since most test species produce numbers of qualitatively normal sperm that greatly exceed the minimum requirements for fertility as evaluated in current protocols, fertility data obtained by natural mating must be coupled with other male reproductive endpoints to provide the most comprehensive insight into an exposed animal's reproductive capability. Since human males appear to function nearer to the threshold for the number of normal sperm needed to ensure reproductive competence, a more sensitive means of evaluating fertility should be used to study exposures in test species.

A number of investigators have used the strategy proposed by Amann [44] that employs AI with a limited number of sperm from both control and treated males. AI by IUI in the rat does indeed increase the sensitivity of detection for toxicant-induced decreases in sperm quality [62,66,68,164,168]. Moreover, Robl and Dziuk [221] have successfully used IUI in three strains of mice. Dose–response curves for fertility as a function of number of sperm inseminated have been developed for both mice [221] and rats [62]. Robl and Dziuk [221] found that the EC_{50} sperm number for fertility varied among the strains: 1.5×10^6 (DBA/2N), 3×10^6 (CF1), and 6.3×10^6 (C57BL/6N) sperm. Klinefelter et al. [62] reported that the EC_{50} for rat sperm was 2.5×10^6 inseminated per uterine horn. However, an insemination dose of 5.0×10^6 sperm per uterine horn was selected to evaluate toxicant-induced alterations in fertility, as this concentration lies within the linear (i.e., sensitive) portion of the sperm dose–response curve and provides optimal control fertility (i.e., 75%); a dose of 2.5×10^6 sperm results in suboptimal control fertility and a dose of 10×10^6 sperm results in 100% fertility with no enhancement of sensitivity. Rather than recovering zygotes to calculate fertilization rates, these studies used vasectomized (sterile) males to cervically stimulate synchronized females and thereby prime them for implantation. Then, the number of postimplantation implants was determined on gestation day 9 or 20 [62,66]. These studies have been used to demonstrate the potential for epididymal toxicants and by-products of drinking water disinfection to produce low experimental dose alterations in fertility, as well as identify a novel sperm protein biomarker of fertility (SP22) mentioned earlier.

Changes in endpoints that measure effects on spermatogenesis (i.e., histopathology, homogenization-resistant spermatid numbers) and sperm maturation (i.e., epididymal sperm number, motility, and morphology) have been related to fertility in several test species, but the ability to predict infertility from these data (in the absence of fertility data) for test species is not reliable. This is in part due to the observation, in both test species and humans, that fertility is dependent not only on having adequate numbers of sperm but also on the degree to which those sperm are qualitatively normal. If sperm quality is high, sperm number must be reduced substantially before fertility by natural mating is affected. Similarly, if sperm numbers are normal in rodents, a relatively large effect on sperm motility is required before fertility is affected. Again, this is because rodents and other test species produce an excess of qualitatively normal sperm. In the presence of adequate numbers of sperm, average sperm velocity must be reduced substantially before fertility is affected [222]. Nevertheless, fertility in other species may be impaired by smaller changes in both number and motility (or other qualitative characteristics). Thus, relatively modest reductions in sperm number or quality may not cause infertility in species with relatively robust reproductive

TABLE 34.2
Selected Indices That May Be Calculated from Fertility Endpoints Following Exposure of Male Rats

Mating index

$$\frac{\text{Number of males or females mated}}{\text{Number of males or females cohabited}} \times 100$$

Fertility index

$$\frac{\text{Number of cohabited females becoming pregnant}}{\text{Number of unpregnant couples cohabited}} \times 100$$

Preimplantation loss

$$\frac{\left(\text{Number of corpora lutea} - \text{Number of implantation sites}\right)}{\text{Number of corpora lutea}} \times 100$$

Postimplantation loss

$$\frac{\left(\text{Number of implantation sites} - \text{Number of fetuses}\right)}{\text{Number of implantation sites}} \times 100$$

Note: Mating is based on evidence of copulation (observation or other evidence of ejaculation such as vaginal plug or sperm in vaginal smear) was obtained.

TABLE 34.3
Summary of Endpoints of Male Reproductive Toxicity Derived from In Vivo Studies

Organ weights	Testes, epididymides, seminal vesicles, prostate, pituitary
Gross examination and histopathology	Testes, epididymides, seminal vesicles, prostate, pituitary
Sperm evaluations[a]	Sperm number (testis and epididymal/ejaculated) and sperm quality (morphology, motility, viability, sperm membrane proteome, nuclear integrity, fertility via IUI, in vitro fertilization)
Sexual behavior[a]	Mounts, intromissions, ejaculations
Hormone levels[a]	Luteinizing hormone, FSH, testosterone, estrogen, prolactin
Developmental effects	Testis descent,[a] preputial separation, sperm production,[a] anogenital distance, structure of external genitalia[a]

[a] Reproductive endpoints that can be obtained or estimated relatively noninvasively with humans.

characteristics, but these changes can be predictive of infertility at higher exposure levels or in species that do not have the same level of excess sperm production.

In summary, sperm measures add considerable information and sensitivity to standard fertility measures in reproductive toxicity testing. Significant changes in any of these measures, even if modest in magnitude, may be considered adverse and used to set the no-effect or benchmark dose, particularly when they predict more severe effects, including infertility, at higher doses. Such changes are proving useful as critical endpoints for risk assessment; for example, sperm motility and morphology helped inform detailed assessments of several male reproductive toxicants (e.g., acrylamide and 1- and 2-bromopropane) by the National Institute of Environmental Health Sciences (NIEHS) Center for the Evaluation of Risks to Human Reproduction [55,223].

Additional Markers of Sperm Function

The functional capacity of sperm can be evaluated in vivo by recovering eggs at the appropriate time after copulation (species dependent) and determining whether fertilization and normal initial development (timing of cleavage divisions) occurred [224,225]. Alternatively, sperm can be collected and cultured in vitro under capacitating conditions and then cocultured with eggs. In vitro fertilization (IVF) assays have been used for years to study basic mechanisms of sperm maturation and function, but only recently have such methods been proposed for use in toxicology studies, where they may be applied after either in vitro or in vivo exposures. For example, the latter approach has been used to evaluate sperm function in rats exposed acutely to the testicular toxicant 1,3-dinitrobenzene [226]. Although labor intensive, such methods may prove valuable to test hypotheses regarding toxicant-induced effects on sperm function specifically.

The extrapolation of IVF data to predict the in vivo condition requires certain considerations. The ease with which IVF can be achieved varies across species, being readily accomplished in the mouse and hamster but rather difficult in the rat. Such test systems do not reflect the dynamic role played by the female reproductive tract in sperm transport, survival, and capacitation prior to fertilization. The conditions required to achieve successful fertilization in vitro may, in some instances, bear little resemblance to the state that exists in vivo [227,228]; for example, IVF typically employs far higher sperm concentrations than those found at the site of fertilization in vivo.

Another direct indicator of sperm function is the ability to undergo the acrosome reaction after in vitro incubation under conditions that support sperm capacitation. Fluorescent probes can be used to label the acrosome and permit determination of acrosomal status; for example, the plant lectin, *Pisum sativum* agglutinin (peanut agglutinin, PNA), can be used to identify acrosome-intact sperm [229]. In combination with viability stains, live acrosome-reacted sperm can be distinguished from live acrosome-intact sperm and from dead sperm that have shed their acrosomes and the percentages of each type quantified using flow cytometry. A combination of MitoTracker, a dye which stains active mitochondria, SYBR14+PI, to assess plasma membrane integrity and FITC-PNA to assess acrosomal integrity was used collectively to identify viable, acrosomal intact sperm following cryopreservation (Table 34.3) [230].

IN VITRO EVALUATIONS OF MALE REPRODUCTIVE TOXICITY

The following discussion highlights in vitro methods in use in male reproductive toxicology. In vitro methods have been proposed with two goals in mind: either as screens that would assist in prioritizing chemicals for in vivo testing or as specific tests for use in elucidating modes of toxicant action. For the latter purpose, it is important to consider linkage to a specific tissue or cell type that has been demonstrated to be altered by in vivo toxicant exposure. Even if appropriate preliminary in vivo data are obtained, one may fail to demonstrate an effect in vitro. This may reflect the need for a toxicant to interact in vivo with another tissue or cell type or to form an active metabolite; thus, it is advisable to ascertain both the metabolic fate and dosimetry of a toxicant and metabolites within the putative target tissue.

Today, given the rising practical and ethical concerns associated with the use of large numbers of animals for research and the growing number of environmental chemicals

requiring risk assessment, there is an increased emphasis on the use of cell lines for screening potential reproductive toxicants. Progress in this direction has been limited because cell lines frequently fail to retain specific aspects of normal differentiated function as mentioned earlier for cell lines used to screen for steroidogenic compromise. Nevertheless, they may prove to have utility in screening assays as long as provisions are made to test positive chemicals further using either a primary cell culture or an in vivo test system. It is also important to recognize that a chemical that tests negative in a cell line screen may actually test positive in a primary cell culture or in vivo system.

Seminiferous Tubule Culture

Toxicology studies that have demonstrated a disruption in the process of spermatogenesis have implicated virtually every cell type in the seminiferous tubule. A toxicant may perturb either one of the early germ cell types in the basal compartment of the seminiferous epithelium or one of the more mature germ cell types in the adluminal compartment. To affect the more advanced germ cells, a toxicant might either exert its effects directly by passing through the blood–testis barrier or indirectly by perturbing Sertoli cell function.

The cell–cell interactions that occur normally within the seminiferous tubule, particularly the interactions between Sertoli cells and germ cells [231], justify the use of in vitro models in which the whole seminiferous tubule is studied. Seminiferous tubule cultures from rats have been used to study the in vitro effects of 1,3-dinitrobenzene and methoxyacetic acid (MAA). These agents are toxic to Sertoli cells and pachytene spermatocytes, respectively [232]. However, the amount of inhibin, a Sertoli cell product, secreted by the seminiferous tubules in vitro was significantly increased by both compounds. More importantly, the stimulation in inhibin secretion was seen in rats exposed in vivo to doses resulting in intratesticular toxicant concentrations that approximated the effective in vitro concentrations. The seminiferous tubule culture reported by Allenby et al. [232] formed the basis of a series of elegant studies designed to determine the role germ cells play in the expression of androgen-dependent protein secretion [233,234]. Seminiferous tubules were isolated 4, 18, and 30 days following exposure to MAA when pachytene spermatocytes, round spermatids, and elongating spermatids, respectively, would be selectively depleted. Specific proteins were secreted selectively by these different germ cells. The effects of androgen depletion were then assessed 4 days following exposure to EDS, with or without exogenous androgen supplementation. Together, these studies present evidence that specific androgen-dependent proteins are synthesized by seminiferous tubules when different germ cell types are present. This may explain why it has been impossible to demonstrate androgen-dependent proteins in isolated Sertoli cell cultures. It is clear from this work that isolated seminiferous tubules can be used to identify in vitro effects on the seminiferous epithelium if a toxicant disrupts spermatogenesis in vivo.

Short-term seminiferous tubule culture has also been employed to elucidate modes of action of certain drinking water disinfection by-products—namely, the disubstituted haloacids—that have been identified as male reproductive toxicants in the rat. Low-dose effects after in vivo exposure include abnormal spermatid head morphology, delayed spermiation, and spermatid fusion, implicating a defect during spermiogenesis that could be mediated through the Sertoli cell or directly through the late germ cells. Stage-isolated seminiferous tubules from adult rats following a combination of ex vivo and in vitro exposures were used to explore the possibility that requisite Sertoli cell–germ cell interactions might be altered via changes in protein synthesis and secretion [235]. At least three specific proteins were diminished by either in vivo or in vitro exposure to dibromoacetic acid.

In the study by Allenby et al. [236] mentioned previously, a comparison was made between the response of optimized cultures of isolated seminiferous tubules and cultures of isolated Sertoli cells. The results clearly showed that inhibin production by isolated Sertoli cells was smaller and more variable in response to both toxicants. This may be attributed to the fact that the isolated Sertoli cells were derived from immature Sertoli cells. It would be useful to know whether the response of Sertoli cells from adult rats would better approximate the response of the isolated seminiferous tubules. Immature cells have been used in most of the Sertoli cell culture/toxicological studies to date, although the effects of various phthalate esters have been compared in cultures of Sertoli cells obtained from both immature and young adult rats [236]. Because differentiated function (i.e., transferrin, androgen-binding protein, and inhibin secretion) of the Sertoli cell changes with sexual maturity [237,238], an in vitro study should use Sertoli cells that are at the same ontogenic stage as those affected in the in vivo studies. This is important if in vitro data are to be useful in risk assessment. Recent advances in culturing adult Sertoli cells may provide promising in vitro models for male reproductive toxicology research [239,240]. For this and any system using testicular cells [241] or combinations of cells in vitro, it will be important to demonstrate the extent to which differentiated endpoints are maintained and can be evaluated following both in vivo and in vitro exposures.

The use of cell lines representing cells in a less differentiated state holds more promise for screening, particularly as it applies to perturbation in development of the male reproductive system. Testicular cells from neonatal rats and novel approaches for culturing cells on matrices have been used recently to generate Sertoli cell–germ cell cocultures [242]. In theory, these undifferentiated cells should be maintained well in culture and be capable of responding to developmental male reproductive toxicants in a manner similar to that observed in vivo. Recently, spermatogenesis has been reportedly maintained in an in vitro organ culture system [243]. In this system, immature testis fragments were used and spermatogenesis proceeded through meiosis to round spermatid development. After multiple refinements, the same laboratory more recently was able to demonstrate that functional sperm

could be produced by these cultures [244]. A challenge will be to demonstrate whether functional (i.e., cell–cell interactions) and molecular (i.e., transcriptional and proteomic) alterations observed following chemical exposure in vitro can be evaluated in neonatal Sertoli cells and spermatogonia following in vivo insult.

Advances in stem cell biology may lead to other approaches, including the use of pluripotent spermatogonial stem cells [245] at different stages of differentiation. Likewise, advances in bioengineering may produce novel systems wherein testis development can be reenacted and studied with respect to toxicant response. For example, dissociated immature testis cells from rats were recently reported to develop into structures resembling immature seminiferous tubules when they were grown in an extracellular matrix gel and then xenografted into a rat. Encouragingly, the xenografts became vascularized and the tubular structures exhibited lumena and a few putative spermatogonia, as well as an interstitium containing putative Leydig cells [246].

Leydig Cells

Today, a variety of well-accepted methods are available to identify and characterize Leydig cell toxicity in vitro. The choice of method depends largely on the available in vivo data, the tissue availability, and the sensitivity desired. As discussed earlier, a variety of Leydig cell lines have been used to screen for chemicals that might perturb steroidogenesis. Each of these cell lines has limitations such as inability to respond to LH, lack of steroidogenic enzymes representative of the entire pathway for testosterone biosynthesis, or simply an inability to produce sufficient amount of testosterone to afford adequate sensitivity. There seems to be no reason not to use highly purified preparations of purified Leydig cells [247]. These cells produce hundreds of nanograms of testosterone in a few hours in response to LH stimulation and more testosterone unstimulated than Leydig cell lines; only a 10^5 cells/well are needed in a 96-well culture format.

Highly purified Leydig cell preparations were used to establish the dose–response, intracellular site of action, and morphological integrity following in vitro EDS exposure [98]. Results from this study demonstrated clearly that the steroidogenic lesion induced by either in vivo or in vitro EDS exposure exists between the second messenger cyclic AMP and cytochrome P450 side-chain cleavage enzyme. In another study, highly purified Leydig cells were incubated with increasing concentrations of EDS and with either a maximally stimulating concentration of LH or with [^{35}S] methionine to discriminate between functional and cytotoxic effects [248]. Similarly, purified Leydig cells were used recently to study the interaction between Sertoli cells and Leydig cells during in vitro exposure to tri-*o*-cresyl phosphate (TOCP). This approach demonstrated that Leydig cells must first metabolize the toxicant for subsequent Sertoli cell toxicity to occur [249].

To evaluate the ability of the active metabolite of methoxychlor to produce differentiation-dependent alterations in the ability of the Leydig cell to produce testosterone during postnatal development, purified Leydig cells were isolated from rats on postnatal days 21, 35, and 90, when progenitor, immature, and adult Leydig cell populations, respectively, are present within the testis [250]. Results indicated that the prepubertal Leydig cell was more sensitive than the adult Leydig cell to disrupted cholesterol side-chain cleavage activity and cholesterol mobilization. More recently, Leydig cells were isolated from both prepubertal rats on postnatal day 21 and adult rats on postnatal day 90 following exposure to soy isoflavones during gestation and weaning [251]. Isoflavone treatment induced proliferative activity in the prepubertal Leydig cells resulting in increased numbers of Leydig cells with compromised steroidogenic capacity in the adult testis.

Another relatively simple approach involves the use of decapsulated testicular parenchyma incubated with or without LH stimulation to identify alterations in testosterone biosynthetic ability under stimulated or basal conditions, respectively. These incubations can be performed using parenchyma derived from testes exposed in vivo or in vitro. An advantage is that data can be easily obtained on a per animal basis, an important consideration when using species such as the rabbit and hamster that contain relatively few steroidogenically active Leydig cells following enzymatic dispersion. This technique has been used to identify chemical effects on both LH-stimulated and basal testosterone production. The effects of EDS on LH-stimulated testosterone production were compared in the rat and hamster [252]. Testosterone production was linear over time in the testis incubations of both species, and the results demonstrated that the hamster Leydig cell is far less sensitive than the rat Leydig cell to the cytotoxic effects of EDS. Chloroethyl methanesulfonate is an example of a toxicant that resulted in significantly reduced basal testosterone production [92].

A similar strategy has been applied in the mouse where either an entire, nicked testis or aliquot of testis parenchyma was studied on postnatal days 24 and 82, respectively [253]. Gestational exposure of mice to EDS resulted in decreased LH-stimulated testosterone production per testis at both time points. There were significantly fewer Leydig cells in the adult testis but these Leydig cells produced more testosterone per cell than control Leydig cells. In addition, there were decreased numbers of sperm in the cauda epididymis and decreases in fertility in these adults.

Epididymal Cells

Because the epididymis is the organ that confers fertilizing ability on maturing sperm, it is important to develop methods that identify epididymal toxicity following in vivo exposure and confirm any direct toxicant action on the epididymis following in vitro exposure. To establish direct toxicant action, a culture system must be used that is capable of maintaining facets of normal epididymal sperm maturation. This means that the culture system must (1) preserve the morphological integrity of the epididymal epithelial cells, that is, tight junctions between adjacent cells and polarized ultrastructure and

(2) synthesize and secrete proteins in an androgen-dependent manner. Few in vitro models meet these criteria. Several epididymal cell lines have been developed. Immortalized epithelial cells from the caput epididymidis of the mouse achieved polarized features and, based on expression of specific genes, retained some degree of differentiation but failed to express androgen receptor and other key markers of epididymal epithelial cell function [254]. Another immortalized epididymal epithelial cell line was shown to exhibit polarity, form tight junctions between adjacent cells, and express the androgen receptor but was not androgen responsive [255]. Recently, epididymal epithelial cells from both prepubertal and adult rats were cultured and maintained normal in vivo expression of both androgen and estrogen receptors but these cells were not contiguous and displayed no polarity [256].

Epididymal epithelial cells from various regions of the boar epididymis have been cultured with reasonable success. Both monolayer and tubule fragment cultures were compared with regard to their ability to support the maturation of cocultured immature boar sperm [257]. The results indicated that functionally, the epididymal epithelial cell monolayers behaved no differently than other epithelial cells, whereas the tubule fragment cultures appeared to secrete factors necessary for facets of sperm maturation. Using small intact fragments of rat epididymal epithelial cells that were cultured within a dilute extracellular matrix on top of a porous membrane, epithelial cells were well maintained and did respond to toxicant exposure, for example, EDS [258]. Not only was the synthesis and secretion of specific proteins decreased, but when caput sperm were cocultured above the underlying epithelial cells, secreted proteins failed to become associated with the sperm membrane and the progressive motility of cocultured sperm was reduced. It was concluded that EDS acts directly on epididymal epithelial cells to disrupt protein secretion and this, in turn, disrupts facets of sperm maturation. Because the biomarker of fertility (SP22) was discovered using a protocol to identify toxicity within the epididymis and is now known to be expressed in the testis around the time of round spermatid formation [208], it is likely that a protein secreted by the epididymal epithelium serves to stabilize SP22 on the sperm surface. To explore this possibility, epididymal epithelial cell–sperm cocultures will be useful. Since gestational exposures to a variety of environmental chemicals perturb reproductive development, including the development and differentiation of the Wolffian ducts, it may be worthwhile to investigate the critical events (e.g., gene/protein expression) that regulate the development of the Wolffian ducts. For this, a system for the isolation and culture of the Wolffian ducts will be required.

PATERNALLY MEDIATED EFFECTS ON OFFSPRING

The concept is well accepted that exposure of a female to toxic chemicals during gestation or lactation may produce death, birth defects, growth retardation, or postnatal functional deficits in her offspring. Sufficient data now exist with a variety of agents to conclude that male-only exposure can also produce deleterious effects in offspring [259,260]. Agents for which such adverse effects in test species have been reported include lead [261], diethylstilbestrol [262], urethane [263], cyclophosphamide [264–273], marijuana [274], and opiates [275]. The dominant lethal test is intended to detect mutagenic effects in the spermatogenic process that are lethal to the embryo or fetus. Dominant lethal protocols may utilize acute dosing (1 to 5 days) followed by serial matings with one or two females per male per week for the duration of the spermatogenic process. An alternative protocol may utilize subchronic dosing for the duration of the spermatogenic process followed by matings. Females are monitored for evidence of mating, sacrificed at approximately midgestation, and examined for incidence of pre- and/or postimplantation loss. The acute exposure protocol of the standard dominant lethal test, combined with serial mating, may allow identification of the spermatogenic cell types that are affected; however, acute dosing may not produce adverse effects at levels as low as with subchronic dosing because of factors such as bioaccumulation. Information from such studies can be useful for identifying site and potential mechanism of action and, thus, facilitate design of subsequent studies. The occurrence of pre- or postimplantation loss is often considered to provide sufficient evidence that the agent has gained access to the reproductive organs and has compromised fertilizing ability or induced mutagenic damage to the sperm, respectively. Such data were informative, for example, in the CERHR's report on acrylamide [223], which is available at http://cerhr.niehs.nih.gov/chemicals/acrylamide-eval.html.

Although a number of human studies have reported associations between a variety of paternal occupations and the occurrence of birth defects or childhood cancer [276–282], others have failed to observe such relationships [283–285]. A large proportion of the chemicals reported to cause paternally mediated effects are considered to exert this effect via transmissible genetic or epigenetic alterations [286–287]. Low doses of cyclophosphamide have resulted in induction of single-strand DNA breaks during rat spermatogenesis that, due in part to the absence of subsequent DNA repair capability, remain at fertilization. The results of such damage have been observed in F2 generation offspring [288] and result in changes evident as early as the pronuclear stage of development [289]. Indeed, paternal cyclophosphamide exposure is now known to result in alterations in both chromatin structure and basic sperm nuclear proteins [290].

Another example of epigenetic transgenerational action was provided by Anway et al. [56]. Brief gestational exposure of pregnant dams to vinclozolin was shown to result in compromised sperm production and altered sperm quality including infertility. The altered male genome was transmitted through several generations and linked to altered DNA methylation. More recently, this laboratory compared the effects of vinclozolin and another well-known antiandrogen flutamide [291]. While vinclozolin has antiandrogenic action, it appears that its ability to induce epigenetic alterations is

independent of this action; only vinclozolin produced transgenerational alterations in male offspring.

Other mechanisms of induction of paternally mediated effects also are possible. Xenobiotics present in seminal plasma or bound to the fertilizing sperm could be introduced into the female genital tract or into the oocyte directly and might also interfere with fertilization or early development. With humans, the possibility exists that a parent could transport the toxic agent from the work environment to the home (e.g., on work clothes), exposing other adults or children. Further work is needed to clarify the extent to which paternal exposures may be associated with adverse effects on offspring. Regardless, if an agent is identified in test species or in humans as causing a paternally mediated adverse effect on offspring, the effect should be considered an adverse reproductive effect.

SEXUAL BEHAVIOR

Sexual behavior is a complex process involving neural and endocrine components of the central and peripheral nervous systems and the reproductive system. For humans, interactions of personality, social, and experiential factors also influence the initiation and performance of these behaviors. Similar factors may exist in other species, but they are more controlled by standardized laboratory conditions; however, the perturbation of sexual behavior in animals suggests the potential for similar effects on humans. Consistent with this position are data on CNS-active drugs that have been shown to disrupt sexual behavior in both animals and humans [292].

Although the functional components of sexual performance can be quantified in rats [293], direct evaluation of this behavior is not typically done in most breeding studies; rather, the presence of copulatory plugs or sperm-positive vaginal lavages has been interpreted as indirect evidence of successful mating. These markers do not demonstrate that male performance necessarily resulted in adequate sexual stimulation of the female. In rats, the degree of sexual preparedness of the female partner can strongly influence the site of semen deposition and subsequent sperm transport in her genital tract [294]. Failure of the female to achieve sufficient stimulation may adversely influence these processes, thereby reducing the probability of successful impregnation. Such a mating failure would be reflected in the fertility index as reduced fertility and could erroneously be attributed to a spermatotoxic effect. Other aspects of current breeding protocols exist that may serve to mask a decline in the fertility potential of a given male.

Although the need and outcome of direct evaluation of sexual behavior routinely for all suspected reproductive toxicants is questionable in rodent studies, likely candidates may be agents reported to exert neurotoxic effects, as several neurotoxicants have also been shown to produce disruptions in copulatory behavior (e.g., trichloroethylene [295], carbon disulfide [296], acrylamide [297]). Chemicals possessing or suspected to possess androgenic or estrogenic properties (or antagonistic properties) also are potential candidates for the evaluation of copulatory behavior, separate from effects on reproductive organs (e.g., chlorinated hydrocarbon pesticides).

The limitations of evaluating sexual behavior using rodent models described earlier can be overcome by using rabbits in male reproductive toxicology studies as the teaser doe need not be sexually prepared to be receptive to a buck as the female rabbit is an induced ovulatory and is always sexually receptive and the male rabbits are easily trainable to ejaculate in an artificial vagina in the presence of a teaser doe. Thus, by timed collection of ejaculates using an artificial vagina, the copulatory behavior as well as erectile and ejaculatory functions can be methodically evaluated. Indeed, valuable information on the detrimental effects of common chemical contaminants in drinking water [71] and the antiandrogenic fungicide vinclozolin [298] on sexual behavior and functions has been obtained utilizing the rabbit as an animal model.

Follow-up studies on the brain as a target organ for the observed sexual dysfunction revealed that vinclozolin significantly increased calbindin expression in the ventral preoptic area and anterior hypothalamus and significantly decreased the number of gonadotropin-releasing hormone (GnRH) neurons selectively in the region of the organum vasculosum of the lamina terminalis [299]. This is the first documentation of a sexually dimorphic region in the rabbit brain and supports the use of this species as a model for studying the influence of vinclozolin on reproductive development with potential application to human systems. Furthermore, it was found that subacute, prenatal vinclozolin exposure is sufficient to create perdurable alterations in the GnRH neuronal network that forms an important input into the reproductive axis. Finally, the effect of vinclozolin on the GnRH neuronal network was not comparable to that of flutamide, suggesting that vinclozolin was not acting through antiandrogenic mechanisms [300].

MALE REPRODUCTIVE EPIDEMIOLOGY

The ultimate goal of the toxicologist is to provide data that might be extrapolated to human exposures. As already discussed, human males are inherently on a threshold for subfertility, and coupled with a myriad of unintended daily occupational and recreational exposures, it is reasonable to assume that environmental exposures are indeed further compromising reproductive competence. Unfortunately, most epidemiology studies are not designed and executed in a manner that allows one to support or refute toxicology data and most toxicology data are derived from studies conducted at exposures that are several orders of magnitude above relevant human exposures. The pitfalls in both scientific arenas have been recently reviewed [43].

In most epidemiology studies, exposure data are inadequate or completely lacking. Levels of a particular chemical(s) in the blood, urine, or semen need to be obtained to correlate with observed effects in semen quality. Additionally, the data typically gathered on sperm quantity and quality inconsistent across laboratories. Some laboratories report sperm

concentration, some sperm count, some both and abstinence is not consistently controlled. Equal variability exists for measures of sperm quality. Finally, usually only one ejaculate is obtained from an individual rather than repeated measures. It has been argued that the only biologically meaningful parameter of sperm production by the testis is total sperm per ejaculate [301,302] and that only by controlling abstinence to an interval of 42–60 h can meaningful data on the rate of sperm production can be gathered [303]. By extension, this is the only manner in which total sperm per ejaculate can be compared across individuals in a meaningful fashion. It is no wonder that we are still debating whether there is plausible scientific data to determine whether semen quality is on the decline, and if so, to what extent environmental exposures may play. Recently, Bonde et al. [304] stated that the fundamental problem with secular trend studies is the lack of comparability of study data across time and that we can now only change and adopt consistency to obtain meaningful data in future studies. Finally, while most epidemiology studies attempt to gather data on semen quality, measures of fertility such as time to pregnancy are rarely incorporated. Aside from irrelevant in vivo exposures in most toxicology studies, another common failing is the use of insensitive measures of fertility. The bottom line—fertility assessments come up short on both fronts.

A TDS has been described to encompass are male reproductive phenotypes that have been reported with increased incidence, namely, cryptorchidism, hypospadias, testicular cancer, and reduced semen quality [305]. Each phenotype has been associated with decreased fetal testosterone production [306]. Obviously, data on the testosterone production by the human fetal testis in males presenting with TDS phenotypes after birth cannot be gathered. Thus, the link to decreased fetal testosterone production was hypothesized based on phthalate studies in the rat that resulted in some of the same phenotypes albeit at high exposure levels. Recent data suggest that testosterone production by the human fetal testis is not decreased by phthalate exposure even at comparable high doses [307]. This clearly illustrates the need to incorporate new predictive markers of altered phenotypes in epidemiology studies.

FUTURE OF MALE REPRODUCTIVE TOXICOLOGY

The field of male reproductive toxicology and risk assessment has been evolving rapidly since reports of adverse effects of pesticides such as dibromochloropropane became evident in the 1970s [18]. Our understanding of modes of action of chemicals on male (and female) reproductive health has expanded with the application of cell and molecular approaches, including genomics and proteomics. The need to develop better interfacing between toxicology and epidemiology has been evident for some time now [308], but far too little progress has been made [43]. The importance of measuring contaminants in the environment and in accessible human tissues and fluids (i.e., blood, urine, semen) to build more precise models of exposure–effect relationships is obvious. The Centers for Disease Control and Prevention has added many reproductive toxicants and endocrine disruptors to its list of substances measured in Americans as part of their biomonitoring program. These are listed in the National Report on Human Exposure to Environmental Chemicals (see www.cdc.gov/exposurereport). While this is valuable effort, more needs to be done to attempt to estimate fetal exposures to improve the quality of prospective study data.

The importance of identifying adverse outcome pathways of male reproductive toxicity has grown in scope to include not only the relationships between exposures and semen quality in adults but also the relationship between exposures during fetal development, or prepubertal development, and adverse phenotypes such as hypospadias, testicular cancer, delayed puberty, and poor semen quality at adulthood. Realizing that there are far too many chemicals in the environment to test in multigenerational protocols, U.S. and international agencies and their industrial partners have gathered to test and implement new screening protocols such as the in vivo and in vitro tests summarized earlier for EDCs. These efforts have now given rise to systems modeling and high-throughput computational toxicology with the end goal being virtual (i.e., in silico) predictive models for toxicity in the male reproductive axis—the hypothalamic–pituitary–gonadal axis throughout reproductive development, from fetus to adult. This is a very attractive approach because it reduces the need for animals and could save much time and effort. However, before such models become an institutional reality, model validation will need to be done, particularly if the data is to be extrapolated to humans.

Although chemicals are usually listed, studied, and regulated singly, their occurrence as components of highly complex mixtures is recognized—for example, disinfection by-products in our drinking water, various products that are widely used by consumers (e.g., pharmaceuticals and personal care products), antibiotics and growth promoters (and their metabolites) used in agriculturally important species, and the rapidly growing list of nanomaterials introduced into our environment. This further complicates the challenge as relevant mixture studies are only beginning to emerge. And the predictive models alluded earlier will be even more abstract and difficult as mixtures are incorporated. Moreover, most high-throughput screening tests are based on a specific mode of action, for example, androgen receptor antagonism. It is quite unlikely that a relevant environmental mixture acts via one or two specific modes of action. It is more likely that many modes of action are involved simultaneously and the resulting adverse outcome or phenotype is dependent on the relative weight given to these multiple paths. As such, it seems prudent to continue research that can evaluate effects in a collective manner. To this end, in recent years, progress has been made in the culture of the testis. It is now possible to produce functional sperm in vitro [243,244]. But like all in vitro systems, their success limited to knowledge of the metabolite(s). So it is really inevitable that animal work continues, but it must be done in a more meaningful fashion [43].

As new adverse outcome pathways and the associated molecular targets are elucidated, we must begin to validate these biomarkers of effect, first in the animal, and then in accessible human tissues and fluids. It's conceivable that someday, we will be able to assay a mother's urine and predict whether the baby she is carrying is at increased risk for hypospadias or testicular cancer.

DISCLAIMER

This document has been reviewed in accordance with the policy of the National Health and Environmental Effects Research Laboratory, U.S. EPA, and approved for publication. Approval does not signify that the contents necessarily reflect the views or policy of the agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

QUESTIONS

34.1 Why is it necessary to evaluate so many different endpoints to assess adult male reproductive toxicity? Why is the harmonized multigeneration test more informative than an adult subchronic toxicity test?

34.2 What cellular and molecular mechanisms are thought to be responsible for male-mediated transmissible (transgenerational) effects?

34.3 How can histology help determine the phase of spermatogenesis implicated with toxicity to a particular chemical exposure?

34.4 What in vitro approaches provide data that best supports what might be occurring in the exposed animal and advance our understanding of path to phenotype?

34.5 Why is it important to correlate multiple endpoints on an individual animal basis?

34.6 Why is it important to assess fertility by a more sensitive means than natural mating?

34.7 How can epidemiology and toxicology studies be improved for better data linkage?

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35 Test Methods for Assessing Female Reproductive and Developmental Toxicology*

Raymond G. York, Robert M. Parker, and Lynne T. Haber

CONTENTS

* We (York and Parker) have worked with and learned from Dr. Mildred Christian. We were greatly saddened with her passing and are honored to pick up her torch to revise and update this chapter on assessing female reproductive and developmental toxicology. While writing these sections, we were constantly reminded to look at all the animal data, not just the group means or statistical significances, because as Dr. Christian would say, "The animals will tell you what is happening. You just need to look."

PURPOSE

This chapter presents practical methods of testing used in performing female reproductive and developmental toxicity studies, as well as approaches for interpreting the test results in a risk assessment context. The evaluation methods presented are those used in studies conducted for regulatory use, that is, in the process of identifying the safety of both small and large (biologic) molecule pharmaceuticals, chemicals, pesticides, fungicides, direct and indirect food additives, and medical devices. The methods described in this chapter deal mainly with in vivo tests conducted for regulatory use in standard laboratory species and touch only briefly on in vitro and in vivo methods used for screening agents and identification of the mechanism or mode of action. Test methods used to evaluate reproductive toxicity in male animals, as well as in depth in vitro methods used to evaluate development, genomic methodology, and in vivo behavioral/functional parameters, are addressed elsewhere in this book.

INTRODUCTION

During ancient times, humans and animals that deviated greatly from their parents at birth were impressive events. Attitudes toward these abnormal progeny ranged from admiration and adoration to rejection and hostility, depending on the culture of the tribe or nation. Historically, human birth defects were usually considered spontaneous or hereditary events.[1,2] By 1925, the effects of maternal malnutrition were known to reduce fertility, reduce birth weight, and cause abortion or death of the conceptus, but were not thought to cause congenital malformation.[3,4] In 1941, the causal relationship between maternal exposure to German measles and congenital cataracts was the first identification of a disease to adversely affect human pregnancy.[5,6] Extreme maternal malnutrition during World War II resulted in congenital malformations, reproductive failure, amenorrhea, abortions, reduced birth weights, and postnatal survival.[7,8]

Research on animals used for food generally focused on enhancing the reproductive performance or the effects of nutrition on these animals. Studies on vitamin A deficiency in animals[9,10] were the first to demonstrate that the observed anophthalmia was associated with a maternal dietary deficiency (vitamin A). Congenital malformations in a conventional laboratory species, diet-deficient rats, were first reported in 1940.[11] Once it was proven that laboratory animals could be used to study congenital malformations, considerable research into teratogenic agents and susceptibility to these agents followed.[12,13]

The development of reproductive testing guidelines in animals followed three human tragedies resulting from in utero exposure: (1) 1961, congenital malformations from thalidomide[14,15]; (2) early 1970s, vagina cancer in young women whose mothers underwent stilbestrol therapy[16,17]; and (3) 1976, behavioral/functional alterations from methylmercury compounds. The first event, the thalidomide tragedy, completely changed the perception of concern regarding consumption of a medicine during pregnancy and potential adverse outcomes. The second event, cancer resulting from in utero exposure to diethylstilbestrol, raised additional concerns regarding adverse effects that were not evident until after sexual maturity. The third, Minamata Bay disease, resulted in additional tests for postnatal behavioral changes.

Increased public concern and regulatory requirements resulted in a large body of research associated with teratogenic insult during the first trimester of gestation.[1,18] Many continue to assume that susceptibility to teratogenesis is limited to this period of organogenesis.[19,20] It is now clearly evident that the *all or nothing* law of recovery or death does not always apply to preimplantation embryos (i.e., surviving embryos may demonstrate growth retardation, malformation, and/or functional impairment), that both the preimplantation and fetal stages of development are also susceptible to toxic insult (i.e., toxic exposure may result in death, retarded growth, malformation, and/or functional impairment), and that many systems continue to develop after birth (e.g., lungs, immune and nervous systems, reproductive organs).

Pregnancy, as well as the outcome, is dependent upon dynamic complex interactive systems. Each pregnancy is dependent upon the entire reproductive process (Figure 35.1), including the development, genetic makeup and nutritional status of the parental generation, as well as the genetic makeup, nutritional status during growth and development, and exposure of the offspring to perturbations in the macro- and microenvironments. Both sensitivity and response to a xenobiotic are sex-, age-, dose-, and tissue-dependent.

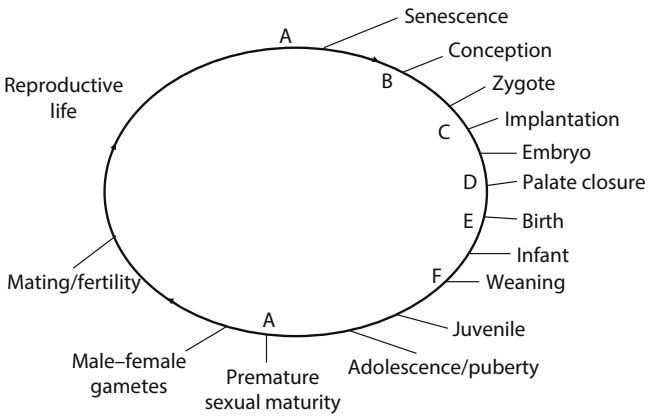


FIGURE 35.1 Reproductive and developmental effects.

NORMAL REPRODUCTION AND DEVELOPMENT

Reproduction in humans, as well as domestic, wild, and laboratory vertebrates, can occur multiple times, beginning at puberty and ending at reproductive senility. During this process, two haploid chromosomes, one from each sex, are joined to produce a diploid state in a new individual. In most mammalian species, phases of the reproductive cycle, during which development of a new individual occurs, principally take place within the uterus of the female. This development of the conceptus from the fertilized ovum to birth is a complex process during which extremely rapid cell proliferation occurs, and apoptosis is normal and required. The multiplicity of xenobiotic exposures, lesion sites, and potential reproductive disorders precludes simple screening of suspected reproductive toxicants in humans, because the effects and the agents causing reproductive dysfunctions or altered development are often impossible to predict.

Normal Reproductive Performance

A high proportion of humans have difficulty getting pregnant or staying pregnant. Infertility occurs in tens of millions married couples in the United States, with noncontraceptive sterility occurring in at least one of the partners in approximately 3 million of these couples[21,22]; ovulatory problems have been reported to be the cause of infertility in approximately 40% of all infertile couples.[23] It has been estimated[24] that as many as 50% of all fertilized ova are lost within the first 3 weeks of human development. The World Health Organization (WHO)[25] estimated that 15% of all clinically recognizable pregnancies end in a spontaneous abortion, defined as 20 weeks from the first day of the last menstrual period for a fetus weighing less than 500 g, and that 50%–60% of the spontaneously aborted fetuses have chromosomal abnormalities.[26,27] Prematurity (defined as birth before the 37th week of gestation) occurs in approximately 7% of births, and low birth weight (defined as 2.5 kg or less) is the most common developmental abnormality in humans and has often been associated with functional/neurological defects and malformations.[28,29] In the United States, of over 4.1 million liveborn infants in 2011, 5.98 per 1000 die before one year of age,[30] and 2%–3% of these liveborn infants are identified to have a congenital malformation within the first postnatal year.[31,32] Based on the current relatively crude criteria, approximately 16% of live births have major or minor malformations.[31]

Causes of Birth Defects

The cause of approximately 50% of human congenital malformations is unknown.[33] As causes for birth defects continue to be identified, this percentage can be expected to decrease. Known causes can be divided into genetic and partially genetic causes originating preconception, and those causes originating postconception, but before birth.

Most birth defects originate preconception and are due to genetic abnormalities in the chromosomes or genes. Genetic abnormalities can be inherited and thus are found in families, or they can occur as isolated events. Chromosomal abnormalities are due to changes in normal structure or in the number of chromosomes. Such abnormalities account for approximately 6% of the birth defects in industrialized nations.[33] Down's syndrome, caused by an extra chromosome 21 (Trisomy 21) or part of chromosome 21, is the most common chromosome abnormality in man and was first described clinically by John Langdon Down in 1866[34] and has an incidence rate estimated at 4.6 per 10,000 births.

Mutations, or single-gene defects, result in abnormal cell function. More than 15,000 single-gene defects had been described by 2004.[35] The first clinical description of a single-gene defect was brachydactyly (short digits) in a family in 1903.[36] All single-gene defects combined account for approximately 6% of the birth defects in industrialized nations.[33]

The concept of inheritance due to complex genetic and environmental factors (multifactorial inheritance) was first put forth by Boris Ephrussi in 1953; and F. Clarke Frazer expanded on multifactorial inheritance in the area of birth defects in 1976.[37] This category accounts for approximately 20%–30% of all birth defects, a number of which are lethal.[33] Modulating factors include, but are not limited to, placental blood flow, placental transport, site of implantation, maternal disease states, infections, drugs, chemicals, and spontaneous errors of development.

Causes of birth defects originating postconception are primarily nongenetic, caused by intrauterine environmental factor(s), and account for approximately 5%–10% of all birth defects.[38] These include teratogens that interfere with normal growth and development of the embryo or fetus, mechanical forces that deform the fetus, and vascular accidents that disrupt normal growth. Teratogens are broadly grouped into five categories: (1) physical agents like radiation; (2) environmental contaminants, such as methylmercury; (3) maternal metabolic disturbances, like diabetes mellitus; (4) maternal infections, such as rubella; and (5) drugs, both legal and illegal.[39]

Human Epidemiology Studies

Epidemiology has the potential to play central and imperative roles regarding identifying how developmental and reproductive toxicants affect human populations. Several inherent problems exist in performing human epidemiology studies.[40] First, they are time-consuming and expensive, especially when dealing with rare disease states. Second, reported findings are often based on anecdotal information, and essentially impossible to prove in the absence of an association. Third, studies may be either experimental or observational, and the more scientifically useful experimental type is precluded by ethical concerns that disallow direct testing of women of childbearing potential. Thus, epidemiology studies in the area of reproduction and development usually are observational, that is, evaluation of events in two groups without intervention or random assignment of subjects to treatments. These epidemiology studies usually involve statistical analyses of medical data to identify associations between toxic agents and adverse outcomes, resulting in identification of relative risks that may or may not identify causal relationships.

The greatest difficulty in reproductive/developmental epidemiology studies is in obtaining adequate sample sizes. To detect a 3.2-fold increase in major malformations with a rare frequency of 3%, a sample size of at least 300 live births is required.[41] Frequent outcomes, such as spontaneous abortion, which occurs at a 15% incidence, would require only a population of 50 pregnancies to detect a threefold increase.[42] Animal studies are most frequently used to detect potential adverse reproductive or developmental effects because of the difficulties and complexities associated with human clinical and epidemiological studies, and the need to prevent potential human exposures. Such studies are, for the most part, the subject of this chapter.

FEMALE REPRODUCTIVE TOXICOLOGY

Indifferent Gonad

An indifferent gonad is a gonad in an embryo that has not differentiated into a definitive testis or ovary. An indifferent gonad becomes a testis if the embryo has a Y chromosome, but if the embryo has no Y chromosome, the indifferent gonad becomes an ovary. The absence of a Y chromosome permits the indifferent gonad to become an ovary, but both X chromosomes are needed for the ovary to function normally.

Soon after gastrulation, the primordial germ cells migrate by ameboid movement to the dorsal mesentery of the hindgut, the coelomic epithelium of the genital ridge proliferates, and epithelial cells penetrate the underlying mesenchyme.[43] Here they form a number of irregularly shaped cords connected to the surface epithelium. Since these cords are impossible to differentiate between the sexes, they are known as the indifferent gonad. The indifferent gonad contains three principal types of somatic cells: supporting, stromal, and steroidogenic. Each type is able to progress in a male or female direction. The supporting cells will provide support to the Wolffian or Mullerian ducts, the steroidogenic cells will differentiate into either embryonic male Leydig cells or female ovarian theca cells, and the stromal cells become involved in forming the vasculature and extracellular matrix.[44]

Gametogenesis and Ovulation

Each sex of most multicellular animals produces specialized cells (gametes), which are joined (fertilization) to form a new individual zygote/conceptus. Gametogenesis is the production of sperm or ova and the peripubertal changes leading up to its onset. The mammalian ovulatory cycle includes multiple interrelated events involving folliculogenesis, ovulation, and preparation of the reproductive tract for fertilization and implantation leading to pregnancy. Ovulation is the central event in the ovulatory cycle.[45]

Ovulation results from interaction of multiple feedback systems, including the hypothalamic regions of the interbrain (diencephalon), anterior pituitary, and the ovary. The hypothalamus releases gonadotropin hormone-releasing hormone (GnRH) and, through this process, regulates anterior pituitary production and secretion of gonadotropin hormones, including luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH released from the anterior pituitary and transported to the ovary initiate and maintain ovarian follicle growth.

The initial phase of positive feedback is that in which the hypothalamic–anterior pituitary axis component signals to initiate growth of the ovarian follicles. The mature ovarian follicle then signals to start ovulation through production and secretion of estradiol and progesterone. Estradiol and progesterone initiate a release of GnRH, which is followed by a release of the ovulating-inducing hormones, LH and FSH. These hormones provide the stimulus initiating the cascade of events in the ovary ultimately resulting in ovulation.[45]

Ovulation of a fertilizable ovum (oocyte, egg, or female gamete) requires formation of a corpus luteum, and growth, maturation, and differentiation of three cell types. Each of these three cell types, the germ cell (oocyte), granulosa cells, and thecal endocrine cells, is susceptible to toxic effects, as are the three major processes that occur during the development of the mature oocyte: (1) mitosis of oogonia and granulosa cells during follicular growth; (2) meiosis of oogonia to form oocytes; and (3) differentiation of granulosa cells and theca cells, allowing response to a surge of LH and subsequent ovulation.

The inseparability of the reproductive process and development of the conceptus is most apparent when one considers that the female germ cells and follicles are formed during prenatal life. Primordial germ cells are first detectable in the yolk sac at 3 weeks of human development. These cells undergo mitosis, migrate to the urogenital ridge, populate the indifferent gonad, and then differentiate into oogonia or prespermatogonia. Approximately 1700 germ cells migrate to the gonads in a human embryo. These increase to approximately 600,000 germ cells by 8 weeks of gestation and peak at approximately

7,000,000 germ cells by 20 weeks of gestation. Oogonia begin to enter meiosis at month 3 of gestation, with all oogonia in early prophase I by the end of month 5, at which time the oogonia are termed primary oocytes.[46] Oocyte atresia then results in decline in numbers to less than 1,000,000 by birth, another sharp decline with the onset of puberty (at around 12 years of age), and continued reductions in numbers throughout the remainder of the reproductive life of the woman until they are all gone by about 50 years of age.[45]

The meiotic stages are prophase, metaphase, anaphase, and telophase. Prophase is the first meiotic division and occurs during the fetal or neonatal period. Within 8 weeks after birth, human oocytes enter a resting phase (diakinesis), in which they stay until puberty begins.[47] Sex differentiation and ovarian germ cell development occurs at different developmental ages in various mammalian species, as shown in Table 35.1.

The majority of germ cells are lost to normal, physiological degeneration of atresia, which occurs during the oogonial and primary oocyte stages. Approximately 60% of the germ cells in a human fetus are lost between 5 months of gestation and birth, with three distinct waves of oogonial degeneration occurring. One wave affects oogonia in mitosis (final interphase), and the other two affect oocytes in the pachytene and diplotene stages of prophase I. This normal apoptosis is synchronous in oogonia connected by cytoplasmic bridges. After the meiotic prophase, simultaneous atresia no longer occurs, although individual oocytes spontaneously degenerate at all stages of development.

Folliculogenesis

Folliculogenesis is the maturation of the ovarian follicle, a densely packed shell of somatic cells that contains an immature oocyte. Granulosa cells surround the oocytes during prophase. Their initial growth is gonadotropin-independent with no LH or FSH support. Follicular growth then becomes dependent on the continuous presence of gonadotropins; follicles that do not grow remain as primary oocytes within unilamellar follicles during prepubertal and reproductive periods. This provides a pool from which groups of small follicles are recruited for further maturation, resulting in preovulatory or Graafian follicles. There are three stages to follicular growth: oocyte enlargement, transition of granulosa cells, and formation of the zona pellucida, an extracellular matrix present between the granulosa cells and oocyte. The first stage is characteristic of small follicles[48] that enter the pool of committed growing follicles. Follicle growth requires five events: continued oocyte enlargement, rapid proliferation and increase in layers of granulosa cells, formation of basal lamina, organization of endocrine thecal cells around a basal lamina, and formation of the antrum. The majority of follicles grow into the preantral stage. A large surge of gonadotropins in the cycle preceding ovulation results in selection of a few antral stage follicles from a pool of preantral follicles.

The preovulatory surge in gonadotropin stimulates conversion of progesterone to androstenedione in theca cells,[49] which is then converted to estradiol in granulosa cells. The estradiol secreted by the growing follicles, in conjunction with FSH, effects differentiation of granulosa cells. Only follicles that can produce estradiol progress to preovulatory follicles. Oocyte atresia can be induced by any agent that inhibits either the theca cell's ability to synthesize androstenedione or the granulosa cell's function to synthesize estradiol. Atresia may also be caused by agents that alter gonadotropin receptors or the functional coupling of the receptor to adenylate cyclase.[49]

Primary oocytes in preovulatory follicles continue to form secondary oocytes through the first meiotic division and remain in metaphase of the second division. The first polar body, containing half of the chromosomes, is extruded. As ovulation nears, the follicle vascularizes and swells, becoming a macroscopically visible blister-like protuberance. The secondary oocyte is ovulated at metaphase II and stays in this stage until fertilization. At fertilization, the second meiotic division is completed, the second polar body extruded, and the female pronucleus formed. Male and female pronuclei combine at fertilization to regain the diploid state.[50]

Fertilization and Implantation

Fertilization is the union of a spermatozoon and an oocyte and occurs in the female reproductive tract in mammals. This restores the diploid number of chromosomes, determines the genetic sex of the zygote, and initiates rapid mitotic division.[51] Following ovulation, vascularization of the granulosa cell layer occurs, and granulosa cells are transformed into luteal cells, which produce the progesterone required for preparation of the endometrial lining of the uterus for implantation of the conceptus.[52] In the absence of fertilization, the process by which a postovulatory ovarian follicle vascularizes and

TABLE 35.1
Ovarian Germ Cell Development in Mammalian Species

Species	Length of Gestation	Gonadal Sex Differentiation	Initiation of Meiosis	Arrest of Meiosis
Mouse	19	12	13	(5)
Rat	21	13–14	17	(5)
Hamster	16	11–12	(1)	(9)
Rabbit	31	15–16	(1)	(21)
Monkey	165	38	56	Newborn
Human	270	40–42	84	Newborn

Sources: Adapted from Goldman, J.M. et al., *Birth Defects Res. Part B*, 89, 84, 2007; Richards, J.S. and Bogvich, K., Development of gonadotropin receptors during follicular growth, in: Maresh, M., Saxena, and Sadler (eds.), *Functional Correlates of Hormone Receptors in Reproduction*, Elsevier/North Holland, Amsterdam, the Netherlands, 1980, pp. 223–244.

Note: Numbers indicate days of gestation. Numbers in parentheses indicate days of postnatal age.

transforms into a corpus luteum occurs (luteinization). This process includes degeneration of the ovulated oocyte, continued LH stimulation, and luteinizing of the empty follicle into a corpus luteum (Latin for *yellow body*), which secretes progesterone. This process continues to occur throughout reproductive life until all primordial follicles are depleted or menopause occurs.

The corpus luteum is dependent on LH to function, and withdrawal leads to luteal failure, decreased estrogen and progesterone secretions, and failure to maintain the pregnancy. In a normal cycle without fertilization, corpus luteal failure occurs after approximately 10 days of functioning. This failure is associated with an increase in the activity of prostaglandins.[53] Once the oocyte is fertilized, the corpus luteum is maintained by secretion of human chorionic gonadotropin (HCG), which is an LH-like molecule synthesized by the trophoblastic tissue of the embryo. Under HCG stimulation, progesterone synthesis continues in the corpus luteum until this steroid is principally produced by the placenta. The corpus luteum is also a source for oxytocin, a mammalian hormone that acts primarily as a neuromodulator in the brain.

Ovulatory Cycles

The process of ovulation is controlled by the hypothalamus of the brain and through the release of hormones secreted in the anterior lobe of the pituitary gland, LH and FSH. Ovulatory cycles vary widely in laboratory animals, farm animals, nonhuman primates (NHP), and humans[45] and among humans.[54] Two major categories exist: (1) animals that spontaneously ovulate and (2) animals that are induced to ovulate by mating. Spontaneous ovulators include laboratory rodents, hamsters, mice, guinea pigs, sheep, pigs, rhesus monkeys, baboons, and humans. Reflex or induced ovulators include rabbits, cats, ferrets, short-tailed shrews, and voles. Mechanical or coital stimulation in these species results in a gonadotropin release of primarily LH within 1–2 h that results in ovulation of follicles that have reached maturity in the ovary. The FSH secreted in the surge may result in the development of new ovarian follicles for estradiol production, which is essential for continued corpus luteum function.

Circadian rhythms and seasonal variation are two other neuroendocrine signals that are important in regulating ovulatory cycles. Humans and guinea pigs are species least affected by circadian rhythms and seasonal variations. In rodents, the suprachiasmatic nucleus in the hypothalamus is identified as the site of signal transduction by light-dark cycles, and lesions at this site, as well as exposure to constant light, can terminate the estrous cycle. Seasonal variations associated with the duration of the photoperiod are known to affect reproduction. Most laboratory species require a minimum of 10 h of light for reproduction to occur. Diets with phytoestrogen content are also known to affect ovulatory cycles and fertility. Phytoestrogens are naturally occurring diphenols that have structural and functional similarities to 17β-estradiol.[54,55]

Oocyte maturation, folliculogenesis, ovulation, and corpus luteum formation are processes under the influence of gonadotropins and occur during each menstrual cycle in humans. Many of these events also occur in rodents, although factors other than gonadotropins appear to be involved in the initial stimulation of growth of the resting follicle. Once the follicle is committed to growth, the selection of preovulatory follicles depends on the LH surge proceeding each cycle.[42]

Ovarian Physiology and Toxicology

Ovarian weights should be recorded at necropsy close to the time of removal and trimming to avoid dehydration. Ovarian weight in the normal rat does not show significant fluctuations during the estrous cycle; however, polycystic ovaries, luteal cysts, decreases in corpus lutea or oocytes, and reproductive senescence can alter weight changes. Evaluation of follicular alterations is difficult because a sexually mature ovary contains a diverse population of resting, maturing, and mature follicles, and the female has only one cluster of follicles that is selected for maturation in each cycle. However, the oogonia enter meiosis during fetal life, and there is no mechanism to replace oocytes, so reduction of the numbers of oocytes has the potential to reduce the reproductive life span of the female. During adulthood, each of the three components of the follicle may be uniquely susceptible to specific toxicants.[56] A xenobiotic agent, for example, might increase the progesterone/estradiol or testosterone/estradiol ratios by FSH stimulation or alter steroidogenesis and androgen production in the thecal cells, thus delaying follicle maturation and ovulation.[57]

Primordial follicle counts are among the most variable reproductive endpoints measured across laboratories.[58] This is probably related to the variability in the number of animals, number of histologic sections, and selection of ovarian sections examined since they are not specified in current U.S. Environmental Protection Agency (EPA) and OECD (Organisation for Economic Co-operation and Development) guidelines.[59] There is an inherent intra- and interanimal variability in the ovarian follicle pool: 30%–40% variability in counts between ovaries taken from the same rodent and even greater variability between ovaries from different rodents of the same strain.[60]

Reproductive Endocrinology and Toxicologic Interactions

An in-depth review of endocrine disruptive chemicals and the field of endocrine disruptors will be presented in the Chapter 36.

Interaction of Reproductive Hormones and Target Tissues

Estrogens increase oviduct secretions and muscular contractions, actions antagonized by progesterone. Thus, cyclic patterns of estrogen and progesterone levels that occur during the menstrual cycle in primates and humans play important roles in regulating sperm transport, through the cervix, into the uterine

lumen and to the oviduct.[61] Of the 150–300 million sperm in an ejaculate, less than 500 (<0.001%) reach the site of fertilization, with the greatest loss of sperm at the cervix and uterotubal junction. Capacitation is the changes sperm undergo in the female reproductive tract, allowing penetration of the ovum, and is normally induced by secretions in the female genital tract that require several hours to occur. Capacitated sperm penetrate the layers of the granulosa cells and bind to a major glycoprotein in the zona pellucida, which is the main barrier to fertilization. The release of proteases and hyaluronidase from the binding sperm is essential for sperm penetration of the zona. Contact of the sperm and oocyte membrane after penetration triggers a cortical reaction in the egg, releasing enzymes that prevent further binding and entry of other sperm.[62]

The fertilized ovum divides and slowly moves down the oviduct. Decreased motility in the oviduct is associated with secretions by the corpus luteum and prevents the ovum from prematurely reaching the uterus. The *morning-after* pill accelerates ovum migration by levonorgestrel, a progestin, resulting in premature reaching of the uterus.[63] Similar effects have been observed with methoxychlor, which mimic estrogens.[64] The embryo floats free in the uterus before implantation, nourished by endometrial gland secretions under the control of progesterone. Combined estrogen and progesterone action result in the uterine endometrium preparing for implantation of the blastocyst and the embryo invading the uterine endometrium. In humans, decidualization of the uterine lining normally occurs during the luteal phase of the menstrual cycle, whether or not implantation occurs (Figure 35.2). Decidualization of the uterine lining contributes the maternal portion of the placenta.[45]

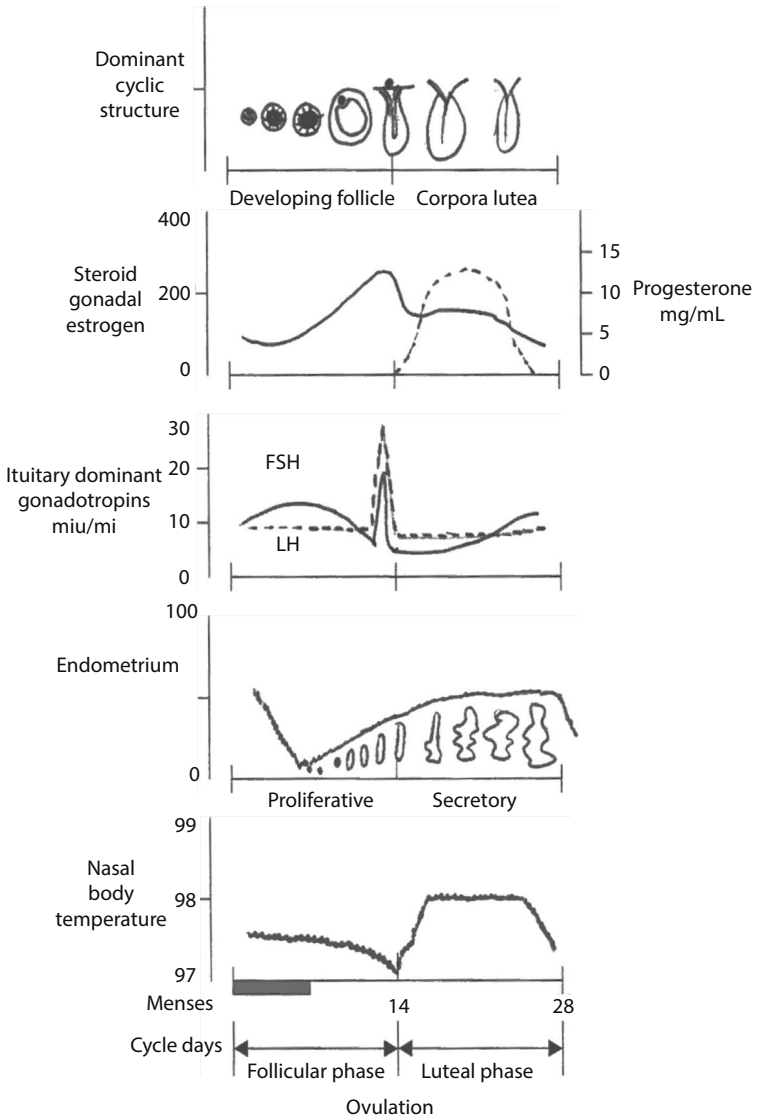


FIGURE 35.2 Progression of ovulatory cycle in humans. Development of the dominant follicle of pituitary gonadotropins, endometrial proliferation, and basal body temperature are depicted. (Adapted from Goldman, J.M. and Cooper, R.L., Normal development of the female reproductive system, in: Kapp, R.W. and Tyl, R.W. (eds.), *Reproductive Toxicology*, 3rd edn., Informa Healthcare, New York, 2010, pp. 36–50; Cohen, G.M., *Biochem. J.*, 326(1), 1, 1997.)

Estrogen and progesterone synthesis and secretion during normal human pregnancy are a cooperative effect of the mother, fetus, and placenta. Large amounts of estradiol, estrone, and estriol are produced during pregnancy, with the placenta being the primary source. A biomarker used to monitor fetal well-being is estriol because one of the precursors for its synthesis by the placenta is dehydroepiandrosterone, which is produced by the fetal adrenal gland.[65]

The placenta is the connection between fetal and maternal systems and has many functions: an endocrine organ; the exchange of gases, nutrients, and metabolic wastes; and protects the fetus from physical and potential chemical harm.[66] It also secretes human placental lactogen, a protein that is similar to human growth hormone and human prolactin, but does not appear to promote body growth. Lactogen prepares the mammary gland for lactation and usually appears in the blood approximately 2 months after fertilization, increases until parturition, and is used as a biomarker to monitor placental size and growth.[61] Prolactin is secreted by the anterior pituitary gland and controls mammary development in women. Prolactin levels continually increase during pregnancy from about 2 months after fertilization, until parturition.[67]

TABLE 35.2
Steroid Source and Mechanism of Action for Preparturition Progesterone in Several Species

Species	Source of Steroids in Late Pregnancy	Fall in Maternal Progesterone	Mechanism
Mouse	Corpus luteum	Yes	Luteolysis
Rat	Corpus luteum	Yes	Luteolysis
Rabbit	Corpus luteum	Yes	Luteolysis
Guinea pig	Placenta	No	—
Goat	Corpus luteum	Yes	P450C17/ Luteolysis
Sheep	Placenta	Yes	P450C17
Monkey	Fetoplacental unit	No	—

Note: P450C17 enzymes are induced in the placenta by an increase in fetal cortisol levels. Luteolysis is provoked by prostaglandin $F_{2\alpha}$ acting on fetoplacental receptors.

Source: Adapted from Soloff, M.S., Endocrine control of parturition, in: Wynn, R.M. and Jollie, W.P. (eds.), *Biology of the Uterus*, 2nd edn., Plenum Press, New York, 1989, pp. 559–607.

Parturition

Parturition, process of labor and delivery, represents the transition of the fetus to the neonate and occurs when the fetus and its associated membranes are expelled to the external environment. The exact mechanisms that initiate parturition in humans are not well understood; however, maturation of the fetal hypothalamic–pituitary–adrenal axis plays an important role, and most current evidence suggests that estrogen, progesterone, oxytocin, and prostaglandins may all be involved.[61] Fetal cortisol elevations activate the placental steroidogenic enzyme system, resulting in decreased progesterone and elevated estrogen prior to parturition.[68] The fall of maternal progesterone is a necessary prerequisite for parturition, but source and mechanism for the endocrine changes are not uniform across species: the source of progesterone varies (placenta or corpus luteum) and is dependent on the mechanism of action (MOA) (luteolysis, the structural and functional degradation of the corpus luteum, or P450C17 metabolism in the placenta enabling progesterone to be converted to estrogen) (Table 35.2). Luteolysis is responsible for the physiological reduction of maternal progesterone levels preceding parturition in the common laboratory species rat, mouse, and rabbit; however, P450C17 metabolism is responsible in the goat and sheep.[69] In the primate and guinea pig, there is no apparent change in maternal progesterone levels before parturition.

As labor approaches, estrogen and oxytocin stimulate uterine muscle contractility.[70] Progesterone antagonizes the effects of progesterone and oxytocin, and the decreasing progesterone levels trigger uterine contractions, although peripheral progesterone concentrations do not fall prior to the onset of labor. The decrease in levels of progesterone is thought to occur before peripheral changes, and that local prostaglandin production may also be involved in initiating labor.[70] The fall in progesterone levels following delivery is one of the triggers for milk production, since it inhibits lactation during pregnancy. Stretching of the uterine cervix and vagina stimulates reflex release of oxytocin from the posterior pituitary, resulting cyclically in increased uterine contractions and stretching, facilitating the delivery process. The increase in oxytocin receptors near term may be the cause of the increased sensitivity of the myometrial response to oxytocin.[71] Physical factors also regulate myometrial activity in the human, of which the increase in uterine volume associated with fetal and placental growth, resulting in stimulation of myometrial contractions, and the inhibition of myometrial contractions by progesterone are the most important. Estrogens, oxytocin, and PGF_2 are stimulatory hormones additionally contributing to uterine contractility and inhibited by progesterone, which blocks estrogen action, oxytocin receptor production, and prostaglandin release. Drugs, such as alcohol, can directly act on uterine smooth muscle or indirectly inhibit oxytocin release.[61]

Lactation

Lactation describes the secretion of milk from the mammary glands and the period of time that a mother lactates to feed her young. Lactogenesis is a two-stage process: (1) the enzymatic and cytologic differentiation of the mammary alveolar cells and (2) the secretion of milk, which normally begins after parturition. The first stage requires growth hormone, aldosterone, prostaglandins, insulin, estrogens, progesterone, placental lactogens, and prolactin and generally occurs in the third trimester of pregnancy. The second stage is initiated by prolactin secretion by the lactotropes located in the anterior pituitary. These hormones modulate the development and secretory capacity of the mammary gland. Prolactin is

the primary hormone responsible for increased growth and differentiation of the alveoli and influences differentiation of ductal structures. Gonadotropin hormones stimulate the ovaries to secrete estrogen that work indirectly on the mammary gland to promote prolactin synthesis.

The posterior pituitary gland secretes oxytocin as the result of stimulation of the nipple by the suckling reflex. While prolactin release from suckling does not cause the milk release at the time of nursing, it is responsible for release of milk (milk letdown) during the subsequent nonsuckling interval. The young obtain this milk at the next nursing. Normal milk secretion also requires other hormones, including ACTH, insulin, growth hormone, parathyroid hormone, and thyroid hormones.[72] Milk letdown in humans is a conditioned reflex phenomenon that can be affected as a response to the presence or crying of a baby.[72] Initiation of milk secretion will not occur until the placenta is removed by abortion or delivery because of estrogen and progesterone secreted by the placenta.[72] Most common oral contraceptives contain estrogen and progesterone and can suppress lactation; therefore, they should not be used during the nursing period.[61]

Thelarche is the onset of secondary (postnatal) breast development in humans and usually occurs at the beginning of puberty in girls 8 years and older. Estrogens are the primary ovarian hormones responsible for mammary gland development and growth, although progesterone is important for alveolar development in some animals. However, there is no evidence that progesterone is required for alveolar development in humans.[72] Initiation of lactation normally involves withdrawal of an estrogen and progesterone block to the stimulatory effect of prolactin on milk secretion.[61]

Lactating women have a diminished response to GnRH as a result of reduced LH and FSH secretion during lactation.[73] The ovaries seem to be refractory to gonadotropic stimulation during lactation, although the mechanisms resulting in lactational amenorrhea are not currently understood. Several conditions are known to result in abnormal lactation associated with increased prolactin levels: pituitary tumors can cause this observation and hypothalamic lesions, abnormal afferent neural input into the hypothalamus, and tranquilizers can also raise prolactin secretion sufficiently to result in galactorrhea, the spontaneous flow of milk from the breast unassociated with childbirth or nursing.[72]

Ovarian Cyclicity

Normal ovarian function is predicted by the appropriate interactions of the various compartments of the ovary and the changes that occur within these compartments during the cycle. In vivo adverse effects may result from active intermediates or metabolites or through modulation of hypothalamic–pituitary axis; such effects would not be identified by in vitro assays.

Distinct differences exist between ovarian cyclicity in humans and rodents that should be considered in designing toxicologic studies. For example, in most mammals, follicular growth continues through the luteal phase. Luteolysis is mediated by uterine prostaglandins in rats and NHP, whereas, in humans, follicular growth does not continue through the luteal phase, and estrogen, rather than prostaglandins, is the luteolytic agent.[74] Other differences among species include maintenance of progesterone production by the corpora lutea throughout pregnancy in the rat, while in humans and guinea pigs, placental progesterone contributes significantly to maintenance of pregnancy during mid- and late gestation.[75] Progesterone levels in rats must be reduced to induce parturition, while humans and guinea pigs have high levels of progesterone until parturition. Deciduoma formation in rats is highly dependent on a correct progesterone/estrogen ratio, and abortion occurs if the serum estrogen level is increased, while humans and guinea pigs are estrogen-resistant.[75] Prostaglandins, however, have abortifacient properties in humans and guinea pigs. Thus, hormonal similarities between guinea pigs and humans and the greater similarities in timing of fetal in utero development suggest that the guinea pig would make a better model for human risk assessment on ovarian function than the rat, although this model has not been as extensively studied.

The typical estrous cycle in rats lasts 4–5 days and varies from 5% to 30% with some rat strains, animal suppliers, and individual shipments.[76] Estrous cycle reporting is also complicated by the fact that the cycling stages are so short in duration, once daily smears may miss stages and infer an incorrect cycle or duration evaluation.[77] Estrous cycle data are generally used to complement other data and do not typically indicate an adverse effect alone.[76] However, in a collaborative study looking at known female reproductive toxicants,[78] effects on estrous cycle were apparent at lower doses than other functional endpoints. An excellent report to consult for background and methods is Goldman et al.[44,79]

Reproductive Behavior

The rat is frequently considered to provide a model system to study central nervous system (CNS) function and behavioral development based on the large amount of normal and abnormal data available. Neural and molecular mechanisms for female reproductive behaviors in the rat have been widely investigated. The components of reproductive behavior can be divided into proceptive and receptive.[80] Proceptive behaviors include the approach and solicitation by the male, followed by the pacing of the mating interaction. Receptive behavior consists of the lordosis reflex (arching of the back, elevation of the head and rump, and deflection of the tail). Although not the model most used for basic research, female monkeys show clear changes in behavior during the menstrual cycle and subsequent to injection of steroid hormones. Copulation in NHP is restricted to the periovulation period. Similar findings have not been made in women, although the absence of clear findings may reflect methodological problems inherent in the conduct of such studies.[81] Humans have sexual behaviors that are dependent on steroid hormone secretion during puberty, and perceptual and sensory behaviors vary within the menstrual cycle, as well as the susceptibility to irritability, anxiety, and depression during the premenstrual

period.[82] The early postpartum period in humans is known to be an interval with emotional alterations in many women and severe disturbances in some women.[83]

Alteration of Hypothalamic–Pituitary Axis/Ovarian Feedback

Effects on female reproduction are generally studied by evaluation of many endpoints (Table 35.3). Toxic effects may be produced by targeting the hypothalamic–pituitary axis, the ovary, or any point in the complex feedback system. Examples of some agents known to adversely affect female reproductive performance are cited in Table 35.4.

Altered hypothalamic and pituitary secretions can adversely affect fertility and estrous cyclicity in female rats.[74] A substituted triazole[84] inhibited ovarian estradiol synthesis, with resultant delay in the LH surge and ovulation. Further study of this fungicide found that pituitaries from treated rats had LH production after GnRH stimulation.[85] This chemical was later identified as an aromatase inhibitor, preventing ovarian synthesis of estradiol.[74]

Ovarian Morphometry

Ovarian endpoints that may be affected by xenobiotic exposure include oocyte number and follicular development, ovulation, estrous cycling, fertility, and maintenance of pregnancy. There are excellent reviews on ovarian follicular development[86] and follicle quantitation and morphometrics.[87] Models used to evaluate dose- and stage-dependent, as well as age-dependent, effects of xenobiotic exposures of the ovary generally utilize morphometry, which can be performed either as part of general toxicity evaluations or within the framework of reproductive toxicity studies. Histopathological evaluation of the three major compartments of the ovary (i.e., follicular, luteal, and interstitial) plus the capsule and stroma should be performed to reveal possible toxicological conditions. Original methods were extremely time-consuming and expensive (30–60 sections per ovary, resulting in 300–600 sections per group of 10 animals) to be evaluated; however, current screening methods generally are restricted to every 10th or 20th section. Even though these methods have been incorporated into some guidelines, they produce highly variable results that should not be used as the sole endpoint in a risk assessment.

Corpora Lutea Count and Preimplantation Loss

Evidence of an adverse event at the ovarian level in in vivo studies is most frequently based on reduced numbers of corpora lutea, often in combination with preimplantation loss. Preimplantation loss reflects the number of eggs ovulated, fertilized, and implanted, as well as the receptivity of the uterus. Rabbits and ferrets are reflex (induced) ovulators, requiring stimulation of the cervix, whereas humans and most laboratory animals are spontaneous ovulators. When artificial insemination procedures are used for breeding rabbits, an intravenous injection of HCG is generally administered to compensate for reduced natural cervical stimulation during mating. Artificial insemination procedures in rabbits result in greater variability in ovulation, numbers of corpora lutea and preimplantation loss values than natural mating because of several factors. First, release of ova depends on the number of mature follicles present when ovulation is induced by the stimulation of natural mating or injected HCG. One major

TABLE 35.3
Scheme for Identifying Effects on Female Rat Reproduction

Step	Treatment	Activity	Potential Adverse Effects
1	None—14 days	Identify estrous cycling.	Abnormal cycling—if incidence is high, consider environmental factors.
2	Precohabitation—up to 14 days	Identify estrous cycling.	Alterations from treatment—assume hormonal changes.
3	Cohabitation through gestation	Mate females with untreated males (1:1). Observe mating behavior (receptivity) and fertility (persistent diestrus, sperm or plug).	Reduced or absent mating behavior or irregular estrous cycling.
4	Through day before sacrifice	Sacrifice 1/2 preselected pregnant animals per group at GD 21. Observe gross lesions, corpora lutea, pregnancy, implantation numbers, resorptions, live and dead fetuses, fetal body weights, and sexes.	Reduced corpora lutea, implantation sites, live fetuses; reduced fetal body weight; altered sex ratios.
5	Through delivery and lactation	Allow remaining 1/2 of animals per group to deliver. Observe durations of gestation and parturition, maternal behavior peri-partum, pup number, viability, body weight, sex and morphology at birth, pup viability, growth, clinical signs and interaction with dam to weaning, and maternal implantation sites.	Reduced implants; altered gestation or parturition durations; reduced maternal care during lactation; reduced total litter size, live litter size, and/or pup viability; reduced or increased pup weight; altered pup sex ratio; altered pup morphology; reduced pup growth and viability; altered function.

Source: Adapted from Chapin, R.E. and Heindel, J.J., Introduction, in: Heindel, J.J. and Chapin, R.E. (eds.), *Methods in Toxicology, Vol. 3B Female Reproductive Toxicology*, Academic Press, San Diego, CA, 1993, pp. 1–15.

TABLE 35.4
Examples of Agents Producing Adverse Female Reproductive Effects

Agents	General Mechanism	Potential Toxicity
Ethanol, DDT, isoflavones	Altered puberty, estrous/ menstrual cycling	Altered ovarian activity and hypothalamic–pituitary feedback
DDT, kepone	Impaired ovulation	Altered endocrine signal
β-Endorphin, naloxone	Altered mating behavior	Altered modulation
Progesterone, diethylstilbestrol, estrogens, methoxychlor	Altered gamete/embryo transport	Increased urine contractions
Estrogens, DDT, methoxychlor, kepone, EGME	Suboptimal endometrial environment	—
Alkylating agents, chemotherapeutic agents (e.g., prednisone, vincristine, vinblastine, 6-mercaptopurine, radiation, methotrexate, adriamycin), alcohol, polycyclic aromatic hydrocarbons, 4-vincyclohexene, cyclophosphamide	Ovarian toxicity/oocyte destruction/atresia	Mimic structure of naturally occurring hormones; general chemical reactivity
Aminoglutethimide, 3-methoxybenzidine, cyanoketone, estrogens, azastene, danazol, spironolactone, epostane, triazole, fungicide	Altered steroid synthesis	Inhibition of steroidogenic enzymes
Clomiphene citrate, cimetidine, spironolactone, opioid peptides	Antagonized steroid action	Inhibited steroid activity
Marijuana	Inhibition of gonadotropins	Alterations at hypothalamic level
Tranquilizers (e.g., imipramine, clomipramine, buspirone)	Altered maternal behavior/lactation	Alterations at hypothalamic level
Tranquilizers (e.g., trifluoperazine, diazepam, thioridazine, fluphenazine)	Impaired lactation	Altered prolactin levels

Sources: Developed from Haney, A.F., Effects of toxic agents on ovarian function, in: Thomas, J.A., Korach, K.S., and McLachlin, J.M. (eds.), *Endocrine Toxicology*, Raven Press, New York, 1985, pp. 181–210; Cummings, A.M. and Perreault, S.D., *Toxicol. Appl. Pharm*., 102, 110, 1990; Elger, W. et al., Interference with hormonal control of rodent reproduction and its implications for human risk assessment, in: Valans, G.N., Sims, J., Sullivan, F.M., and Turner, P. (eds.), *Proceedings of the Fifth International Congress of Toxicology*, Taylor & Francis, New York, 1990, pp. 445–456; Pfaff, D.W. and Schwartz-Giblin, S., Cellular mechanisms of female reproductive behaviors, in: Knobil, E. and Neill, J.D. (eds.), *The Physiology of Reproduction*, Raven Press, New York, 1988, pp. 1487–1568; Bolon, B. et al., *Fundam. Appl. Toxicol*., 39, 1, 1997.

consequence of HCG priming of the female is the possibility of *superovulation*, that is, a large number of eggs ovulated, including ones not completely mature, and subsequent inability of these eggs to be fertilized or implanted, resulting in a large percentage of preimplantation loss.[88] Second, fertilization depends upon multiple factors, including the quality and quantity of both the eggs and the sperm, as well as the timing of priming, ovulation, and insemination. Artificial insemination is generally performed using one introduction of diluted sperm into the primed female's vagina.

Natural breeding may involve one or more intromissions and inseminations, generally increasing the number of sperm inseminated, cervical stimulation of the female, associated hormonal changes and ovulation of appropriately aged eggs, and the number of egg fertilized and implanting. A summarization of the placental, uterine, and other gestational parameters of humans and six commonly used laboratory mammals for reproductive toxicology studies is presented in Table 35.5.

DEVELOPMENTAL TOXICOLOGY

As early as 1973, Wilson[20] expressed the concept of developmental toxicology: "The unborn is not only the embryo and the fetus in utero but also the as yet unconceived individuals whose potential for future development is represented in the germ cells residing in the parental gonads. Also of interest….are those who have already been born but who are incompletely developed." Wilson also expanded the definition of teratology (study of congenital malformations) to "the study of the adverse effects of environment on developing systems, that is, on germ cells, embryos, fetuses, and immature postnatal individuals," and stated, "a more comprehensive definition is that teratology is the science dealing with the causes, mechanisms, and manifestations of developmental deviations of either structural or functional nature.[89]" Based on these definitions, developmental toxicology can be considered to be the study of the entire reproductive process, with special emphasis on the developing conceptus, but including the development and function of that conceptus throughout its entire life span.

Wilson's basic principles[20] remain valid. All investigators in the field should be familiar with their details (see the following; to comply with current usage, *teratogenesis* has been made synonymous with *developmental toxicity*):

1. Susceptibility to developmental toxicity depends on the genotype of the conceptus and the manner in which this interacts with adverse environmental factors.
2. Susceptibility to developmental toxicity varies with the developmental stage at the time of exposure to an adverse influence.
3. Developmental toxins act in specific ways (mechanisms) on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis).

TABLE 35.5
Historical Values for Mean% Preimplantation Loss in Hra: (NZW)SPF Rabbits

Facility and Mating Procedure	Number of Studies, Time Period, Number of Does	Mean, Standard Deviation, and Range per Study
Artificially inseminated—hCG-primed rabbits		
RTI—Tyl and Marr[320]	88 does Years and number of studies Not reported	30.90% ± 2.9 (range not reported)
Charles River, Pennsylvania	98 studies (1980–1989)	17.8% (SD not reported)
Feussner[a]	1463 does	Range = 8.2% to 38.4%
Naturally mated rabbits		
RTI—Tyl and Marr[320]	117 does Years and number of studies Not reported	11.34% ± 1.49
Charles River, Pennsylvania (historical control data)[b]	30 studies June 2009–June 2011 624 does	5.8% (SD not reported) Range = (1.6% to 15.2%)

[a] Feussner EL, Lightkep GE, Hennesy RA, Hoberman AM, Christian MS. A decade of rabbit fertility data: Study of historical control animals. *Teratology* October 1992;46(4):349–365.

[b] http://www.criver.com/en-U.S./ProdServ/ByType/Preclinical/ToxServ/Pages/Developmental_and_Reproductive_Toxicology.aspx.

4. The access of adverse influences to developing tissues depends on the nature of the influence (agent).
5. The four manifestations of deviant development are death, malformation, growth retardation, and functional deficit.
6. Manifestations of deviant development increase in frequency and degree as dosage increases, from the no-effect to the totally lethal level.

Schmidt and Johnson[90] provided an elegant refinement of Wilson's principles, including that while "teratogenesis *per se*, has its primary focus on embryogenesis, developmental biology, and molecular genetics, the field of developmental toxicology also involves basic principles of toxicokinetics, dose-response relationships, target organ toxicity and exposure assessment."

Normal Development

The following information is provided as an overview of the complex process of normal development and also as an introduction to differences in developmental events in laboratory animal species and humans. For additional information on specific facets discussed, the reader is recommended to reviews by DeSesso,[91] Klinefelter and Gray,[74] Rogers and Kavlock,[92] and Shield and Mirkes,[93] as well as to basic texts[94] on embryology and developmental biology. Much of the following information is excerpted from these sources. For the interrelationship of the reproductive process in the maternal animal, vis-à-vis the developing conceptus, the reader is referred to the prior sections in this chapter.

As noted by Rogers and Kavlock,[92] development is characterized by changes in size, biochemistry and physiology, and form and functionality. The overall process is orchestrated by a cascade of gene transcription regulating factors, the first of which are present in the egg before fertilization. These factors activate regulatory genes in the embryonic genome, with sequential gene activation continuing throughout development.

Development of the Conceptus

Preimplantation

The development of a post-fertilization mammalian conceptus occurs within the uterine tubes and fundus of the uterus. After fertilization, there is a fusion of genetic material of haploid egg and sperm to form a diploid zygote. Sperm activate the egg's metabolism to start development. The development of the conceptus from the zygote to neonate is a complex process of extremely rapid cell proliferation and apoptosis (programmed cell death). In most laboratory species, the time from sperm penetration to first cleavage requires approximately 12 h. As the fertilized oocyte (zygote) travels down the uterine tube to the uterus, cleavage continues with growth to the morula stage. During this time, the zygote is surrounded by an acellular mucopolysaccharide layer, the zona pellucida, which previously prevented sperm penetration and now prevents premature implanting. In most mammals, the zona pellucida disappears at the blastocyst stage and the morula cavitates between 5 and 8 days of gestation. The preimplantation embryo has remarkable regulative (restorative) growth potential,[95] and it has been shown that one cell from an eight-celled rabbit embryo can produce a normal offspring.[96] The preimplantation period was generally identified historically as a period during which toxic insult generally results in embryo death or absence of effect, because of the regenerative powers of the embryo.[97] Currently, the preponderance of evidence shows all or nothing concept of *either death or recovery* does not always apply to preimplantation embryos (i.e., surviving embryos may demonstrate growth retardation, malformation, and/or functional impairment). It has been shown that preimplantation exposure to some agents affects the ultimate growth and development of the embryo, for example, actinomycin D and methotrexate, among others.[19,97,98] Therefore, the entire embryonic and fetal stages of development are susceptible to toxic insult, and because many systems continue to develop after birth (e.g., lungs, immune system, reproductive organs), the postnatal period is also susceptible to toxic insult.

Implantation and Placentation

Cavitation is followed by attachment of the blastocyst to the uterine wall (nidation) and subsequent invasion of the uterine wall (implantation) by the syncytiotrophoblast, which erodes the endometrium. Placental circulation is subsequently established.[99] Each blastocyst includes two different cell populations, the outer layer (trophoblast), which becomes the placenta and fetal membranes, and the inner cell mass (cluster of cells within the blastocyst), which becomes the embryo. Although sometimes not considered in the overall development of the embryo, the trophoblast serves an important function: the extraembryonic membranes protect the conceptus, while the placenta provides a means to supply nutrients and remove metabolic waste. There are multiple diverse mechanisms for placental transport of molecules, including both simple diffusion and carrier-mediated mechanisms (active transport, facilitated diffusion, receptor-mediated endocytosis). The placenta is clearly not a barrier, but rather a means by which a substance in the maternal system will, at some rate and by some mechanism, be transported into the embryo. Remarkable differences exist across species with respect to the layers of embryonic and maternal tissues interposed between the respective circulations, and differences in the duration and functions of the yolk sac among species, which can affect the rate and access of a test material to a conceptus. In the addendum to ICH S6 (May 2012),[100,101] these differences have been used to alter the classical design of developmental and reproductive toxicology (DART) studies in NHP.[101,102] In the NHP and human, IgG placental transfer is low during organogenesis and begins to increase in early second trimester, reaching highest levels late in the third trimester.[103] Therefore, standard embryo–fetal studies in NHPs, which are dosed from early pregnancy gestation day (GD) 20 to GD 50, might not be of value, although effects on embryo–fetal development as an indirect result of maternal effects can be evaluated. Furthermore, maternal dosing in NHP after delivery is generally without relevance as IgG is only excreted in the milk initially (i.e., in the colostrum), and not later during the lactation and nursing phase. Rodents differ from the NHPs and humans, as IgG crosses the yolk sac in rodents by neonatal Fc receptor (FcRn) transport mechanisms and exposure can occur relatively earlier in gestation than with NHPs and humans. In addition, delivery of rodents occurs at a stage of development when the pups are not as mature as the NHP or the human neonate. Therefore, rat/mouse dams should be dosed during lactation in order to expose pups via the milk up to at least day 9 of lactation when the offspring are at an equivalent stage of development as human neonates.

Embryogenesis

The rapid growth of the conceptus continues through embryonic, fetal, and neonatal stages. The duration of these stages differs in various species, as shown in Table 35.6.[104] Comparative development of organ systems (e.g., renal, lung, heart, bone, gastrointestinal tract, male and female reproductive systems, immune system, and CNS) of common laboratory species are included in *Birth Defects Research* (Part B) Volume 68.

TABLE 35.6
Timing of Early Development in Some Mammalian Species

	Times of Early Development (Days from Ovulation)			
Mammal	**Blastocyst Formation**	**Implantation**	**Organogenesis Period**	**Length of Gestation**
Mice	3–4	4–5	6–15	19
Rats	3–4	5–6	6–15	22
Rabbits	3–4	7–8	6–18	33
Sheep	6–7	17–18	14–36	150
Monkeys (rhesus)	5–7	9–11	20–45	184
Humans	5–8	8–13	21–56	267

Source: Adapted from Brinster, R.L., Teratogen testing using preimplantation mammalian embryos, in: Shepard, T.H. and Miller, J.R. (eds.), *Methods for Detection of Environmental Agents That Produce Congenital Defects*, American Elsevier, New York, 1975.

The period of embryogenesis (organogenesis) is generally identified as the interval between implantation (and formation of the neural plate in the ectoderm) and closure of the hard palate. Most organ systems are formed during this period, requiring cell proliferation, cell migration, cell–cell interactions, and morphogenetic tissue remodeling. Each forming structure has a period of maximum susceptibility, with peak susceptibility to insult coinciding with the time key developmental events occurring in these structures. Wilson's classic diagram (Figure 35.3)[105] demonstrates the pattern for the rat embryo. The varying susceptibilities in the hamster, mouse and guinea pig have been demonstrated.[106] In toto, these investigations demonstrate that peak sensitivity may not only differ for a given tissue/organ, but also with the administered dose, reverting to Wilson's concepts.[18,20,105] In addition, the same insult may affect the growth of concurrently developing

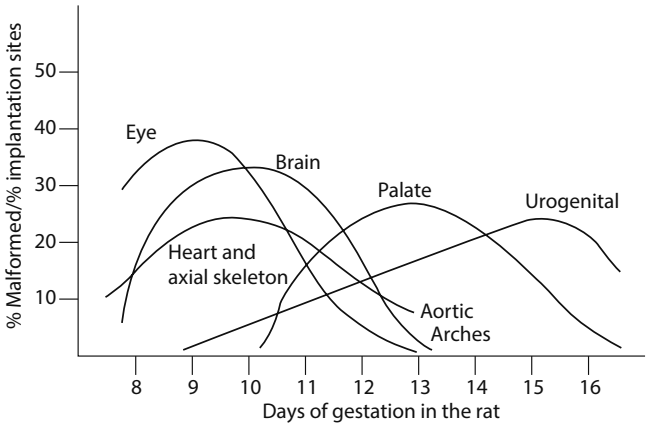


FIGURE 35.3 Hypothetical pattern of susceptibility of embryonic organs to teratogenic insult. (Adapted from Japan Ministry of Agriculture Forestry and Fisheries, *Guidelines for Screening Toxicity Testing of Chemicals*, 59 NohSan No. 4200, 209, 1985.)

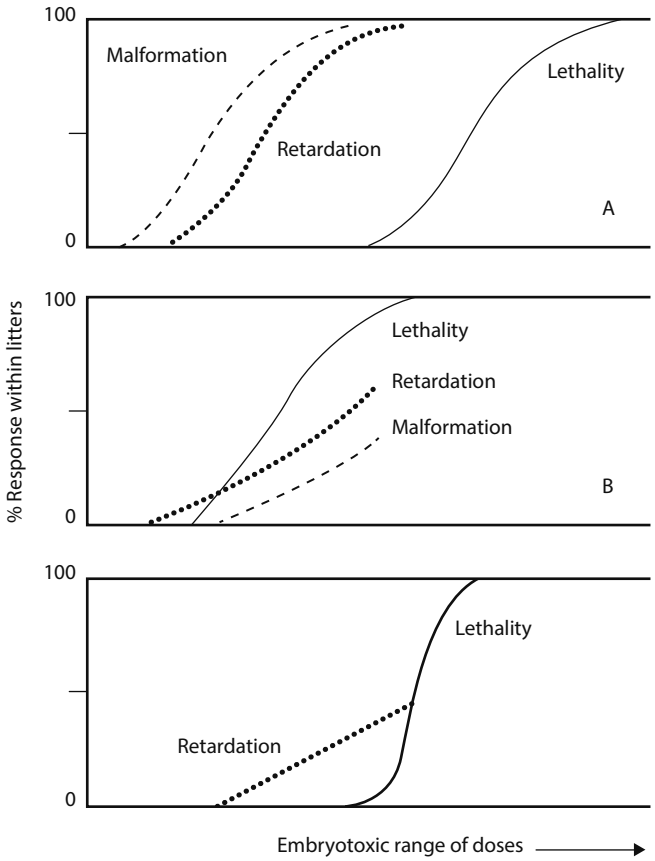


FIGURE 35.4 Dose–response patterns (A–C) for different types of developmental toxicant. (Adapted from Daston, G.P., Relationship between maternal and developmental toxicity, in: Kimmel, C.A. and Buekle-Sam, J. (eds.), *Developmental Toxicology*, 2nd edn., Raven Press, New York, 1994, pp. 189–212.)

systems. Thus, insult during the period of organogenesis is most likely to result in gross structural malformations,[20,105] although all endpoints of developmental toxicology have been shown affected, although the dose–response pattern for agents often differs (see Figure 35.4), and the interrelationships of the responses often confound apparent dose-dependency. In general, the type of agent that is of most concern is that causing malformation at doses that are less than those associated with growth retardation or lethality. As discussed later, regardless of the dose–response, current practice in risk assessment is to base the developmental toxicity effect level on the lowest dose level where any of the four potential endpoints are adversely affected, whether the effect occurs alone or in combination.

Fetogenesis

The fetal period is characterized by tissue differentiation, growth, and physiological maturation. Almost all organs are present and grossly recognizable, and further development of these organs proceeds with the fetus attaining required functions before birth. These include fine structure morphogenesis (e.g., synaptogenesis, neural outgrowth, branching morphogenesis of the bronchial tree and renal cortical tubules) and biochemical maturation (e.g., induction of tissue-specific enzymes and structural proteins). Insult during the period of fetogenesis is most likely to affect growth and functional maturation of the CNS, reproductive organs (including behavioral and motor deficits and reductions in fertility), the pulmonary system, and the immune system. Although gross structural changes can occur during the fetal period, such observations are generally secondary to deformations (changes in previously normal structures, such as clubbed or bent limbs), rather than malformations (abnormal growth).

Apoptosis

Apoptosis (controlled cell death) has been recognized for over 60 years to be as important to development of the conceptus as are cell proliferation and differentiation.[93,107–109] Apoptosis occurs in the developing embryo and in normal healthy adult tissues, as well as in many pathological settings. It is genetically directed, usually requires ongoing protein synthesis,[110] continues through life, and provides the central mechanism for removal of surplus, unwanted, damaged, or aged cells. Apoptosis occurs in almost every tissue during development,[111,112] including palate formation,[113] body sculpting (e.g., digit formation[114]) gastrointestinal development, sexual organ development and gamete formation and number,[109,110,112,115–118] and in the homeostasis of normal tissues, especially the gastrointestinal tract, immune system, and skin.[110] Establishment of normal craniofacial pattern requires apoptosis of the neural crest,[119] with apoptosis perhaps most well studied in development of the nervous system. It is critical in the development of both neuronal and nonneuronal cells in the peripheral nervous system and CNS[120,121] and occurs both pre- and postnatally. As noted by Mazarakis et al.,[118] apoptosis is seen in the developing nervous system as early as neural tube formation and persists throughout terminal differentiation of the neural network, with more than 50% of the neurons lost during development.

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. A full description is beyond the scope of this chapter. Readers are referred to two recent review articles (and the reference within) on this topic.[122,123] There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway.[122] However, these two pathways are linked and molecules in one pathway can influence the other.[124] There is another pathway that involves T-cell-mediated cytotoxicity and perforin–granzyme-dependent killing of the cell. The perforin–granzyme pathway can induce apoptosis via either granzyme B or A. The extrinsic, intrinsic, and granzyme B apoptotic pathways all converge on the same terminal (execution) pathway.

Extrinsic pathway: Tumor necrosis factor (TNF) receptor family members share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids that has been called the *death domain*.[125] This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways involving a multistep process leading to the formation of a

death-inducing signaling complex that results in the autocatalytic activation of procaspase-8 to caspase-8, triggering the execution phase of apoptosis.[126]

Intrinsic pathway: The intrinsic apoptotic signaling pathways involve a diverse array of nonreceptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals such as the absence of certain growth factors, hormones, and cytokines can lead to failure to suppress death programs, thereby triggering apoptosis. Other stimuli (e.g., radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals) can cause changes in the inner mitochondrial membrane that result in an opening of the mitochondrial permeability transition pore, loss of the mitochondrial transmembrane potential, and release of two main groups of normally sequestered proapoptotic proteins from the intermembrane space into the cytosol.[127] The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins, which govern mitochondrial membrane permeability and can be either proapoptotic or antiapoptotic.[128] The tumor suppressor protein *p53* has a critical role in regulation of the Bcl-2 family of proteins.[129] It is thought that the main MOA of the Bcl-2 family of proteins is the regulation of cytochrome *c* release from the mitochondria via alteration of mitochondrial membrane permeability.

Execution pathway: Caspases are widely expressed in an inactive proenzyme form in most cells. Activated caspases can often activate other procaspases, allowing initiation of a protease cascade, which amplifies the apoptotic signaling pathway leading to rapid cell death. Once caspases are initially activated, there appears to be an irreversible commitment toward cell death. To date, 10 major caspases have been identified and broadly categorized into inflammatory caspases (caspase-1,-4,-5), initiators (caspase-2,-8,-9,-10), and effectors or executioners (caspase-3,-6,-7).[130,131] The other identified caspases include caspase-11 (which regulates apoptosis and cytokine maturation during septic shock), caspase-12 (which mediates endoplasmic-specific apoptosis and cytotoxicity by amyloid-β), caspase-13 (probably a bovine gene), and caspase-14, which is highly expressed in embryonic tissues but not in adult tissues.[132–135]

It is the activation of the execution caspases that begins the execution phase of apoptosis. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 cleave various substrates that ultimately cause the morphological and biochemical changes seen in apoptotic cells.[136] Caspase-3 is considered to be the most important executioner caspase and can be activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). In apoptotic cells, activated caspase-3 cleaves ICAD to release the endonuclease CAD,[137] which then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies. Phagocytic uptake of apoptotic cells is the last component of apoptosis. Apoptotic cells express surface markers (e.g., annexin, calreticulin, thrombospondin-1) that result in the early phagocytic recognition by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue.[138–142] Phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells and their fragments is the hallmark of this phase. The appearance of phosphatidylserine on the outer leaflet of apoptotic cells then facilitates noninflammatory phagocytic recognition, allowing for their early uptake and disposal.[143] This process of early and efficient uptake with no release of cellular constituents results in essentially no inflammatory response.

Necrosis: Necrosis, in contrast to apoptosis, is an uncontrolled and passive process that usually affects large fields of cells while apoptosis is controlled and energy-dependent and usually affects individual or clusters of cells. Using standard histology, it is often difficult to distinguish apoptosis from necrosis.[122] Apoptosis and necrosis can occur simultaneously depending on factors such as the intensity and duration of the stimulus, the extent of adenosine triphosphate (ATP) depletion, and the availability of caspases.[144]

Changes associated with necrosis include cellular swelling, organelle dysfunction, mitochondrial collapse and ultimately cellular disintegration, release of the cellular contents in the extracellular milieu, and a marked host inflammatory reaction. Necrotic cell injury is mediated by two primary mechanisms: (1) interference with the energy supply of the cell and (2) direct damage to cell membranes. It is the loss of cell membrane integrity with the subsequent release of the cytoplasmic contents into the surrounding tissue that initiate chemotaxic signals, which eventually cause recruitment of inflammatory cells. Because apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue and are quickly phagocytosed by macrophages or adjacent normal cells, there is essentially no inflammatory reaction.[145] It is also important to note that pyknosis and karyorrhexis are part of the spectrum of cytomorphological changes that occurs with apoptosis and necrosis.[146]

Endpoints of Developmental Toxicology

One or more endpoints of developmental toxicity, alone or in combination, have been identified as affected for scores of agents, despite the fact that *teratogenicity* remains the endpoint of greatest interest. Compendiums of *animal teratogens* are regularly updated[147–149] and provide overviews of effects of test agents, generally in animal studies conducted at high multiples of human exposures. Despite the concept that most, and possibly all, agents are toxic to development when administered to the appropriate species, at the required dose, during the appropriate period of sensitivity,[150] many researchers continue to perceive teratogenicity as a unique property of an agent and incorrectly categorize agents as *teratogenic* or *nonteratogenic*[151] or induced by *maternal toxicity*, when only

an association of toxicity at a given dose can be identified on the basis of the study design.[152–155] Agents that result in toxic effects in the maternal and paternal animals may be expected to result in adverse effects on the developing conceptuses from which the parents but not necessarily the conceptuses may recover, and that cross-species extrapolation of the affected developmental endpoint does not necessarily occur, because of multiple timing, exposure, and species-specific differences.

Mechanisms of Developmental Toxicity

As early as 1977, Wilson[18] identified six general categories for mechanisms resulting in developmental toxicity. These include mitotic interference, altered membrane function/signal transduction, altered energy sources, enzyme inhibition, altered nucleic acid synthesis, and mutations. The current increased knowledge regarding molecular mechanisms of normal development results in addition of perturbations in gene expression and programmed cell death to Wilson's general categories.[156] Six teratogenic mechanisms of medical drugs have been identified by van Gelder et al.[157]: (1) neural crest cell disruption, (2) endocrine disruption (sex hormones), (3) folate antagonism, (4) vascular disruption, (5) oxidative stress, and (6) specific receptor- or enzyme-mediated teratogenesis. In their review, van Gelder et al. used three methods to identify the teratogenic mechanisms of medications: first, the MEDLINE and EMBASE databases; second, two recent books on teratogenic agents; and third, a list of drugs classified as U.S. Food and Drug Administration (FDA) class D or X. Mechanisms were included only if they are associated with major structural birth defects and medications that are used relatively frequently by women of reproductive age. A brief discussion of each mechanism follows. Readers are referred to van Gelder et al.[157] (and references within) for greater detail.

Neural Crest Cell Disruption

The neural crest, a pluripotent cell population originating in the neural folds, can be divided into two major populations: the cranial and truncal neural crest. During neurulation, the neural crest cells detach from the neural folds and migrate into the embryo to give rise to numerous structures. In the craniofacial region, various cell types and structures, including intramembranous bone, cartilage, nerves, and muscles, are derived from the cranial neural crest. The cardiac neural crest, a cranial neural crest subpopulation, migrates into the cardiac outflow tract to mediate septation. Therefore, neural crest–related cardiovascular malformations include aortic arch anomalies and conotruncal defects.[158] Membranous ventricular septal defects are also neural crest–related, since the membranous part of the interventricular septum originates from the cardiac neural crest, whereas the muscular part originates from the mesenchyme.[159] The cardiac neural crest also migrates into other derivatives of the pharyngeal arches (e.g., thymus, thyroid, and parathyroids).[160] Neural crest, noncardiovascular defects include craniofacial malformations,[161] esophageal atresia,[162,163] and abnormalities of the pharyngeal glands.[164] The truncal neural crest produces important components of the peripheral nervous system.[165]

Proper induction, migration, proliferation, and differentiation of neural crest cells are tightly regulated. A variety of molecular signals and receptors are implicated in neural crest cell development. Therefore, drugs that interfere with these molecular pathways may induce neural crest–related malformations. In addition, in vivo and in vitro experiments suggested that altering levels of folate and/or homocysteine cause abnormalities of cardiac neural crest cell migration, differentiation, and cell cycle progression,[166] thereby connecting this teratogenic mechanism with folate antagonism. Retinoic acid, the biologically active form of vitamin A, is one of the most important signaling molecules in neural crest cell development. Excesses as well as shortages[167,168] of retinoic acid appear to cause neural crest–related malformations, indicating that proper retinoid homeostasis is necessary for normal development.

Endocrine Disruption: Sex Hormones

Since the 1940s, a number of drugs have been developed to mimic or inhibit the actions of hormones. These drugs include diethylstilbestrol (DES), oral contraceptives, and hormones used in fertility treatment. These drugs and other endocrine disrupting chemicals (EDCs, such as bisphenol A and phthalates) may interfere with the physiologic functions of endogenous hormones by affecting their release, binding, or metabolism. Their actions may depend not only upon the estrogen and/or androgen receptors binding affinity or specificity but also upon their ability to activate or inhibit other receptor-mediated actions. The in utero actions of EDCs have been of concern because of their possible impact on the developing reproductive systems, especially since treatment of pregnant women with the synthetic estrogen DES led to an increased risk of vaginal adenocarcinoma in their female offspring[16] and has also been associated with an increase in reproductive disorders in sons[169] and grandsons.[170,171]

In addition to drugs that influence endocrine homeostasis as their primary MOA, some oral medications (e.g., mesalamine and omeprazole) have coatings that contain phthalates, which may be a source of EDC exposure.[172] These enteric coatings may affect human male reproductive development due to their anti-androgenic properties.[172] Additionally, other products also contain phthalates as plasticizers, but it should be noted that phthalates do not bioaccumulate and are rapidly excreted in contrast to some other EDCs. The susceptibility to EDCs may also vary greatly between individuals due to genetic factors.[173] Therefore, it is questionable whether the levels of phthalates *in medications* are great enough to produce male reproductive tract anomalies in humans. In epidemiologic studies, the two medications with phthalate containing enteric coatings, omeprazole, and mesalamine have not been associated with an increased risk of major birth defects.[174,175]

Male development is more susceptible to endocrine disruption than female development because of its hormone dependence.[176] The sexual differentiation of males generally depends on a balanced androgen/estrogen ratio. In mice, estrogens impair fetal Leydig cell development and reduce

testosterone production.[177] In rats, phthalates induce male reproductive disorders through inhibition of steroidogenesis by the fetal testis.[178,179] In contrast, this does not occur in vitro with human fetal Leydig cells.[180]

However, since EDCs may affect endocrine homeostasis in multiple ways, the underlying teratogenic mechanisms are often difficult to discern. There are considerable species differences and markedly different estrogen levels in normal human pregnancy when compared with normal rodent pregnancy. Whether many of the described mechanisms elucidated in rodent and in vitro studies are applicable to humans remains debatable.[157]

Folate Antagonism

Folate is the generic term for a water-soluble B vitamin that occurs in certain natural foods (fruits, leafy green vegetables, beans, and liver) as polyglutamate, while folic acid, the synthetic form with a higher bioavailability than food folate, is used in vitamin preparations and food fortification. Folate is converted through two reduction reactions by dihydrofolate reductase (DHFR) to the naturally bioactive form tetrahydrofolate (THF), which is converted into 5-methyltetrahydrofolate, which is the main form of folate in the blood. 5-Methyltetrahydrofolate is transported into cells, and once inside the cell, 5-methyltetrahydrofolate acts as an essential coenzyme by being an acceptor or donor of one-carbon units in many biochemical reactions (e.g., purine and pyrimidine synthesis and DNA methylation reactions). Because rapidly proliferating tissues require DNA synthesis, folate-dependent reactions are essential for growth and development of the conceptus and that folate requirements increase during pregnancy. In addition, DNA methylation is known to be involved in the epigenetic control of gene expression during development.

The teratogenicity of folate antagonists in humans was first suggested by reports of women who were given aminopterin in the first trimester of pregnancy to induce abortion.[181] Some antiepileptic drugs, for example, carbamazepine and valproic acid, are generally known to increase the risk of folate-sensitive birth defects, such as neural tube defects, orofacial clefts, and limb defects. Currently, the exact mechanism by which disturbances of the folate metabolism increase the risk of neural tube defects is unclear.

Several drugs disturb the folate metabolism and may have a teratogenic effect through inhibition of the folate methylation cycle. Two general groups of drugs act as folate antagonists. The first group blocks the conversion of folate to THF by binding irreversibly to the enzyme DHFR. This group consists of competitive inhibitors of DHFR and includes methotrexate, sulfasalazine, triamterene, and trimethoprim.[182] The second group antagonizes other enzymes in the folate metabolism, impair folate absorption, or increase folate degradation. This group primarily consists of antiepileptic drugs, including valproic acid, carbamazepine, and phenytoin.

Vascular Disruption

Vascular disruption defects are structural birth defects resulting from interference with or extrinsic breakdown of an originally normal prenatal development of the arteries, veins, and capillaries (vasculature).[183] Vascular disruption refers to disturbances in the blood circulation (e.g., hyperperfusion, hypoperfusion, hypoxia, obstruction) in the uterine–placental unit, the placental–fetal unit, or the fetus itself. They may be caused by acute or chronic decreases in uterine blood flow, vascular infections, or an abnormal anatomy in the uterine–placental unit. Factors such as placental insufficiency, amnion rupture, and umbilical cord obstruction may cause failures in the vascular supply in the placental–fetal unit. In the fetus, abnormal regulation of vessel formation, premature regression of embryonic vessels, disruption of newly formed vessels, external compression, embolic events, and occlusion with venous engorgement can lead to vascular disruption.[184] Vasoconstriction of maternal and fetal vessels, hypoperfusion, and obstruction may cause a reduced supply of nutrients to the embryonic tissues, which can affect development and growth of embryonic structures or result in tissue loss. These disturbances may create a state of hypoxia, which is involved in the formation of reactive oxygen species (ROS) and oxidative stress.[185] The types of structural anomalies that may be caused by vascular disruption are determined by the timing during gestation, the location and severity of tissue damage, and the possible presence of secondary adhesion of necrotic tissue with adjacent organs or the amnion.[183] During embryogenesis, vascular disruption results in aberrant differentiation and distortion of contiguous tissues, loss of tissue, and incomplete development of structures within the same or a secondary embryonic developmental field. Anomalies resulting from vascular disruption during the fetal period are usually limited to the areas with disturbed blood supply, to which the peripheral vasculature is most susceptible.[184] Thus, the majority of defects caused by tissue damage through vascular disruption during the fetal period occur in structures supplied by the most peripheral vasculature, such as the distal limbs and the embryonic intestine. Vascular disruption–related birth defects include terminal limb reductions, hydranencephaly/porencephaly, gastroschisis, small intestinal atresia, and Poland anomaly. The majority of evidence in support of this mechanism comes from case reports with suspected vascular events such as occlusion, emboli, amnion rupture, and twin placental vessel anastomoses.[183]

During pregnancy, exposure to vasoactive substances, especially vasoconstrictives, has been hypothesized to play a causal role in vascular disruption defects. These substances could decrease placental or embryo–fetal blood flow or affect the angiogenesis, thereby altering the vasculature structure.[181] The following vasoactive therapeutic drugs have shown associations in epidemiologic studies with the vascular disruption: misoprostol, aspirin, ergotamine, and pseudoephedrine. However, all drugs with vasoconstrictive or vasodilating effects may have the potential to cause birth defects due to vascular disruption.

Oxidative Stress

In vivo, several drugs (redox cycling agents) used in the treatment of epilepsy, cardiac arrhythmias, and cancer undergo

single electron reduction reactions yielding radical species.[186] In redox cycling reactions that involve oxygen, ROS such as hydrogen oxide, alkyl peroxides, and various radicals are generated (e.g., hydroxyl and superoxide).[187] The creation of ROS is induced by internal and external agents, such as phagocytes, cytochrome P450 mono-oxygenases (CYP), irradiation, and exogenous chemicals. The generation of ROS can be decreased or reversed by various enzymes, for example, superoxide dismutase, catalase, and glutathione reductase, and by antioxidants.[188] Endogenous ROS serve as second messengers in signal transduction[189] and are thought to be important in ion transport, immunological host defense, transcription, and apoptosis of unwanted cells.[190,191] Conversely, ROS can also be harmful by either covalently or irreversibly binding to cellular macromolecules. Oxidative stress is an imbalance between ROS generation and antioxidant defense mechanisms of a cell or tissue. It can cause irreversible oxidation of DNA, proteins, and lipids, leading to inactivation of many enzymes and cell death. In addition to damaging cellular macromolecules, oxidative stress may affect gene expression by interfering with the activity of redox-sensitive transcription factors and signal transduction by oxidizing thiols.[192] During the gestational period, this may result in birth defects and growth retardation and, in severe cases, in in utero death.[189,193,194]

Because of its weak antioxidant defense, the embryo is especially susceptible to high levels of ROS; however, placental enzymes play a role in protecting the fetus against oxidative stress. Increased ROS in embryos are usually the result of embryonic metabolic changes rather than exposure to ROS of maternal origin.[183] Enzymatic bioactivation of proteratogens may cause increased embryonic ROS. However, the ontogeny and functionality of most of the CYP family occurs after birth. Those CYPs present during the embryonic period are expressed at relatively reduced levels. Only some isoforms are expressed at levels that could be significant in ROS-induced teratogenesis.[195,196] In contrast, the prostaglandin H synthases have a relatively greater expression during the embryonic and fetal period than after birth.[197,198] The peroxidase component of this enzyme can bioactivate exogenous substances, including phenytoin and related teratogens,[199] to toxic reactive intermediates that initiate ROS formation.[200] There is evidence that lipoxygenases, which oxidize proteratogens yielding free radical intermediates, are substantially expressed in embryonic tissues. Based on this information, it is inferred that bioactivation of proteratogens by embryonic prostaglandin H synthases and lipoxygenases is necessary for the formation of ROS and subsequent macromolecule damage in the developing embryo.[191] Additionally, embryonic ROS formation and subsequent oxidative stress may be induced by hypoxia. There is considerable evidence that hypoxia followed by reperfusion is teratogenic in animal studies.[201] In addition to embryonic ROS generation, maternal determinants may play an indirect role in ROS-mediated teratogenesis. Embryonic exposure to proteratogens is reduced by maternal pathways that eliminate these compounds or their metabolites before they can cross the placenta. However, deficiencies in those pathways may increase the maternal plasma concentration of proteratogens and, therefore, increase the concentration that reaches the embryo. Furthermore, maternal production of factors that interfere with embryonic ROS-mediated signal transduction or alter embryonic determinants of oxidative stress may also contribute to the risk of teratogenicity.[202]

Oxidative stress is postulated to be involved in the pathogenesis of a wide spectrum of birth defects, including neural tube defects, cleft lip/palate, cardiovascular defects, limb defects, and skeletal malformations. Several drugs are known to induce oxidative stress, which is suspected to be their main teratogenic mechanism. Among these drugs are phenytoin, class III antiarrhythmic drugs, thalidomide, iron supplements, valproic acid, and various chemotherapeutic drugs.

Specific Receptor- or Enzyme-Mediated Teratogenesis

Many medical drugs and chemicals act on a specific receptor or enzyme in the human body, leading to a particular MOA. Inhibition or stimulation of these specific receptors and enzymes can result in effects on fetal development (e.g., angiotensin-converting enzyme and angiotensin II receptors, hydroxymethylglutaryl-coenzyme A reductase, histone deacetylase, cyclooxygenase-1, *N*-methyl-D-aspartate receptors, 5-hydroxytryptamine receptors and transporters, γ-aminobutyric acid receptors, and carbonic anhydrase). A discussion of these specific receptor- and enzyme-mediated teratogens is beyond the scope of this chapter; the reader is refereed to van Gelder et al.[157] and references cited within.

Structure–Activity Relationships, In Vitro Assays, Computational Biology, and Other Considerations

Reproductive and developmental effects are caused at the molecular and/or cellular level. While some MOAs are known for some toxicants (e.g., thalidomide, phthalic acids, valproic acid), many of the mechanisms are currently unknown or only partially elucidated, and the structure–activity relationships remain weak for elucidating mechanisms of developmental toxicity.[203] Along with carcinogenicity studies, reprotoxicity studies are among the most difficult, time-consuming, and costly experimental procedures. Furthermore, reprotoxicity testing requires the highest number of test animals. For all these reasons, the development of alternative (nonanimal) methods for reprotoxicity assessment has become a high political priority. Although a detailed discussion of structure–activity relationships of reproductive and developmental toxicant agent interaction is beyond the scope of this chapter, a state-of-the-art review of available models for developmental and reproductive toxicity is available online.[204] The 2010 review by Piparo and Worth consists of three sections: (1) software (both freely available and commercial) that can be used to predict reproductive and developmental toxicity, (2) databases that can be used to build the models, and (3) literature available models.

As stated earlier, the reproductive cycle combines a highly diverse multitude of biological processes and mechanisms, each of which has its own time-related sensitivity to

xenobiotic exposures. It is therefore a significant challenge to mimic all aspects of the reproductive cycle with in vitro and in silico assays, which may be considered necessary in order that reproductive toxicity can be predicted reliably on the basis of alternative assays alone. The classical aim of *one to one* replacement of in vivo protocols by alternative tests is clearly not feasible for the complex reproductive and developmental toxicity animal study protocols. However, a *battery of assays* approach in which a limited array of sensitive endpoints are reproduced by a set of in vitro and in silico assays could provide sufficient background for hazard identification and risk assessment. A significant number of alternative in vitro and in silico assays have been developed; however, their implementation in regulatory toxicity testing has not yet been totally achieved. Although a detailed discussion of in vitro and in silico assays is beyond the scope of this chapter, a state-of-the-art review of available assays in the OECD armamentarium for developmental and reproductive toxicity is available online.[205] Details concerning these assays are available at the OECD and EPA websites.

Bioinformatics and computational biology are rooted in life sciences as well as computer and information sciences and technologies. Both of these interdisciplinary approaches draw from specific disciplines (e.g., biology, computer science, mathematics, statistics, physics, engineering, and behavioral science). Bioinformatics and computational biology each maintain close interactions with life sciences to realize their full potential. Bioinformatics involves information theory and data management. Bioinformatics apply principles of information sciences and technologies to make the vast, diverse, and complex life sciences data more understandable and useful, while computational biology uses mathematical and computational approaches to directly address biological hypotheses (theoretical and experimental). The terms *bioinformatics* and *computational biology* are often used as synonyms, but they have also been viewed as having distinct characteristics (NIH Working Definition of Bioinformatics and Computational Biology, July 17, 2000).

Computational biology involves the development and application of data-analytical and theoretical methods, mathematical modeling, and computational simulation techniques to the study of biological, behavioral, and social systems. The field is broadly defined and has as its foundations: computer science, applied mathematics, statistics, biochemistry, chemistry, biophysics, molecular biology, genetics, ecology, evolution, anatomy, neuroscience, and visualization.

Established in 2005, the U.S. EPA's National Center for Computational Toxicology (NCCT) coordinates computational toxicology research on chemical screening and prioritization, informatics, and systems modeling. NCCT is involved in the following areas: (1) applying mathematical and advanced computer models to help assess chemical hazards and risks to human health and the environment; (2) using advances in informatics, high-throughput screening technologies and system; (3) using biology to develop robust and flexible computational tools that can screen the thousands of chemicals for potential toxicity. The NCCT has several databases available, (1) ACToR (Aggregated Computational Toxicology Resource), (2) DSSTox (Distributed Structure-Searchable Toxicity Public Database Network), and (3) the ToxRefDB (Toxicity Reference Database), as well as several programs, (1) ExpoCast™ program, (2) ToxCast™ program, (3) Toxicological Priority Index (ToxPi), (4) Tox21, and (5) the Virtual Embryo Project (v-Embryo™), the one that is most important to this chapter .

Scientists in the U.S. EPA's computational toxicology research program are developing new ways to answer the question: "What role does a pregnant woman's exposure to chemicals in the environment play in disrupting the development of an embryo?" And in the process, they are helping revolutionize the field of developmental toxicology. The traditional method of exploring how chemicals affect a developing embryo is conducting controlled tests on pregnant laboratory animals. While these practices provide valuable information, they can be both costly and slow. Reliance on them is one reason why the pace of testing has not kept up with the development of commercial chemicals, leaving significant data gaps.

The v-Embryo™ is aimed at developing new methods that use high-tech computer modeling and vast collections of data and biological knowledge bases in place of traditional lab tests. As stated by Thomas B. Knudsen, an EPA systems biologist who is leading the project,

> The ultimate objective is to build a suite of screening models, a toolkit, that researchers can use to look at embryonic development and make scientifically-based predictions on how that development might be affected by different chemicals …We are building models that properly integrate all the relevant data and information known about various organ systems and birth defects.[206]

Using a selection of everyday chemicals with known health effects in animals, Knudsen and an interdisciplinary team of reproductive toxicologists, computer engineers, programmers, bioinformaticians, biologists, mathematicians, and other experts have continued to work on building computer-based virtual models of embryo development.

> Computer models can handle this complex information, and it is feasible with current math and engineering practices to use these models as a new approach for predicting the potential for environmental chemicals to affect a developing embryo.[206]

Computer models have been developed for embryonic systems that are susceptible to environmental factors, and they are simple enough that computer engineers and programmers can simulate them. Three embryonic systems developed were embryonic eye, blood vessel, and limb development. The predictive value of these embryo systems models is currently being tested using new and existing data from standardized tests conducted in embryonic stem cells (ESCs), zebrafish embryos, and ToxCast™ program. For more information on the EPA's Virtual Embryo program and the NCCT, readers

are referred to the U.S. EPA's website (www.epa.gov). For more information on the international programs, the reader is referred to the website of the *International Society of Computational Biology* (http://www.iscb.org).

Mixtures: The potential for interaction of multiple chemicals has been known to exist for developmental and other toxicities, and that such interactions may enhance or reduce general toxicity of the agents and their potential developmental toxicity, although not necessarily equally or by the same mechanism. For example, early on, it was identified that caffeine potentiates the activity of multiple agents,[207] and while initial emphasis was on the pharmacologic activity of the putative agent, in this case, the vasoconstrictive effect of caffeine,[208] studies are now investigating the interaction of the agent with gene expression. The U.S. EPA has an active program for evaluating multiple chemical combinations[209,210] that are potential water contaminants, often using modified reproductive or developmental toxicity protocols.[211–215]

METHODS USED IN REPRODUCTIVE (FEMALE) AND DEVELOPMENTAL TOXICOLOGY

Testing Procedures and Guidelines for Regulatory Use

The purpose of this section is to provide an overview of the methods used in collection and interpretation of data obtained from reproductive and developmental toxicity studies, when conducted for regulatory use. The methods described are those used in several laboratories and were developed from the literature and practical experience. Because it was not always practical to provide in-depth details, useful publications have been referenced, when possible. Multiple laboratory species are used in these types of studies. However, for practical reasons, most studies are performed using rats and rabbits and sometimes mice and hamsters. In general, the described techniques can be applied to ferrets, guinea pigs, mini-pigs, dogs, and NHP, using species-specific considerations. As previously noted, there are often special reasons to use alternative species (e.g., prolonged in utero development and comparable in utero CNS development in the guinea pig theoretically make this species ideal for evaluation of neural development). Important caveats are that studies conducted for regulatory use should provide evidence that the agent is pharmacologically active in the species, absorbed, if administered orally or topically, and, if possible, similarly handled metabolically by the test species and humans.

The Guidelines for Toxicity to Reproduction of Medicinal Products,[216] an effort of the International Conference for Harmonization (ICH), are accepted by FDA,[217,218] European Union (EU), and Japan.[219] Although the United States, the EU, and Japan have worked together to harmonize their test guidelines, the ICH Guidance M3(R2),[220] the timing of the conduct of the reproduction toxicity studies is based on two factors: (1) the population in which the clinical trials are going to be performed and (2) the ICH countries that the regulatory dossier will be submitted. Recently, a series of review articles concerning the three principle ICH DART study designs were published and have also been incorporated into the following discussion.[221–223] The Nonhuman Primate Developmental and Reproductive Toxicity study designs have significantly changed, and this topic has been thoroughly reviewed in recent publications.[224–227]

DART study guidelines have been promulgated by the FDA,[228–233] the EPA,[89,234–236] the Ministry of Agriculture, Forestry and Fisheries (MAFF),[219,237] Canada,[238,239] Great Britain,[240] WHO,[241] and the OECD.[242–246] The ICH[228,229,247] has been concerned with unifying the study guideline requirements for pharmaceutical safety assessment for the EU, Japan, and the FDA. Guideline-compliant reproductive and fertility studies must be performed in accordance with associated Good Laboratory Practice (GLP) Regulations[248–250] and biosafety[251] and animal welfare guidelines.[252,253] Comprehensive comparisons of the ICH guidelines with other guidelines have been published.[254,255] The Guidelines and Guidance for Industry documents are available on the U.S. FDA (http://www.fda.gov), U.S. EPA (http://www.epa.gov), European Medicines Agency (EMA) (http://www.emea.europa.eu), OECD (http://www.oecd.org), or ICH (http://www.ich.org) websites.

For the purpose of this chapter, the ICH guidelines were used as the reference for identifying the various stages and interrelationships of female reproductive functions and development of the offspring. These guidelines segment the reproductive cycle into six *ICH stages* (ICH stages A through F) that may be tested separately or in combination, generally using a 1-day overlap of treatment. The recommendations of ICH guidelines were often unidentified in other guidelines, including the use of (1) scientific justification of flexible study designs, (2) kinetics, (3) expanded male reproductive toxicity evaluations, (4) a requirement for mechanistic studies, and (5) essentially equal emphasis on all endpoints of developmental toxicity (death, malformation, reduced weight, functional/behavioral alterations), rather than emphasizing malformation as the most important outcome. Flexible testing strategies are to be based on (1) anticipated drug use, especially in relation to reproduction; (2) the form of the substance and route(s) of administration intended for humans; and (3) consideration of existing data on toxicity, pharmacodynamics, kinetics, and similarity to other compounds in structure/activity. In toto, the purpose of these studies is to identify any effect of an active substance on mammalian reproduction, to compare this effect with all other pharmacologic and toxicologic data for the agent, and ultimately, to determine whether the human risk for reproductive and developmental effects is the same, increased, or reduced, in comparison with the risks of other toxic effects of the agent. As should occur for all toxicologic observations, additional pertinent information should be considered before the results of the animal studies are extrapolated to humans. This information includes human exposure considerations, comparative kinetics, and the mechanism of the toxic effect.

Because this chapter emphasizes toxic effects on the female animal and its offspring (through completion of the lactation period), sections of the ICH guidelines relevant to male reproductive performance and postnatal development of the offspring are discussed only when relevant to the primary subject area. Assessments of male reproductive effects, functional/behavioral effects in the offspring, and pediatric effects are more fully described in other chapters.

General Procedures for Pharmaceuticals

The ICH stages are identified in Figure 35.1. Conduct of a study, including two full reproductive cycles, is equivalent to a two-generation study. As previously noted, rodents and rabbits are the most commonly used laboratory animals. Common protocol designs used for evaluation of these species are provided in Figures 35.5 through 35.8. The most common practice used in developing pharmaceuticals is to evaluate ICH stages A (premating to conception) and B (conception to implantation) in a fertility study, with treatment of the female animals initiated 2 weeks before cohabitation and continuing through day 7 of gestation with a pregnancy evaluation usually around GD 13/14. Embryo–fetal toxicity studies treat through ICH stage C (implantation to closure of the hard palate; rats—days 6/7 through 17 of gestation). ICH stage C is also evaluated in a nonrodent species (generally the rabbit; treatment on days 6/7 through 19 of gestation) early in product development, although depending upon the proposed use of the agent, some investigators chose to delay this test until after the early clinical trials are completed (metabolism, kinetics, safety, and efficacy). Based on the proposed use of the agent, pre- and postnatal toxicity studies are conducted that evaluate ICH stages C (implantation to closure of the hard palate), D (closure of the hard palate to parturition; rats—day 17 through 22 of gestation), E (birth to weaning; rats—day 0/1 through 21 postnatal), and F (without drug exposure—weaning to sexual maturity; rats—day 22 to 60 postnatal).

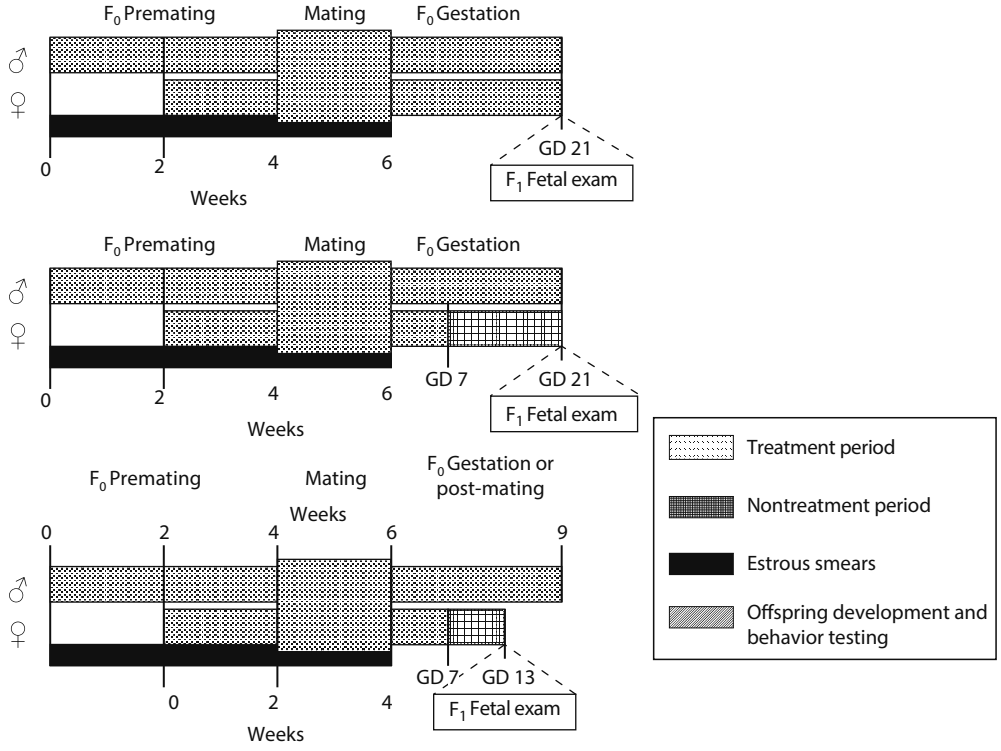


FIGURE 35.5 ICH 4.1.1. The fertility and general reproductive performance study (stages A–B).

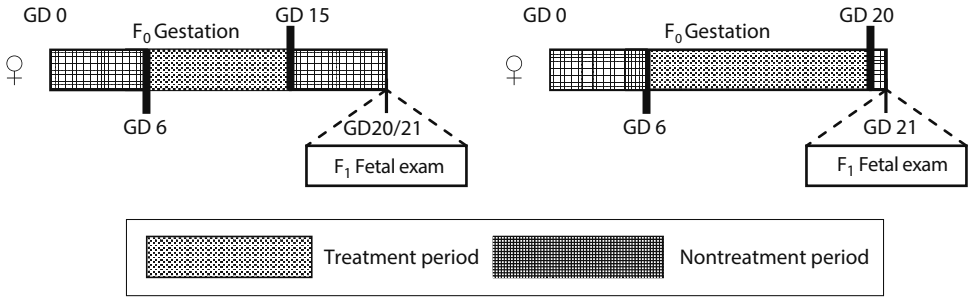


FIGURE 35.6 ICH 4.1.3. The rat developmental toxicity or embryotoxicity study (old segment II; stages C–D).

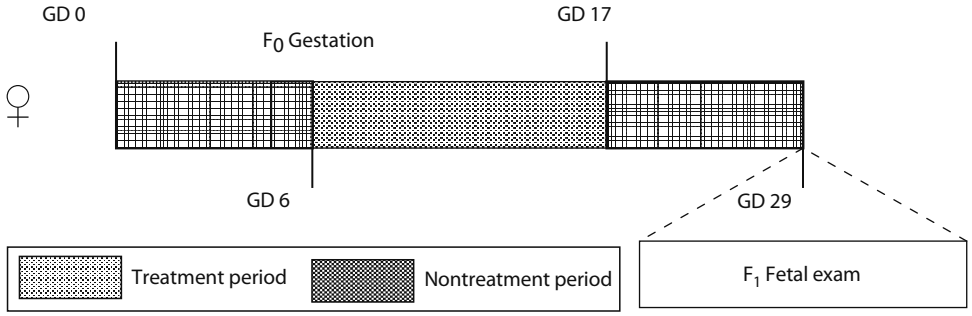


FIGURE 35.7 ICH 4.1.3 The rabbit developmental toxicity or embryotoxicity study (stages C–D).

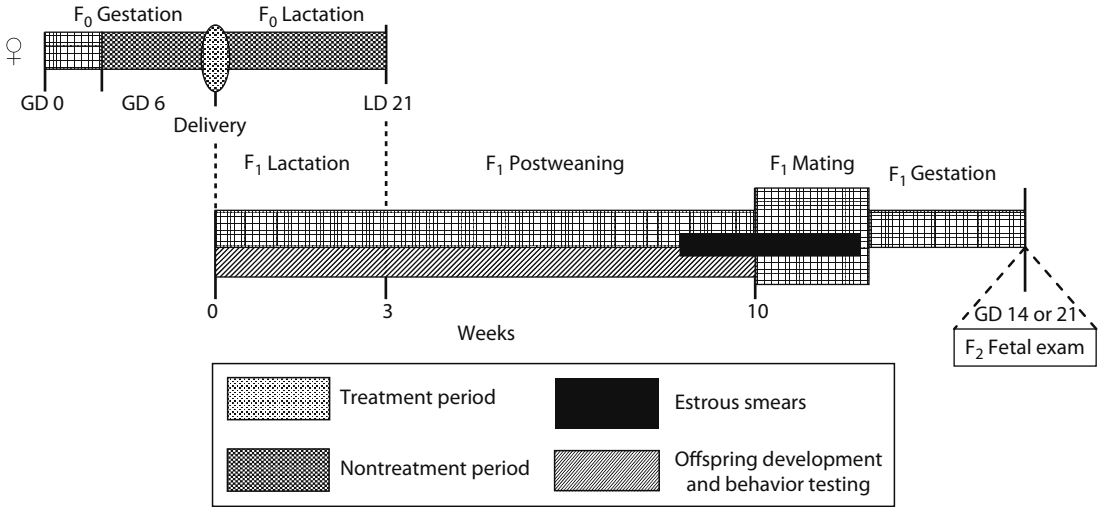


FIGURE 35.8 ICH 4.1.2 The prenatal and postnatal study (old segment III, stages C–F).

General Procedures for Indirect Food Additives

The FDA *Toxicological Principles for the Safety Assessment of Food Ingredients* (*Redbook 2000*), last updated on July 2007,[216,256] is the new name for *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food* that was originally published in 1982 (*Redbook I*) and a draft revision was published in 1993 (*Redbook II*). Major changes were made in this revised guidance (*Redbook II*). This document provides guidance to industry and other stakeholders (e.g., academia, other regulatory groups) regarding toxicological information submitted to the Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety (OFAS) regarding food ingredients. It is a guidance document that is intended to assist in (1) determining the need for toxicity studies; (2) designing, conducting, and reporting the results of toxicity studies; (3) conducting statistical analyses of data; (4) the review of histological data; and (5) the submission of this information to the FDA as part of the safety assessment of food ingredients. The toxicity studies with female reproductive toxicity evaluations included in this guidance document are located in Section IV.C.9.a. *Guidelines for Reproduction Studies*, Section IV.C.9.b. *Guidelines for Developmental Toxicity Studies*, and Section IV.C.10. *Neurotoxicity Studies*, which contains *Developmental Neurotoxicity Study Design*.

General Procedures Specific to EPA OPPTS Protocols

Developmental toxicity studies are required in two species (one rodent and one nonrodent).[257] Treatment is administered from implantation to delivery (GDs 6 through 20 in rats and 6 through 29 in rabbits), with 20 pregnant animals per group assigned to study. It is acceptable to provide treatment throughout the entire gestation period (GDs 0 through 20 in rats and 0 through 29 in rabbits).

The EPA multigeneration (two-generation) protocol[258] has multiple endpoints, incorporating many endpoints traditionally performed in pharmaceutical development, although differing from the ICH guidelines in that the protocols are set, rather than open to scientific judgment regarding design (see Figure 35.9). Treatment of the parental generation (P_0/F_0 male and female rats) is initiated 10 weeks before mating and continues through a 3-week mating period (the male rats can be terminated after the cohabitation period). Treatment of the P_0/F_0 and F_1 generation male and female rats continues until termination. Additional endpoints assessed include estrous

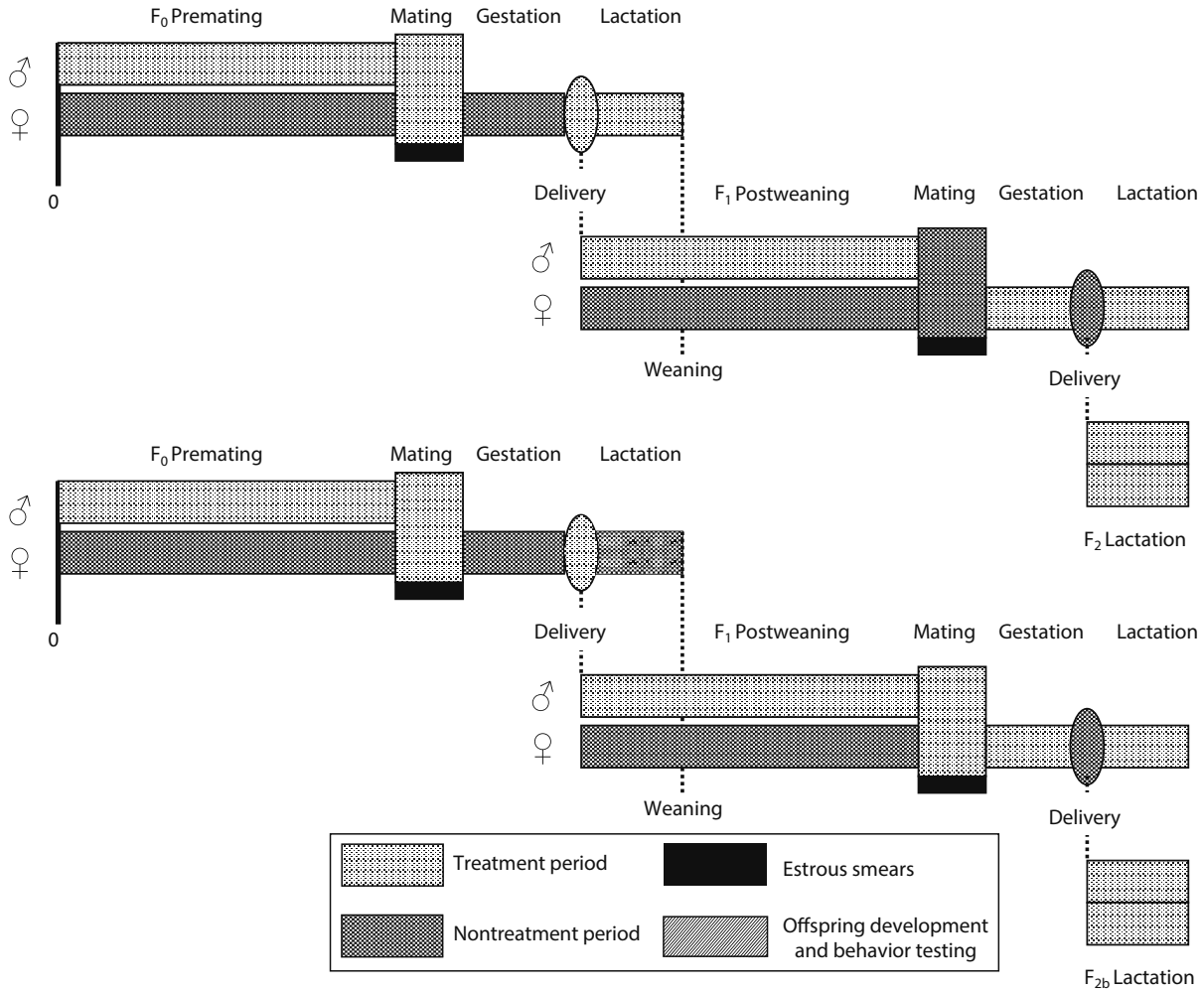


FIGURE 35.9 Two-generation reproduction study schematics (OPPTS 870.3800 and OECD 416).

cyclicity data (vaginal smears) for 3 weeks prior to mating, during mating, and at termination; sperm parameters (total number of sperm, percent of progressively motile sperm, and sperm morphology); developmental milestones (age of vaginal opening and preputial separation for the F_1 generation, anogenital distance for the F_2 generation, if triggered by a treatment-related effect on the sexual maturation of the F_1 generation); gross pathology and selected histopathology of weanlings; and adult organ weight data (uterus, ovaries, testes, one epididymis, including the total weight and cauda epididymal weight, seminal vesicles with coagulating glands and fluids, prostate, brain, liver, kidneys, adrenal glands, spleen, thymus, and all known target organs). Histologic evaluation of the ovary is to include a minimum of 10 sections, randomly selected from one completely sectioned ovary per female in the control and high-dosage groups. Examination of the intact epididymis is to include the caput, corpus, and cauda regions. For the F_1 generation, organ weight data are to be collected for one pup per sex per litter from all dose groups for the ovaries, testes, brain, liver, kidney, adrenal glands, spleen, thymus, and known target organs, with histopathology triggered by treatment-related effects in the F_1 high-dosage group histopathology.

The EPA developmental neurotoxicology protocol may be incorporated within the multigeneration study design or conducted separately. A potential schematic for this very complex study is presented as Figure 35.10. In this study, treatment occurs from implantation through postnatal development of the majority of the CNS (rats—GD 6 through lactation day 12), although treatment can be continued through lactation to day 21, when weaning occurs. Direct dosing of pups may be necessary when lactational exposure through the milk has either been documented as not occurring or is unknown.

REACH and the Extended One-Generation Study: The new European chemicals legislation REACH (Registration, Evaluation, and Authorization of Chemicals) has been estimated to require several million experimental animals until the year 2018, more than 60% of which will be necessary for reproductive and developmental toxicity testing.[259] Testing for REACH can only proceed after approval from the agency. Current OECD activities include the designing of an extended one-generation study, which is aimed at omitting the second-generation offspring whenever justified. The Agricultural Chemical Safety Assessment (ACSA) Committee of HESI (ILSI Health and Environmental Sciences Institute) protocol

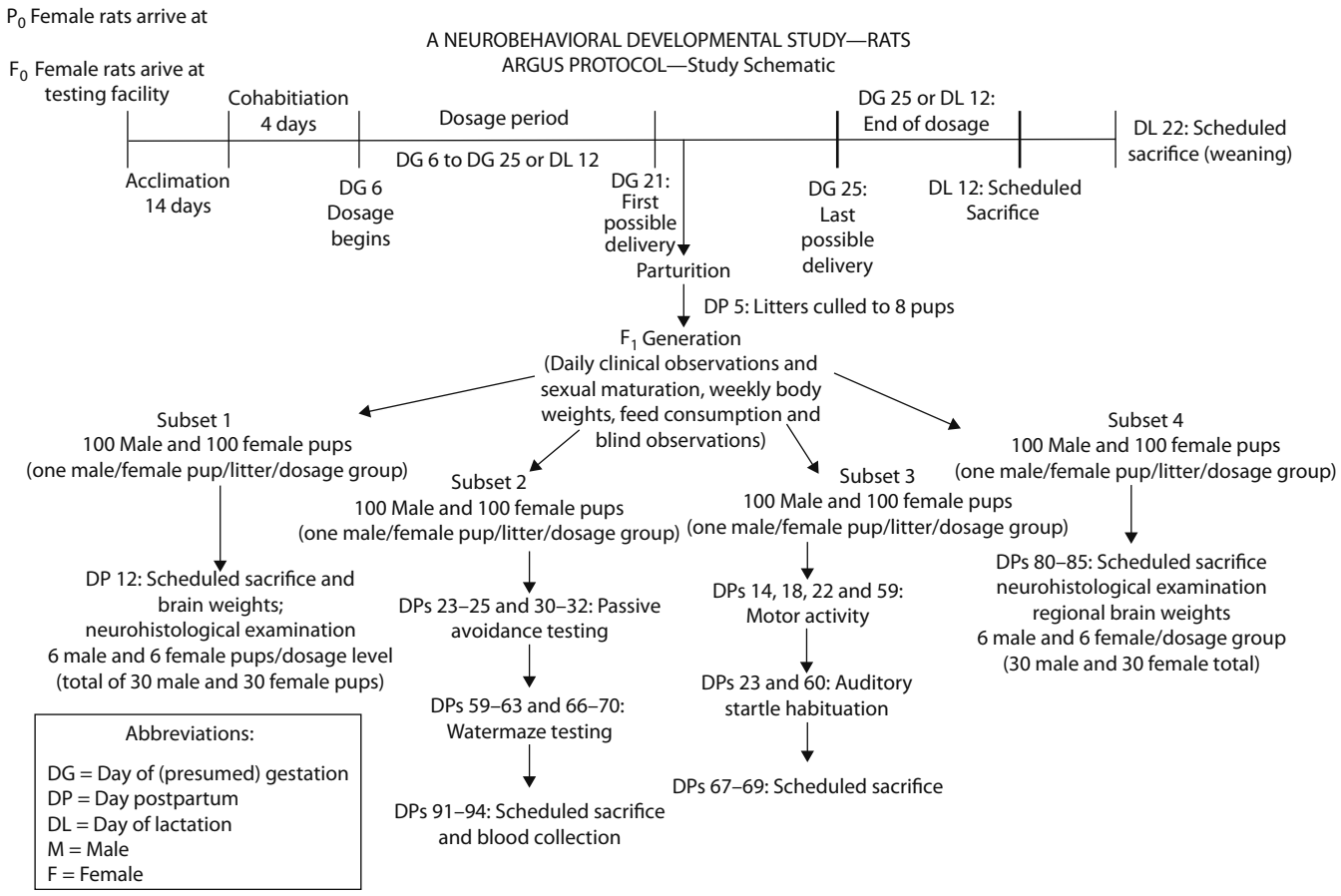


FIGURE 35.10 Neurobehavioral developmental toxicity study—rats.

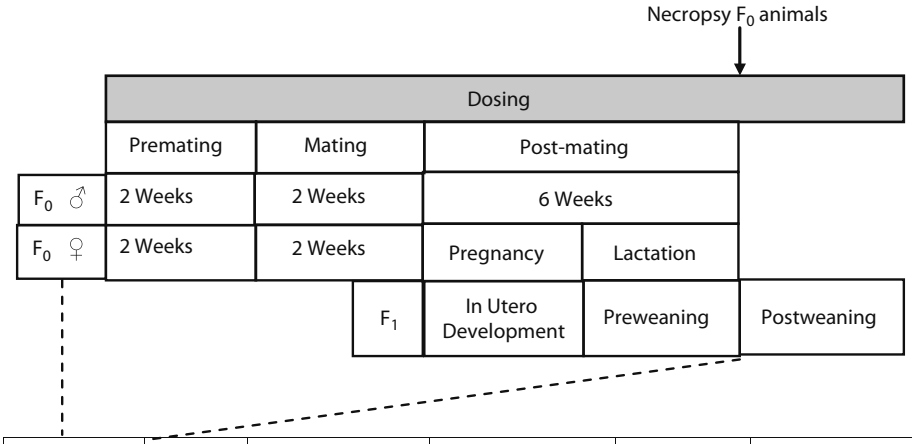
designed by Cooper et al.[260,261] was put forward as a replacement of the two-generation study. Proponents stated that it would significantly reduce animal use as well as cost and duration of the study. The Extended One-Generation Reproductive Toxicity Study Testing Guideline OECD 443 (July 2011) has been promulgated by the OECD, and it is similar to the Agricultural Chemical Safety Assessment (ASCA) protocol. The guideline could replace the current two-generation study and would not require mating of the animals of the second (F_1) generation and assessment of their reproductive performance unless triggered by study findings.

Young adult F_0 males would be treated for 2–4 weeks before pairing and young adult F_0 females treated for 2 weeks before pairing (see Figure 35.11). F_0 males are further treated at least until weaning of the F_1. They should be treated for a minimum of 10 weeks. Treatment of the F_0 females is continued during pregnancy and lactation until termination after the weaning of their litters (i.e., 8–10 weeks of treatment). Pups would be selected to form the second (F_1) generation, and these animals would be treated from weaning until 70 days of age. The F_1 Cohort 1 would be used to assess reproductive/developmental endpoints and could be extended to include an F_2 generation if the data warrant it. The F_1 Cohort 2 and 3 would be used to assess the developing nervous system and immune system, respectively. At this point, the study would be terminated unless there was a trigger to extend the study to include mating of the animals of the second (F_1) generation and assessment of their reproductive performance. There would be no routine testing of the reproductive performance of animals exposed as juveniles.

The *triggering signals* required and the importance of the assessment of the reproductive and developmental endpoints of the second (F_1) generation (as done in a two-generation study) are currently being debated.[262–264] There have been several reviews that are attempting to determine the relative importance of mating the second (F_1) generation versus the first (F_0) generation in identifying treatment-related adverse effects on reproductive performance. Decisions on whether to assess the second generation and to omit the developmental neurotoxicity cohort and/or developmental immunotoxicity cohort would also reflect existing knowledge for the chemical being evaluated, as well as the needs of various regulatory authorities. Additional information concerning this testing guideline can be found on the OECD website.[265]

General Considerations Regarding Reproductive/Developmental Toxicology Studies Conducted for Submission to Regulatory Agencies

These studies should be conducted in conformance with appropriate GLP regulations.[248–250,266,267] Whenever possible, the same species and strain of animal should be used



Parental Generation	Cohort	Designation	Animals/cohort	Sexual maturation	Approximate age at necropsy (weeks)
Target is 20 litters per group	1A	Reproductive	20 M + 20 F	Yes	13
	1B	Reproductive	20 M + 20 F	Yes	14 or 20 if assessed
	2A	Neurotoxicity	10 M + 10 F[a]	Yes	9
	2B	Neurotoxicity	10 M + 10 F[a]	No	3
	3	Immunotoxicity	10 M + 10 F[a]	Yes	8
	Surplus	Spares		No	3

[a] One per litter and representative of 20 litters in total where possible.

FIGURE 35.11 Extended one-generation toxicity study schematics.[340]

as tested in other toxicology studies and in the kinetics studies. The kinetics, pharmacological, and toxicological data should support the position that the selected species provides a model relevant for use in human safety assessment.

Reproductive toxicology studies are generally conducted in one species (usually the rat), with some subsegments (e.g., ovarian toxicity, testicular toxicity) obtained from companion subchronic studies in the same species and strain. Developmental toxicology studies are generally conducted in two species, one rodent (usually the rat) and one nonrodent (usually the rabbit). Each study usually includes a vehicle control group and three or more groups administered dosages of the test agent at arithmetic multiples of the highest clinical dosage. Ideally, the low dosage is a multiple of the highest clinical dosage, based on blood serum levels. Each group should include 16–20 animals or litters. It is axiomatic that animals should be at comparable ages, weights, and parity when assigned to a study.

Reproductive toxicology studies may be conducted by treating animals of only one sex and mating these animals with untreated animals of the opposite sex or by treating both male and female animals. A common refinement of the test is to use double-sized control and high-dosage groups during the premating period, with cross-mating of one-half of the animals in each dosage group, in order to determine whether the administration of the agent to animals of only one sex causes different effects from those observed when both sexes are administered with the agent.[244]

Positive control groups are not considered necessary when reproductive toxicology studies are conducted for regulatory use, because consistent sensitivity to xenobiotics has already been demonstrated in most laboratory species by historical experience and GLP compliance mandates training and expertise of personnel. However, historical control data of abnormalities in fertility, fecundity, fetal morphology, resorption, and consistency from study to study should be available for comparative use with any new study data. Historical data for common laboratory species are available from many published sources, as well as recent updates to the Charles River Historical Control Data (www.criver.com/sitecollection-documents/rm_rm_r_tox_studies_crlcd_br_rat.pdf) and the soon to be rolled out MARTA Historical Control Database (Personal communication with Dr. Hew (2012)), but the most relevant data are those generated at the testing facility in the same animal strains, using the same nomenclature and technical conditions.[268,269] A rule of thumb is that a positive control agent should be evaluated before conducting a new test paradigm or examining a new endpoint, to document appropriate training and expertise.

In general, regulatory studies require that the high dosage be maternally and/or developmentally toxic and that the low dosage should be a no-observable-adverse-effect level (NOAEL). In the past, depending upon the regulatory body, no more than 10% mortality and/or 10% reduction in maternal body weight gain were usually considered sufficient to identify a maternally toxic dosage. Conformance with these criteria and the associated testing of excessively high dosages resulted in both maternal toxicity and developmental toxicity.[270] In contrast, the ICH guidelines indicate that the high dosage should produce minimal maternal (adult) toxicity

and be selected on the basis of data from all available studies, including pharmacologic, acute, and subchronic toxicity and kinetic studies. These guidelines allow evidence of maternal toxicity to include reduced or increased weight gain, specific target organ toxicity, changes in hematology or clinical chemistry parameters, and exaggerated pharmacological response, which may or may not be reflected as marked clinical reactions (e.g., sedation, convulsions). Additional justification for high-dosage selection includes (1) physicochemical properties of the test substance or dosage formulation, which, in combination with the route of administration, can be additional justification for limiting the administered amount (generally 1–1.5 g/kg/day provides an adequate limit dosage); (2) kinetics, which can be very useful in determining exposure; and (3) a marked increase in embryo–fetal lethality in preliminary studies. Although often contested and infrequently pursued, the author's experience is that good science can also be used successfully to justify dosage selection and as a negotiating point with regulatory bodies.

Methods for Testing Reproductive/ Developmental Toxicity

According to the ICH S5(R2) document revised in November 2005, *Guideline for Industry, Detection of Toxicity to Reproduction for Medicinal Products*,[231] a reproductive and developmental toxicity testing regimen should be selected that would "allow exposure of mature adults and all stages of development from conception to sexual maturity. To allow detection of immediate and latent effects of exposure, observations should be continued through one complete life cycle, i.e., from conception in one generation through conception in the following generation." The document further suggests that an integrated test sequence can be subdivided into the following stages (see Figure 35.1):

- (Stage A): Premating to conception (adult male and female reproductive functions, development and maturation of gametes, mating behavior, fertilization)
- (Stage B): Conception to implantation (adult female reproductive functions, preimplantation development, implantation)
- (Stage C): Implantation to closure of the hard palate (adult female reproductive functions, embryonic development, major organ formation)
- (Stage D): Closure of the hard palate to the end of pregnancy (adult female reproductive functions, fetal development and growth, organ development and growth)
- (Stage E): Birth to weaning (adult female reproductive functions, neonate adaptation to extrauterine life, preweaning development and growth)
- (Stage F): Weaning to sexual maturity (postweaning development and growth, adaptation to independent life, attainment of full sexual function)

Nevertheless, in most cases, these six stages are assessed by means of just three studies: ICH 4.1.1. The Fertility and General Reproductive Performance Study (*Segment I*; Stages A to B); ICH 4.1.2. The Prenatal and Postnatal Study (*Segment III*; Stages C to F); and ICH 4.1.3. The Developmental Toxicity or Embryotoxicity Study (*Segment II*; Stages C to D). In addition, in recent years, increased emphasis has been placed on direct toxicity studies in juvenile animals[271,272] and developmental immunotoxicity.[273] All of these study types are discussed in some detail in other chapters in this book. Common protocols for use in evaluation of the various ICH segments are identified in Figures 35.5 through 35.8.

ICH Stage A

Premating to conception (reproductive functions in adult animals, including development and maturation of gametes, mating behavior, and fertilization).

Evaluation of the Ovary

Numerous articles,[274,275] chapters,[276–278] reviews,[86,279] and books[87,280] have been written concerning the ovary and ovulation, and a detailed description of ovarian histology and ovulation is beyond the scope of this chapter. The ovary serves a number of functions that are critical to reproductive activity, including ovulation of oocytes and production of hormones. The developing ovarian follicles produce estrogen, while the corpora lutea formed after ovulation produce progesterone. Delayed ovulation can alter oocyte viability and cause trisomy and polyploidy in the conceptus.[281–283] Altered follicular development, ovulation failure, or altered corpus luteum formation or function can result in disruption of cyclicity and reduced fertility. Therefore, significant increases in follicular atresia, evidence of oocyte toxicity, interference with ovulation, or altered corpus luteum formation or function should be considered adverse reproductive effects.

Ovarian Weight

Ovarian weights are recorded using the same techniques as those generally used for recording organ weights. The tissue should be trimmed and weighed close to the time removed from the animal, to avoid dehydration. An alternate method is to weigh the ovaries after trimming and fixation. Ovarian weight in the normal rat does not show significant fluctuations throughout the estrous cycle. However, persistent pathologies such as polycystic ovaries, oocyte depletion, decreased corpus lutea formation, luteal cysts, and altered pituitary–hypothalamic function, as well as reproductive senescence, may be associated with ovarian weight changes. Ovarian gross morphology and histology should be examined to detect alterations associated with ovarian weight changes.[284]

Follicle Number and Size

Methods described in the following can be used to quantify ovarian follicular development. Histopathological evaluation of the three major compartments of the ovary (i.e., follicular, luteal, and interstitial) plus the capsule and stroma should be performed to reveal possible toxic effects.[285–287] The U.S.

EPA OTTPS 870.3800 *Reproductive and Fertility Effects Guideline* and the Female Pubertal Assay[288] require a quantitative evaluation of the primordial follicle population of the ovary. A postlactational ovary should contain primordial and growing follicles, as well as the large corpora lutea of lactation. Ovarian histology, oocyte quantitation, and differential follicle count methodology have been described.[289] Ovarian follicles have been classified into 10 categories,[48] and these categories have been reduced into three classes for the purposes of quantification: (1) primordial follicles, (2) growing follicles, and (3) antral follicles. The primordial follicles can be described as early with the oocyte having no surrounding granulosa cells or late with the oocyte having a single layer of surrounding granulosa cells. The growing follicle can be described as early with the oocyte that is starting to enlarge and the surrounding cells having formed two layers (granulosa and theca) or late with the enlarging oocyte and multiple layers of granulosa and thecal cells but with no evidence of antral formation. The antral follicle can be described as early with a large oocyte, multiple cell layers, and a developing cavity (antrum), while the late antral (Graafian) follicle has mature multiple layers of granulosa and thecal cells and a large antrum.

Routine histopathological examination should detect qualitative depletion of the primordial follicle population; however, current reproductive study guidelines require a quantitative evaluation of primordial follicles of the F_1 females. The number of animals, ovarian section selection method, and section sample size should be statistically appropriate for the evaluation procedure used. The guidelines cite ovarian follicular-counting methodologies[60,290,291] that use a differential count of the primordial follicles, growing follicles, and antral follicles. The current EPA Multigeneration Protocol modification[235] requires ovaries from 10 randomly selected females from highest dosage and control groups. Five 5-µm ovarian sections are made from the inner third of each ovary, at least 100 µm apart. The number of primordial follicles, growing follicles, antral follicles, and corpora lutea is counted from the highest treatment and control groups' ovaries. If statistically significant differences are found, the next lower treatment group is evaluated.

The Female Pubertal Assay[288] requires ovarian histology following hematoxylin and eosin (H&E) staining that includes an evaluation of follicular development (including presence/absence of tertiary/antral follicles, presence/absence of corpora lutea, changes in corpus luteum development, and changes in the number of both primary and atretic follicles) in addition to any abnormalities/lesions, such as ovarian atrophy. Five random sections are evaluated, using the method of Smith et al.[292]

The Society of Toxicological Pathology's Position Paper (2005)[59] on ovarian follicular counting stated that the publications by Bolon et al.[290] and Bucci et al.[60] provided the most thorough comparisons of follicle-counting techniques in mice. These follicular-counting methods have been adopted and/or modified for use as first-tier screening methods in some regulatory guidelines for reproductive toxicity studies. A current proposal for assessing the ovarian histology and counting ovarian follicles (D. Creasy, personal communication, 2011) is as follows:

1. One ovary is fixed in 10% neutral buffered formalin.
2. The middle third of the ovary is sampled and embedded in paraffin.
3. Five-step serial sections at least 100 microns apart are mounted on a slide and stained with proliferating cell nuclear antigen (PCNA) (to visualize the primordial follicles).[293] PCNA is preferred over routine H&E stain.
4. The ovarian sections are qualitatively evaluated for follicular development (including presence/absence of tertiary/antral follicles, presence/absence of or changes in corpora lutea development) in addition to any abnormalities/lesions, such as ovarian atrophy.
5. Quantitative analysis of the number of primordial and primary follicles (follicles with intact oocytes surrounded by a single layer of granulosa cells) are made and recorded.

Differential follicular counts are associated with age, number of animals evaluated, number and location of the ovarian sections, number of sections per ovary, section thickness, differences between evaluators, and the criteria for defining primordial follicles. Ovarian follicle count appears to be a more sensitive endpoint than ovarian weight. In 18 Reproductive Assessment by Continuous Breeding studies using Sprague-Dawley rats,[292] the number of small follicles counted had a remarkably wide variation, ranging from 147 ± 57 to 556 ± 144, and another investigator reported 301 ± 13 small follicles.[87] A review of follicle count historical control data in rats[294] suggests that (1) with a large number of animals, there is relatively good replication within a generation (*when evaluated by the same person*); (2) there is great variability within individual ovaries and sections; (3) random selection of ovarian sections has a high probability of biasing effects; (4) ovarian follicle counts do not appear to be correlated with either ovarian weight or body weight at termination; and (5) ovarian follicle counts should not be the sole endpoint used in risk assessment. However, notwithstanding the great variability between reported values, a detectable decrease in follicle count in a treated group when compared to the control group is considered an adverse effect, because no recovery is possible.

Corpora Lutea Number

The corpus luteum is a transitory endocrine organ formed from the thecal and granulosa cells of the postovulatory follicle.[295] Progesterone is the major hormone produced by the corpus luteum and is necessary for implantation and the maintenance of pregnancy. Each corpus luteum appears as a round, slightly pink, discrete swelling on the surface of the ovary. Corpora lutea can be counted with the naked eye or under a dissecting microscope to determine the number of eggs ovulated. The number of corpora lutea should be

recorded for each ovary. In a litter with no obvious signs of resorption, the number of corpora lutea should be equal to or exceed the number of total implantation sites. For partially resorbed litters, the number of corpora lutea may actually be less than the total number of implantations. When some of the corpora lutea are no longer required to support the remaining conceptuses, some of the corpora lutea involute to become corpora albicantia. However, the concordance between the number of corpora lutea that involute and the number of resorbed implants is not precise. Corpora lutea count either can be discounted (excluded), when the count made is less than the number of implants, or be set to correspond to the number of implants to correct for involuting corpora lutea in partially resorbed litters. The method of handling this issue should be described in the Standard Operating Procedure.

The number of corpora lutea can be affected by many factors, including enhanced ovarian atresia, injection of hormones, a procedure common in mating rabbits; handling, especially in reflex ovulators; and litter size and hormonal feedback mechanisms, some of which are species-specific.[296,297] The corpora lutea count is used in determining pre- and postimplantation losses. Identification of preimplantation loss on the basis of comparison of the number of implants at Caesarean sectioning of the dams is often quite imprecise, especially in mice and rabbits. Generally, in these types of studies, comparison of litter sizes is a more representative indicator of preimplantation loss. As regression of the corpora lutea occurs during lactation, inclusion of this parameter in postlactational evaluations is unnecessary. Even though imprecise, a reduction in the number of corpora lutea or an increase in the pre- and/or postimplantation loss should be considered an adverse reproductive effect.

Hormone Integrity and Function

Sexual maturation begins in the hypothalamic–pituitary–ovarian (HPO) axis, leading to the development of female secondary sex characteristics and fertility. A complete discussion of hormones involved with ovarian function is beyond the scope of this chapter. However, several review articles[298–300] are available. The interrelationships within the HPO axis are already functional during gestation. The basic mechanisms involved in the progression of sexual maturation are conserved across most species. Many of the endocrine-mediated events that are involved in this progression in the rat are similar to those of other mammalian species, including humans. For example, the control of GnRH, the release of gonadotropins from the pituitary, and the steroid positive and negative feedbacks are relatively consistent.[301]

The three primary controllers in the HPO axis are as follows: (1) the neurosecretory neurons within the hypothalamic arcuate nucleus that produce GnRH, (2) the pituitary gonadotropes (which secrete LH and FSH) and lactotropes (which secrete prolactin [PRL]), and (3) the ovarian tissue secreting estradiol and inhibin (granulosa cells of the follicle) and progesterone (the corpora lutea). Germ cells and gonadotropins are not necessary for the development of the female genital tract. Puberty coincides with the maturation of the HPO–uterine axis, as does the onset of vaginal opening (described earlier), ovulation, and estrous cycles. Vaginal opening and vaginal cornification are dependent on increased estrogen levels. Ovulation and estrous cyclicity are dependent on increasing estrogen but are also a function of the developing HPO axis.

Female reproductive cycles are a manifestation of complicated endocrine, paracrine, and autocrine hormonal positive and negative interactions between and within the hypothalamus, pituitary, ovary, and uterus. Sampling of blood for female hormone level determinations must take into account the pulsatile nature (within hours), the diurnal nature (within the day), and the cyclical nature (usually over 3–5 days in rodents) of the female reproductive hormones. A detailed description of the hormonal activity during the estrous cycle is beyond the scope of this chapter [302]; however, there are numerous reviews.[76,303–305]

While numerous hormones and other factors are involved with the HPO axis, few preclinical toxicology studies require the evaluation of hormones. Direct measurements of hormone levels are usually second-level evaluations. These can most effectively be performed using commercially available antibody kits or by sending samples to contract analytical laboratories for evaluation.

Evaluation of the Uterus

Uterine Weight

Uterine weight measurements are a historic method of measuring potential estrogenicity of a test material. Uterine weight fluctuates throughout the estrous cycle, peaking at proestrus when the uterus is distended with watery fluid (sometimes misdiagnosed as hydrometra) in response to increased estrogen secretion. Compounds that inhibit steroidogenesis and cyclicity can cause the uterus to become small and atrophic, thereby decreasing the uterine weight. Uterine weight change has been used as a basis for comparing relative potency of estrogenic compounds in bioassays.[306] Both juvenile[289] and ovariectimized[307] models are used, with the intact model demonstrating both primary and secondary effects and the ovariectimized model identifying uterine-specific effects. The text within the uterotrophic assay guideline fully describes the methodology for the dissection and measurement of uterine weight.

Regardless of the duration of the treatment period, responses can vary with the strain, source, and specific population and age tested, requiring characterization of historical data at the testing laboratory. The common use of limited numbers of animals (10 and 6 animals per group in repeated-dose toxicity and uterotrophic studies, respectively) often results in statistical significance between groups when outlier values (increased and reduced uterine weights) are included, with inappropriate identification of false positives and negatives.[308] However, because of the sensitivity of the assay, the screen is a good predictor of absence of effect. When a positive effect is observed, it should be further characterized. When uterine weights are evaluated in animals in subchronic or chronic tests, it is helpful to identify the estrous cycle stage shortly before sacrifice, as cyclicity greatly affects uterine

weight. The estrous stage identified from a vaginal lavage and the uterine estrous stage identified by histopathology will differ by approximately 1/2 day.

Note: The oviducts and vagina are not routinely weighed in reproductive toxicity studies. The oviductal weight fluctuates depending upon the stage of the estrous cycle. The oviductal lumen contains a variable amount of fluid, which is greatest at metestrus, the period of ovulation.[309] Gross evaluations can be of value in detecting morphologic anomalies, such as agenesis and segmental aplasia. Vaginal weight changes usually parallel uterine weight changes during the estrous cycle, although the magnitude is less. The vaginal and cervical epithelia show cyclic changes in morphology and thickness associated with stages of the estrous cycle.

Evaluation of Estrous Cycling

Reprotoxicity screening studies are usually performed in rodent species, with the female animals treated for the first 14 days before cohabitation and then until mating occurs. Ideally, sexually mature female rodents are evaluated for estrous cycling[77] for 14 days before treatment, to establish a baseline for regularity of cycling, and animals that irregularly cycle should be excluded from evaluation before the treatment phase. Animals housed in the same room tend to have synchronous estrous cycles, with resultant cyclic matings.

Vaginal lavage for the determination of the stage of estrous can be performed at any time of the day; however, it is important that the samples be collected at approximately the same time each day over the course of a study. A pre- and post-check of the saline should be made. (*Note*: If glass pipettes are used, the rubber bulbs can be saved and washed for future use, while the glass stems should be discarded after each sampling. If plastic pipettes are used, the pipettes should be discarded following each sampling. Dropping pipettes should be held with the tip pointing down whenever they contain a vaginal lavage or saline sample, to avoid contaminating the bulb. Contaminated bulbs should be discarded.)

The female rat is removed from the cage, and her identification is verified. One or two drops (approximately 0.25 mL) of physiological saline are drawn into a new, clean dropping pipette, and the tip of the pipette is gently inserted into the vaginal canal. The pipette bulb is firmly but gently depressed to expel the saline into the vagina. The saline is gently drawn back into the dropping pipette, and the pipette is removed from the vaginal canal. If the flush is very clear, the lavage should be repeated. As previously mentioned, care must be taken not to insert the tip too far into the vaginal canal, which can result in pseudopregnancy. The animal is returned to its cage. The contents of the pipette are then delivered onto either (1) a clean glass slide or (2) a ring slide containing designated areas for the placement of each sample (vaginal smear). Each slide should be numbered, the top and bottom of each slide clearly identified, and a corresponding record made, indicating the animal number and location for each vaginal smear. Technicians should record the date, the start and end times of sample collection, and their initials on appropriate forms. All slides should be handled carefully to avoid mixing vaginal lavage samples from different animals. If samples become mixed, they are discarded, and the sampling procedure is repeated for those animals. The smear of the vaginal contents is then examined (wet and unstained) by using a microscope at 100–200×.

The cellular characteristics of vaginal smears reflect structural changes of vaginal epithelium and follow a predictable course during the estrous cycle. The stage of the estrous cycle is determined by recognition of the predominant cell type present in the smear at each daily evaluation. An estrous cycle in rats and mice is typically 4–5 days and may be influenced by multiple factors, including light, temperature, humidity, noise, nutrition, and social relationship. In general, cycles are more easily influenced in mice than in rats.

Cycling changes are usually divided into four stages: estrus, metestrus, diestrus, and proestrus (Figure 35.12). Estrus lasts approximately 10–15 h in rats and approximately 21 h in mice; it is the only stage in the cycle when the female will copulate with the male. As shown in Figure 35.12A, cornified squamous epithelial cells are the dominant cell type in the vaginal smear and are few in number or absent. The squamous epithelial cells vary from flat (occurring singly during early estrus) to curled (occurring in large sheets in late estrus). Metestrus lasts from 6 to 14 h in rats and approximately 22 h in mice. It is characterized by the presence of numerous cornified epithelial cells together with irregularly shaped nucleated epithelial cells. Leukocytes are present in considerable numbers (Figure 35.12B). Diestrus lasts 60–70 h (approximately 1/2 of entire cycle) in rats and 22–33 h in mice. It is dominated by leukocytes, although some nucleated epithelial cells may be present. In late diestrus, the nucleated epithelial cells become more spherical, and occasional cornified cells and erythrocytes are present (Figure 35.12C). Proestrus has a 12–18 h duration in rats and approximately 21 h in mice and denotes the beginning of the next cycle. The vaginal smear is dominated by numerous nucleated epithelial cells, usually arranged in grape-like clusters (Figure 35.12D).

Measurement of estrous cycle length is done by selecting a particular stage and counting until the recurrence of the same stage (usually day of first estrus, day of first diestrus, or day of proestrus). The estrous cycle lengths should be calculated for each female. It is often easier to visualize the estrous cycle by plotting the data (e.g., Figure 35.12E). The group mean estrous cycle length should be calculated, and the number of females with a cycle length of 6 days or greater should be calculated. There is a general consensus that a single cycle with a diestrus period of 4 days or longer or an estrus period of 3 days or longer is aberrant. Cycles that have three or more days of estrus during most of the cycles are classified as showing *persistent* estrus. Cycles that have four or more days of diestrus during most of the cycles are classified as showing *persistent* or prolonged diestrus. Pseudopregnancy is a persistent diestrus (approximately 14 days in duration). Constant estrus and diestrus, if observed, should be reported. Persistent diestrus indicates at least temporary and possibly permanent cessation of follicular development and ovulation,

and thus at least temporary infertility. Anestrus or persistent diestrus may be indicative of toxicants that interfere with follicular development, that deplete the pool of primordial follicles, or that perturb gonadotropin support of the ovary. The ovaries of anestrous females are atrophic, with few primary follicles and an unstimulated uterus. As expected, serum estradiol and progesterone are abnormally low. The presence of regular estrous cycles after treatment does not necessarily indicate that ovulation occurred, because luteal tissue may form in follicles that have not ruptured. However, that effect should be reflected in reduced fertility. Altered estrous cyclicity or complete cessation of vaginal cycling in response to toxicants should be considered an adverse reproductive effect. Subtle changes of cyclicity can occur at doses below those that alter fertility. However, subtle changes in cyclicity without associated changes in reproductive or hormonal endpoints would not be considered adverse.

Evaluation of Mating Behavior and Fertilization

Techniques for evaluating mating performance and fertility are used both for impregnating animals for use in developmental toxicity studies and when mating performance is evaluated in a reproductive toxicology evaluation. In the latter case, treatment is usually begun in the female animal at least 2 weeks before cohabitation and continued until necropsy. The timing of necropsy is dependent on the endpoint(s) to be evaluated, occurring at some interval within the gestation period. If the purpose of the screen is to observe mating behavior and any associated impairment of fertility or implantation, necropsy is usually performed at 13–15 days of presumed gestation. For alternate uses, treatment continues to the day before termination, either approximately 1 day before expected parturition, with Caesarean sectioning and evaluation of uterine contents, or at completion of a 21 day lactation period, with evaluation of uterine contents and litter sizes and viability.

In general, mating ratios of one male per female are ideal for use in rodents, because it allows easy identification of the sire and dam, history of reproductive performance, and excludes potential reductions in performance associated with excess use of the same male. The most effective way to produce pregnancies is to pair the animals when the female is in proestrus (usually late in the afternoon, dependent upon the light cycle used), as it will soon enter estrus and be receptive. However, assuming a large population of animals is available (approximately 125 breeder males and 125 virgin females), sufficient numbers (approximately 100 mated females) can be obtained within 4–5 days by simply cohabiting the entire

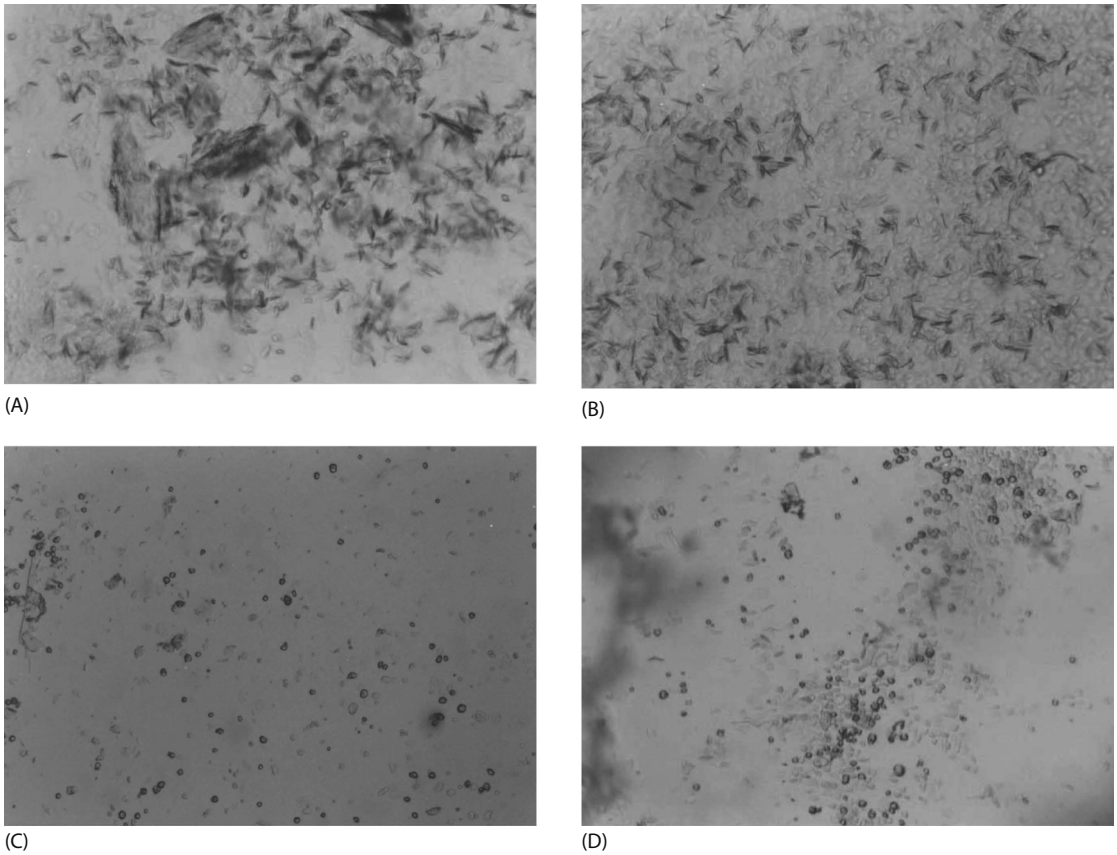


FIGURE 35.12 (A) Estrus cycle stage 1: estrus. (B) Estrus cycle stage 2: metestrus (C) Estrus cycle stage 3: diestrus. (D) Estrus cycle stage 4: proestrus.

(*continued*)

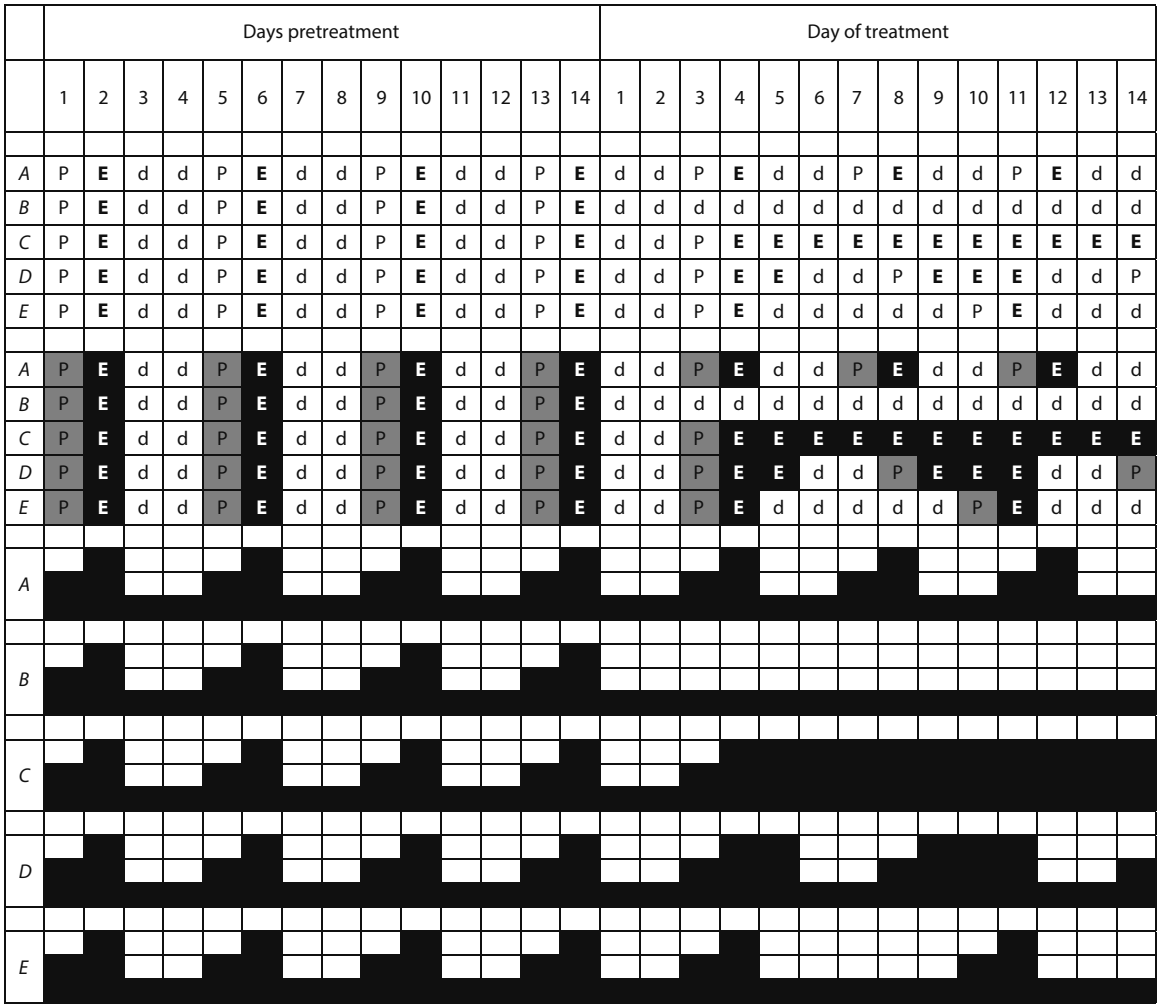


FIGURE 35.12 (continued) (E) Graphical display of vaginal smear data. Three methods of depicting the estrus cycle data are presented in the following. The first method is presenting the data on a spreadsheet with the estrus in bold letter. The second method is shading the estrus and proestrus. The third method is using stacked bars (estrus, 3 bars; proestrus, 2 bars; and diestrus, 1 bar). Cycle A represents a normal cycle. Cycle B shows the pattern in constant diestrus or pseudopregnancy. Cycle C shows constant estrus. Cycle D shows persistent or prolonged estrus. Cycle E shows persistent or prolonged diestrus. (From Parker, R.M., Testing for reproductive toxicity, in: Hood, R.D. (ed.), *Handbook of Reproductive Toxicology*, 3rd edn., CRC Press, Boca Raton, FL, 2011.)

population simultaneously. Timed-mated female rats can also be purchased from animal vendors. The pregnancy rate for timed-mated females is greater than 95%, which is similar to in-house breeding colonies.

Although estrous cycling is not usually evaluated when animals are bred for use in developmental toxicity studies, this parameter is very helpful in identifying potential effects in female animals in reproductive toxicity evaluations, when perturbations in estrous cycling may predict reduced female receptivity and fertility. Male mating performance should be considered in evaluation of any observed effects, as altered estrous cycling during cohabitation and reduced female fertility may reflect male-mediated effects (e.g., naivete, general health, altered/reduced mating performance, testicular lesions, and abnormal sperm characteristics).

Rats and mice generally have multiple intromissions in one copulatory interval. If there is an indication that aberrant copulatory behavior may be present, the animals can be observed (videotaped) and graded for the number of copulations, number of intromissions, duration of intromission, as well as expected female receptivity (lordosis). Mating (copulation) is confirmed by the presence of spermatozoa in the contents of the vaginal smear and/or the observation of a copulatory plug in situ; these findings designate day 0 of presumed gestation. It is prudent to note when less than 10 sperm are present in a smear, because this information can assist in interpreting mating and fertility data. For mice, the presence of an expelled copulatory plug in the pan is often considered an adequate proof of mating, although only approximately 85% of the mice identified in this fashion actually become pregnant.

Female mating performance can be measured by several endpoints. These include identifying the number of days in cohabitation before mating (this value can be adjusted to the number of days after the first estrus), the number of mated (inseminated) female animals per group, and pregnancy

incidences based on the total population per group (% pregnant/number cohabited) and on the inseminated population per group (% pregnant/number inseminated). These formulas can be found in several textbooks.[94,285] The number of females assigned to an alternate male and the duration of cohabitation (routinely 7 days) with the second male should always be identified. Care must be taken to ensure that all parameters based on group values are calculated on the basis of observations for the initial cohort pair, and data regarding any subsequent matings should be identified as such.

Vendor-Supplied Timed-Pregnant Rabbits

Vendors (e.g., Covance Research Products, Denver, PA) offer timed-mated rabbits and have done extensive studies with numerous laboratories that show the fertility rate based on gestational day of shipment. Unless the facility doing the study is in close proximity to the vendor, it is not possible to measure food consumption from GD 0. The advantage of purchasing timed-mated rabbits is eliminating the upkeep of a large number of bucks and the technical time expended for mating procedures and recordkeeping, but there is no on-site quarantine period prior to GD 0 with these animals. Usually, the period between the arrival of the purchased timed-mated does on GD 1, 2, or 3 and the first data collection (GD 4) is considered the stabilization (quarantine) period. During this period, technicians monitor the does for viability and observe feed consumption. Body weights and clinical observations as well as beginning food consumption measurements occur on GD 4.

Artificial Insemination Procedures (Rabbits)

Artificial insemination is an alternate method of mating rabbits. It has the advantages of better control over fewer male breeders (up to five females may be inseminated with one sample) and reductions in possible cross-infection and technical time requirements. Another advantage of artificial insemination is that the semen sample can be evaluated for sperm count, viability, and motility before insemination to ensure that an optimal sample is introduced into the female. However, the downside is a slightly reduced pregnancy rate and limited genetic background of the sires, although this can prove a benefit, should a malformation be traceable to the sire. The methodology of semen collection and evaluation is not a subject for this chapter and can be found in the literature. The standard sperm concentration used for insemination is 6.0×10^6 spermatozoa per 0.25 mL. Saline is then used to dilute the semen sample.

Before insemination, each doe is given an intravenous injection (ear vein) of HCG (20–25 U.S.P. units/kg HCG), to induce ovulation (larger dosages tend to result in superovulation and do not increase fertility or litter sizes). At this concentration, each injection volume is 0.10 mL/kg of body weight (e.g., a rabbit weighing 5.0 kg is administered 0.50 mL for a dosage of 20 U.S.P. units/kg, which is a total dose of 100 U.S.P. units/animal).

Approximately 3–6 h after HCG-induced ovulation of the female rabbits, each doe is inseminated with approximately 0.25 mL of diluted semen. The technician gently restrains the doe with the head downward and facing away from the technician. A glass insemination tube (with an inside diameter of 4 mm, length of 7.5 in.) that is bent at a 45° angle 1.5 in. from one end is used; a rubber bulb covers the opposite end. The tube is filled to the bend with the diluted semen (approximately 0.25 mL). The tail is pulled, exposing the vulva, which should appear pink and glistening (evidence of receptivity). The semen-filled glass tube is held with the short end of the glass tube parallel to the rabbit's spine and guided into the vagina to the depth of the bend (at the pelvic brim). The tube is inserted until resistance is no longer felt, because the tube has passed into the cervix. The tube bulb is squeezed, depositing the dilute semen, and the tube is then gently rotated 90°–180° (stimulating the cervix) and subsequently removed (continued squeezing of the bulb occurs during removal). If the doe urinates during insemination, another semen sample is injected approximately 30 min later. The day of artificial insemination is usually designated GD 0. One major consequence of hormonally priming the female (necessary for artificial insemination) is the possibility of *superovulation*. A large number of eggs can be ovulated, but many are not implanted, resulting in a large percentage of preimplantation loss for this animal.

ICH Stage B

Conception to implantation (reproductive functions in the adult female, preimplantation, and implantation stages of the conceptus).

Evaluation of Preimplantation Loss and Impaired Implantation

This process is based on comparison of the number of corpora lutea with the number of uterine implantation sites identified at Caesarean sectioning of the dams. As previously described, this parameter is highly variable and tends to be of minimal value in mice, rabbits, and guinea pigs and is highly associated with fetal survival and hormonal feedback in rats.

ICH Stages B, C, and D

- B: Conception to implantation—reproductive functions in the adult female and preimplantation and implantation stages of the conceptus
- C: Implantation to closure of the hard palate—adult female reproductive functions and development of the embryo through major organ formation
- D: Closure of the hard palate to the end of pregnancy—adult female reproductive function, fetal development and growth, and organ development and growth

Caesarean-Sectioning Procedures

When the purpose of the reproductive or developmental toxicity study is to screen for preimplantation loss and early implantation, the animals are killed near the beginning of the fetal period, as this allows easy identification of viability,

based on a beating heart. In a full developmental toxicity screen, the animals are killed on the day before or the day parturition is expected, to preclude effects associated with delivery complications (e.g., prolonged gestation and associated pup mortality and maternal cannibalization of stillborn or malformed pups). Regardless of the day of gestation, balanced termination of animals across groups should occur, to preclude differences associated with gestational ages. Termination of large populations of animals on the day parturition is expected tends to result in some dams delivering litters. This is particularly common in mice and hamsters and occurs occasionally in rabbits. Such events produce complications in data capture because the animals can be included in some analyses (e.g., pregnancy incidence, implantation numbers), but should be excluded from others (e.g., fetal body weights, fetal ossification site observations). One benefit of termination of the dam on the day parturition is expected is that there is less variability in the degree of skeletal ossification. General practice is to terminate mice, rats, rabbits, and hamsters on days 18, 20 or 21, 29, and 15 of gestation, respectively.

Gross Necropsy of Maternal Animals

Euthanasia of the rodent dams is easily accomplished by asphyxiation with carbon dioxide gas, and animals are terminated at intervals, ensuring that the time between death and necropsy is no more than 20 min. After external examination, the animal is placed on its dorsal surface and an incision made through the skin and/or musculature, extending from the inguinal area to the anterior portion of the neck. The abdominal viscera are exposed by making an incision through the abdominal muscles and peritoneum up to the sternum. The thoracic viscera are exposed by making an incision along one or both sides of the sternum up to the level of the clavicles.

Gross examination of the thoracic, abdominal, and pelvic viscera of the dam and retention of gross lesions in neutral buffered 10% formalin is a standard procedure that often provides evidence of maternal effects. When required, the brain may also be examined. In some cases, target organs, for example, liver, kidneys, and brain, are weighed and retained for further evaluation. It is also common to collect maternal blood, placentas, and embryos or fetuses for use in pharmacokinetics evaluations at Caesarean sectioning. Obviously, data from nonpregnant animals should be excluded in the pharmacokinetics studies relevant to pregnancy. According to present ICH guidelines, pharmacokinetic studies should be conducted in pregnant animals to determine if pharmacokinetic differences occur in the pregnant state versus the nonpregnant state and whether the fetus is exposed to appreciable amounts of the test material. These studies are usually conducted twice (first and last dosage) during pregnancy in rats and rabbits and are frequently performed in satellite animals or as separate studies, in order to preclude the occurrence of developmental abnormalities that may be associated with the stress of multiple blood collections and/or unusually high dosages of test material in the dams assigned to full reproduction or developmental toxicity studies. Although it is occasionally appropriate to perform detailed kinetics studies, such is not usually done. Common practice in the toxicokinetics animals is to identify only pregnancy (presence of live conceptuses), to avoid potential legal or liability actions based on the identification of possible fetal malformations associated with nondrug-related manipulations of the dam.

Evaluation of Ovaries and Uterus

Evaluation of the uterus and its contents can be performed at any time during the gestation period, depending upon the purpose of the evaluation. The uterus with attached ovaries is removed from the abdominal cavity and placed on a dissection blotting paper. The number of corpora lutea in each ovary is counted (with ovaries attached to oviduct), and the number is compared with the number of implantation sites, which can be identified through the uterine wall. Figure 35.13A and B show rat and rabbit ovaries with corpora lutea, respectively. The uterus of female rodents that do not appear pregnant can be examined effectively by pressing the uterus between glass plates and examining it for the presence of implantation sites or by staining it with ammonium sulfide.[310]

Evaluation of Uterine Weight

When a uterine weight is desired, the right ovary is removed, and the left ovary remains on the oviduct of the uterine horn (for identification of right and left uterine horns). An alternate method is to remove both ovaries and attach a hemostat (weight tared before weighing) to the right uterine horn for identification. Care is taken throughout the process to not cut the uterus or express any fluid. The same procedure can be used to evaluate nonpregnant uterine weights of rats or mice used in assays for estrogenicity, as described previously.

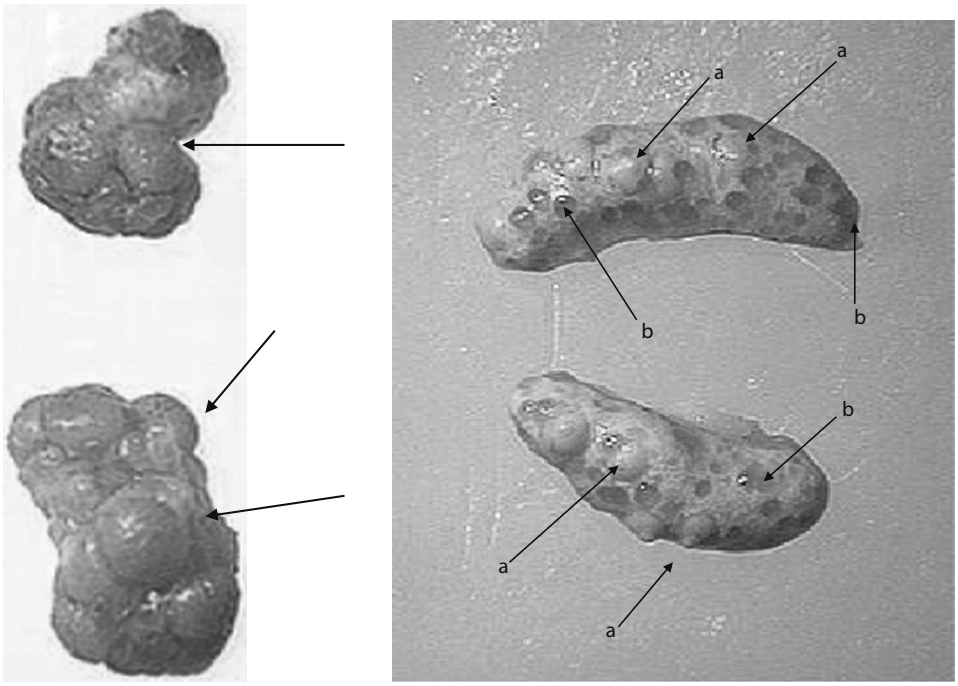
Evaluation of Uterine Contents

After weighing, the uterine horns are opened along the greater curvature, and the number and distribution of implantation sites are recorded (see Figure 35.14A and B). Each implantation site is consecutively numbered in both uterine horns, beginning with the ovarian end of the right horn and counting toward the cervix, and continuing with the ovarian end of the left uterine horn and again counting toward the cervix. Each implantation site is described as follows.

Early or Late Resorption

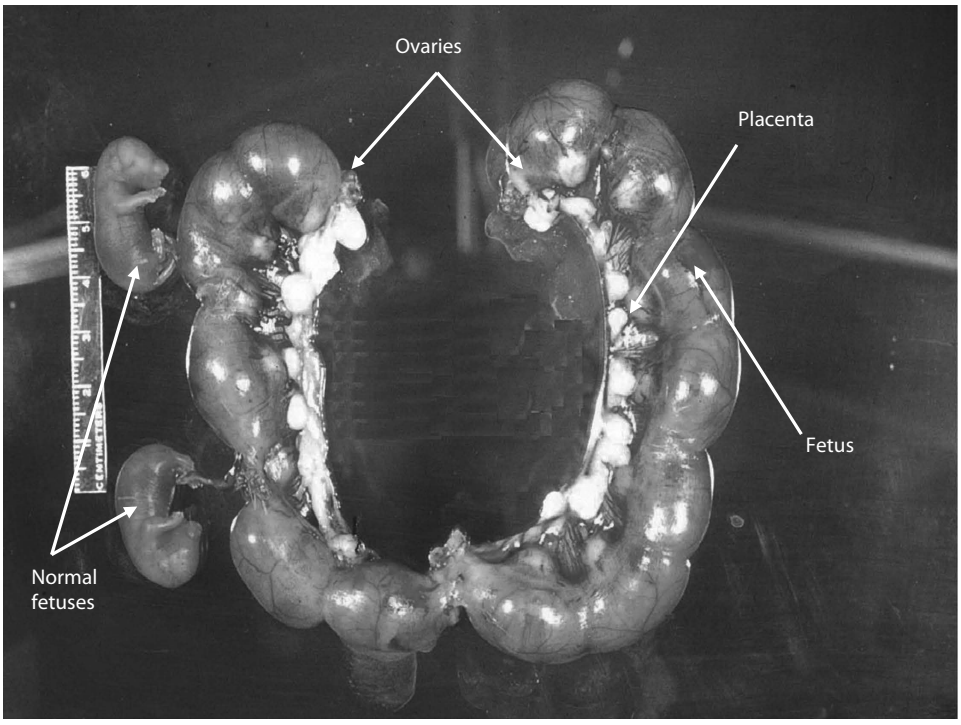
An implanted conceptus that is undergoing autolysis is termed a resorption.

- An *early resorption* is defined as a conceptus that has implanted but has no recognizable embryonic characteristics evident upon examination. An early resorption is usually visualized by ammonium sulfate staining or by observation through pressed glass plates.



(A) (B)

FIGURE 35.13 (A) Rat ovaries—corpora lutea. (B) Rabbit ovaries—(a) corpora lutea; (b) empty follicles.



(A)

FIGURE 35.14 (A) Rat uterus—day 20 of gestation.

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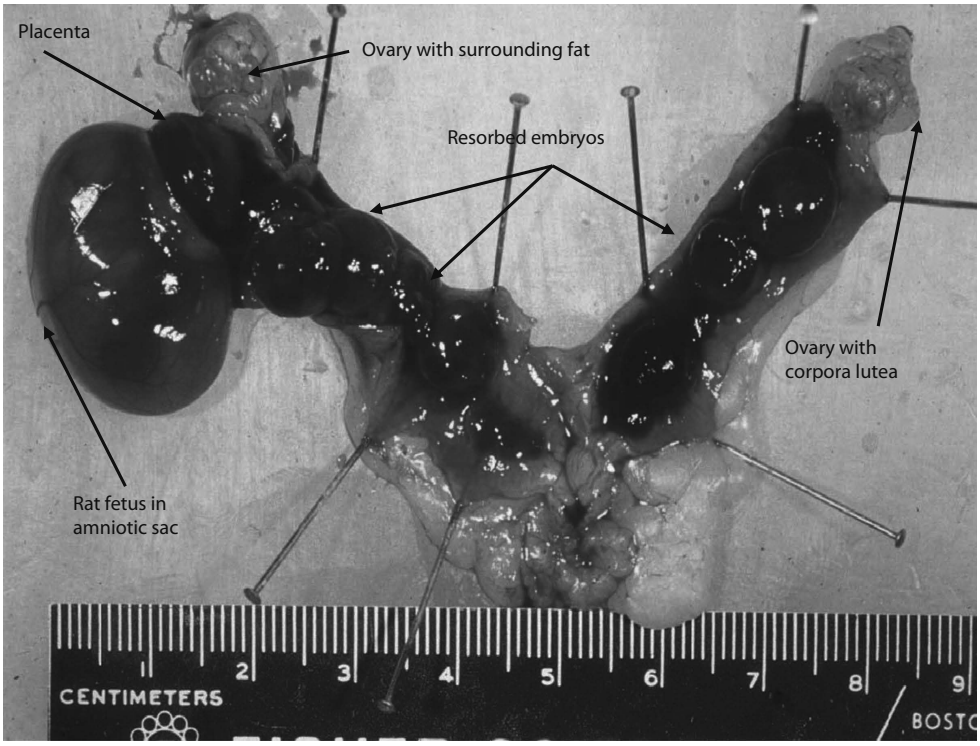


(B)

FIGURE 35.14 (continued) (B) Rat uterine contents—day 20 of gestation.

- A *late resorption* is defined as fetal remains or tissues that have recognizable fetal characteristics (such as limb buds with no discernable digits present) and are undergoing autolysis. Fetuses with marked to extreme autolysis are considered to be late resorptions.
- A *live fetus* is defined as one that has a beating heart or a fetus that responds to stimuli.
- A *dead fetus* is defined as one that has tissues with recognizable fetal characteristics (such as limb buds with no discernable digits present) and usually undergoing autolysis. A dead fetus is defined as a term fetus not demonstrating marked to extreme autolysis.

In some fertility studies, in which the necropsy is performed near term, fetuses are classified as live (fetus exhibits normal appearance, spontaneous movement, heartbeat, etc.) or dead (nonliving full-sized fetus with discernable digits, with a body weight greater than 0.8 g for rats).

Viable or Nonviable Embryos (Day 10, 13/14/15 of Gestation for Rats)

In fertility studies in which the pregnant female rodents are necropsied around day 10–15 of gestation, the conceptuses are categorized as live (normal appearance, heartbeat, etc.) or dead (discolored, undergoing autolysis). The dead classification in these studies is analogous to a resorption. The numbers and uterine positions of any early and late resorptions and viable and dead fetuses are recorded:

- A viable embryo is oval or crescent shaped, pink, firm, and enclosed in an amniotic sac filled with clear fluid.
- A nonviable embryo is amorphous, small, pale pink to tan or deep red to black, soft, and enclosed in an amniotic sac filled with clear or cloudy fluid; it is unnecessary to open the uterine horns to determine viability of day 10 or day 13 rat embryos.

Assessment of Implantation in *Apparently Nonpregnant* Dams

When dams appear to not be pregnant at necropsy, a thorough examination of the uterus must be performed. If no implantation sites are observed, the opened uterus is stained with a 10% aqueous solution of ammonium sulfide.[310] The results will be recorded (under maternal gross findings) that the uterus was placed in stain, and if implantation sites are found after staining, the pregnancy status must be updated.

Gross Examination and Weight of Placenta

The placenta and attached amniotic sac containing the fetus are removed from the uterine horn. The placenta is separated from the amniotic sac, and the placenta and fetus are placed in the compartmentalized box for the litter. Each placenta is observed for gross changes (e.g., white spots, areas of necrosis, abnormal size). Individual placental weights are often recorded. Altered placental weights might reflect an estrogenic effect, although the relatively high variability in placental weight associated with fetal size and blood status at maternal death makes this endpoint relatively variable.

Fetal Evaluations

The following description is a representation of the activities involved in the evaluation of the fetus but should not be construed as the only acceptable methodology. During the fetal examinations, all unusual (notable) observations for each individual fetus are recorded or described by the examining technician. The general considerations for designation of a finding as an "**M**" or a "**V**" are imprecise, may vary from teratologist to teratologist and study to study, may be relatively arbitrary, and are not always generally accepted. This lack of consistency was noted in the Terminology of Developmental Abnormalities in Common Laboratory Mammals,[311] which does not attempt to differentiate between malformations and variations. However, the observations are often coded by many laboratories as malformations or variations based on previously established criteria and designations (usually defined by the study director and laboratory supervisor).

Fetal alterations are generally considered *malformations* when

- The alteration is incompatible with or severely detrimental to postnatal survival (e.g., exencephaly, anencephaly, ventricular septal defect [VSD], diaphragmatic hernia, spina bifida, and cleft palate)
- The alteration is irreversible without intervention (e.g., hydroureter, hydronephrosis, VSD). Although, if small, VSDs may resolve postnatally in children
- The alterations result from partial or complete failure of cellular migration, closure, or fusion (e.g., cleft palate, cleft lip, spina bifida, facial clefts, renal agenesis, and ectopic organs)
- The alteration involves replication, reduction (if extreme), or absence of essential organs or structures (e.g., brain region(s), appendages, major blood vessels, heart chambers or valves, lungs)
- The alteration exhibits a concentration- or dose-dependent increased incidence with a qualitative and/or quantitative increase in severity across dose groups (e.g., missing distal limb bones/missing distal and some proximal limb bones/amelia (missing limb)
- Several alterations occur together comprising a recognized syndrome of otherwise minor anomalies

An alteration is considered a *variation* when

- The alteration is nonlethal and not detrimental to postnatal survival
- The alterations are generally transitory and/or reversible (e.g., wavy ribs or the reduced ossification in a cephalocaudal sequence that is frequently seen associated with immaturity or delayed development due to toxicity)
- The alteration may occur with a measurable frequency in controls and/or not always exhibit a dose-related increased incidence in treated litters
- The alteration is a small detectable change in size of specific structures

Experience and expertise are pivotal requirements for accurate evaluations of fetus specimens. Technical training should include evaluation of a positive control group (e.g., aspirin or vitamin A), as well as evaluation of several untreated control group litters (co-evaluation of two or three studies, with an experienced investigator), particularly for soft tissue and skeletal evaluations, to ensure comparable levels of expertise, when multiple technicians perform these evaluations in the same laboratory. It is also recommended that identified alterations be confirmed by a senior level investigator, to ensure comparable evaluation within a study, as well as across studies.

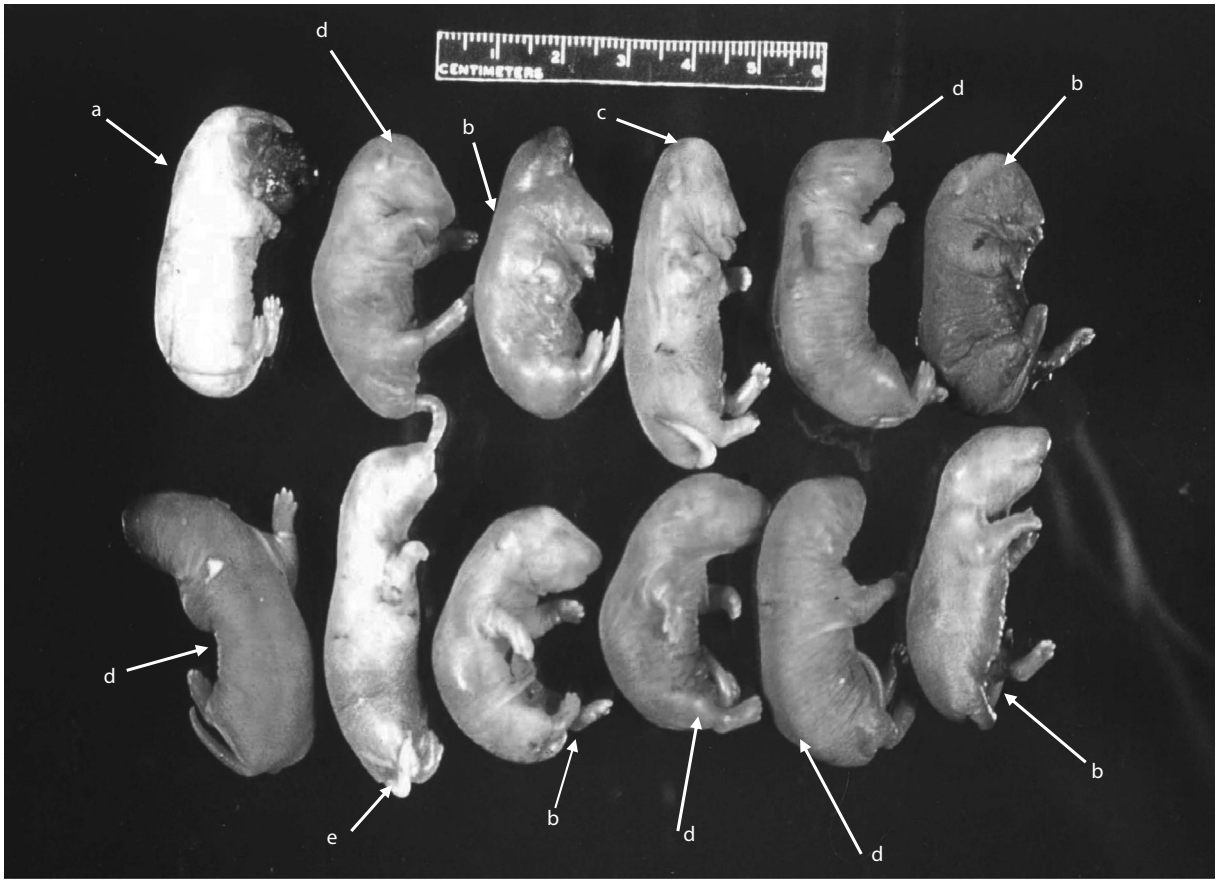
The UK Industrial Reproductive Toxicology Discussion Group (UK IRDG) and the Society of Biology have established an International Register of Fetal Morphologists. There are excellent teaching images of external, visceral, and skeletal evaluation of the rat and rabbit on the UK IRDG website (http://www.irdg.co.uk).

Gross External Fetal Examination

After removing the fetus from the uterus, excess blood and amniotic fluid are removed from the fetus by rinsing and/or blotting on absorbent paper. Individual fetuses are deposited in multicompartmented boxes, each compartment of which contains a tag that will ultimately be tied to the fetus and will remain with the fetus throughout processing, evaluation, and archiving. The tag cites the study number, dam number, uterine placement of the fetus, and appropriate fixative to be used for further processing, if required. When evaluations are to be made without the investigator's knowledge of the dosage group of the fetus (i.e., *blind*), the box containing the fetuses is given a white label bearing only the study number and animal number and with a Caesarean-sectioning processing tag. Although some investigators believe that *blind* evaluations enhance the quality of the data, this procedure is not always valid. In many cases, knowledge regarding the specimen evaluated enhances the ability of an experienced investigator to identify alterations associated with treatment. In addition, conducting evaluations under blinded conditions increases the probability of error in data processing and storage of the specimens. It also increases the difficulties of data collection, should an automated data collection system not be available.

To the extent possible, observations made during gross external examination of the fetus should be confirmed in soft tissue and skeletal examinations and the related changes described. Because the degree of ossification increases very rapidly shortly before birth, any errors regarding day 0 of gestation for the dam should result in exclusion of the litter from summarizations of delays in ossification.

Examples of typical externally identifiable abnormalities in rat fetuses are provided in Figure 35.15A through F. All fetuses are examined for gross external alterations, externally sexed, weighed, and tagged. Each fetus is euthanized by intraperitoneal injection of pentobarbital or euthanasia solution (preferred method of euthanasia for fetuses assigned to skeletal evaluation). Hypothermia is no longer an acceptable method of euthanasia for fetuses.[312] Decapitation may



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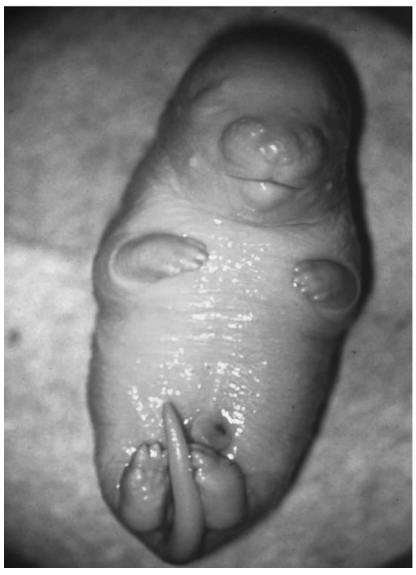

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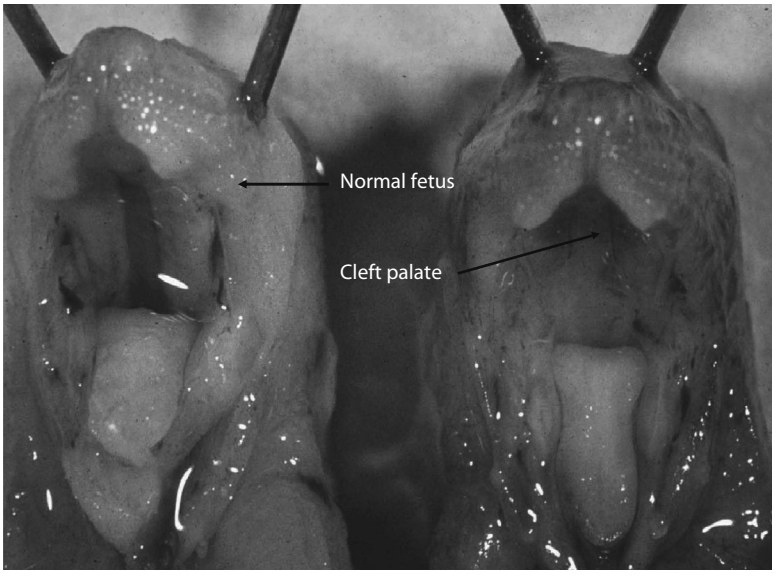
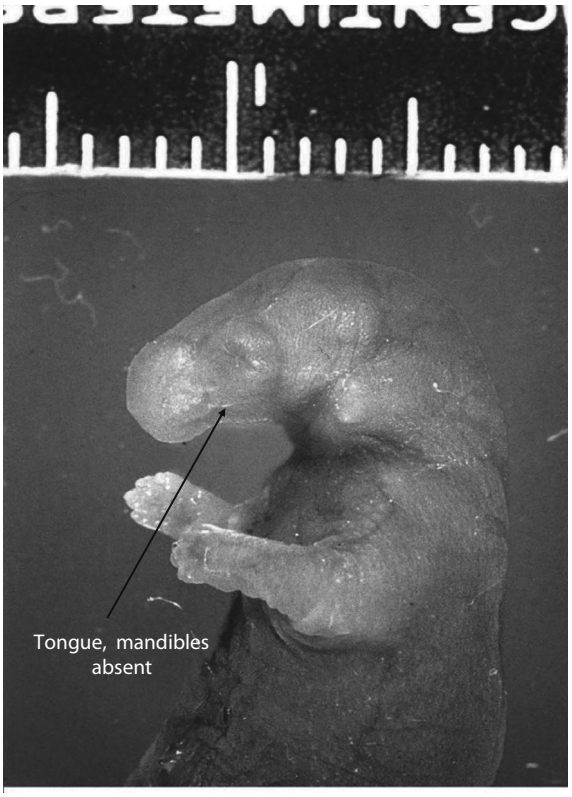


(C)

FIGURE 35.15 (A) Rat fetuses—day 20 of gestation. Example of differential effects on litter: a = dead, macerated; b= malformed; c = dead, compressed; d = live, externally normal, e = dead. (B) Rat fetus—day 20 of gestation—anasarca (edema of entire body); note apparent clubbing of limbs. (C) Rat fetuses—day 20 of gestation—cleft palate.



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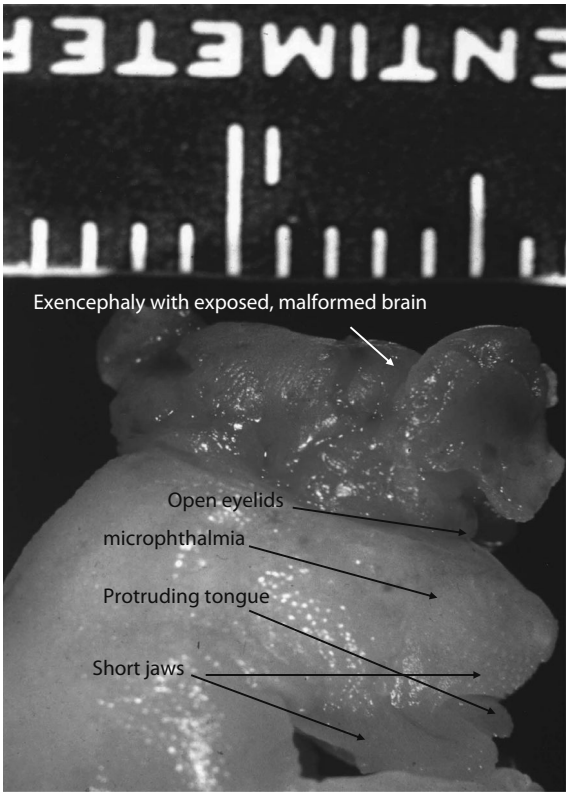

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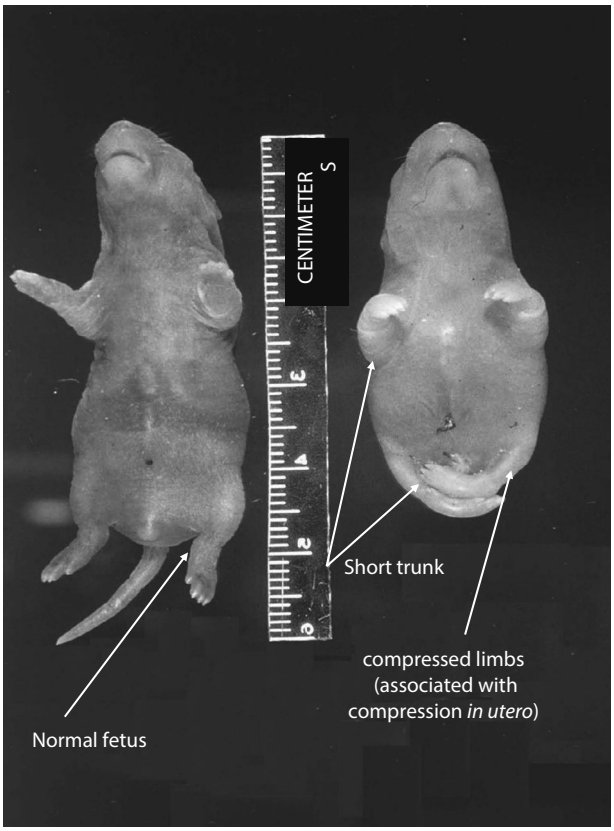


(F)

FIGURE 35.15 (continued) (D) Mouse fetus—day 18 of gestation—Agmathia (absence of tongue and mandibles). (E) Rat fetus—day 20 of gestation: exencephaly, short jaws, protruding tongue, microphthalmia. (F) Rat fetuses—day 20 of gestation—normal fetus and fetus with short trunk and compressed limbs.

be used for fetuses assigned to evaluation using Wilson's free-hand sectioning technique[105] for the head and visceral dissection of the body. After completion of the gross external fetal examinations, a fresh visceral examination can be performed.

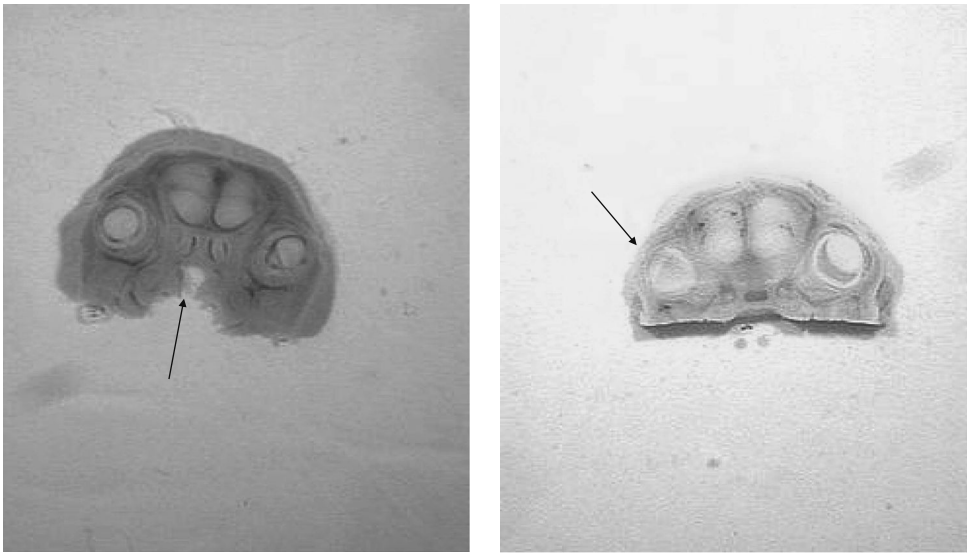
The general practice for rabbits and other large mammals with relatively small litter sizes is to evaluate all fetuses for gross external, soft tissue, and skeletal alterations; for small rodents (rats, mice, hamsters, relatively large litter sizes), the usual practice is to evaluate half of the fetuses in each litter for soft tissue alterations and the remaining fetuses for skeletal alterations. If all fetuses must be evaluated for both soft tissue and skeletal examinations, dissection procedures, such as those described by Staples[313] and Stuckhardt and Poppe,[314] are performed before fixation of tissues and processing for skeletal examination.

Although some investigators recommend random assignment of fetuses to either evaluation, this procedure complicates an already technically difficult process. The most common practice is to assign fetuses to skeletal or soft tissue evaluation on an every-other-fetus basis, beginning with the first fetus at the ovarian position of the right uterine horn and ending with the last fetus at the vaginal position of the left uterine horn. The first fetus is assigned to skeletal evaluation, although the use of the reverse procedure would not affect the overall outcome of the evaluation. Occasionally, it may be desirable to evaluate a fetus with a specific gross external alteration to the alternate fixative, in order to achieve a more meaningful evaluation.

Soft Tissue Evaluation

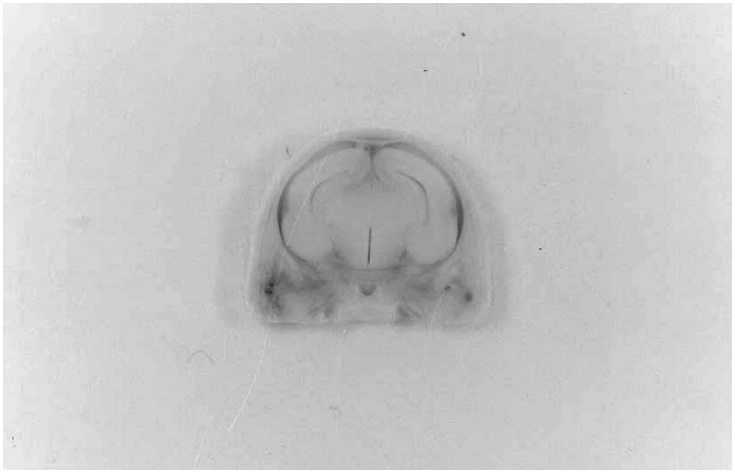
All nonrodent fetuses are evaluated for soft tissue alterations by using gross dissection techniques. Soft tissue evaluation in rodents can be performed by using either Wilson's free-hand cross-sectioning,[105] or gross dissection.[313] Examples of normal sections and a few abnormalities in rat fetuses are provided as Figure 35.16A through J. As noted previously, approximately half of the rodent fetuses in each litter are generally assigned to soft tissue evaluation after gross external evaluation. Fixation of these specimens in Bouin's solution and subsequent evaluation by Wilson's method,[105] rather than dissection of the fetuses directly after Caesarean delivery, allow the use of fewer personnel and reduce the time required to perform all observations associated with Caesarean sectioning. Evaluation of the modified Davidson's- or Bouin's-fixed fetuses can occur at any time after hardening and decalcification of the specimens (a process usually requiring approximately 1–2 weeks). In the absence of high incidences of multiple abnormalities in the fetuses, an experienced individual can generally evaluate all specimens (approximately 1000 fetuses) from a study over five working days. The cross sections are easily preserved and are ready for processing for histopathological evaluation, if required. The disadvantages of this method are that it tends to require more intensive training and experience than fresh dissection. This is because the evaluator must have an extensive knowledge of normal appearance of each cross section and be aware of potential artifacts associated with fixation (e.g., potential shrinkage, tracks from slicing), biased slicing, and small differences in the thickness of the slices and the tissue present, events associated with individual fetal size.

In contrast, the Staples' gross dissection[315] allows identification of both gross external and soft tissue alterations on the same day Caesarean delivery occurs, shortening study time by approximately 1–2 weeks. It requires less training, because the viscera are evaluated in situ, an aspect that is relatively easy to conceptualize. Gross dissection provides an overall view of the major organs, blood vessels, and the heart, as well as a relatively easy identification of most common cardiac and great vessel alterations. It also eliminates



(A) (B)

FIGURE 35.16 (A) Rat fetus—day 20 of gestation—Bouin's fixation—cleft palate. (B) Rat fetus—day 20 of gestation—unilateral microphthalmia.



(C)

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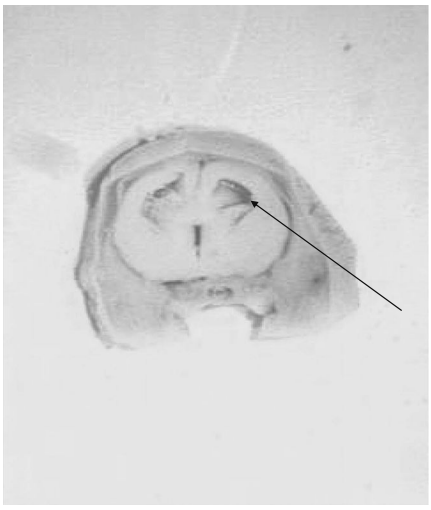

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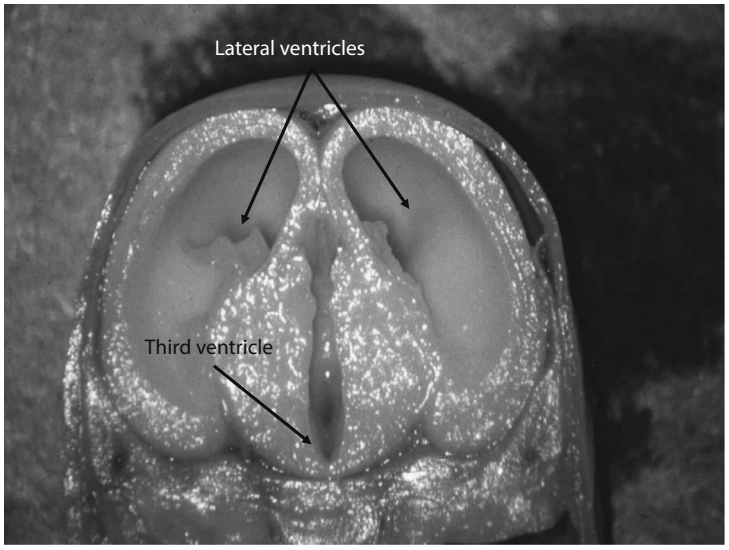
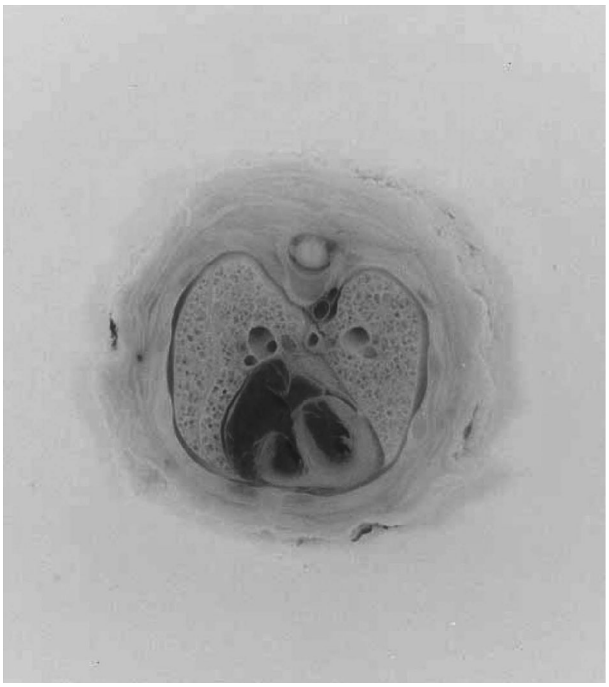


(F)

FIGURE 35.16 (continued) (C) Rat fetus—day 20 of gestation—Bouin's fixation—normal brain. (D) Rat fetus—day 20 of gestation—slight dilation of lateral ventricles in brain. (E) Rat fetus—day 20 of gestation—Bouin's fixation—marked dilation of lateral ventricles in brain. (F) Rat fetus—day 20 of gestation—Bouin's fixation—extreme dilation of lateral ventricles and moderate dilation of third ventricle in brain.

(*continued*)



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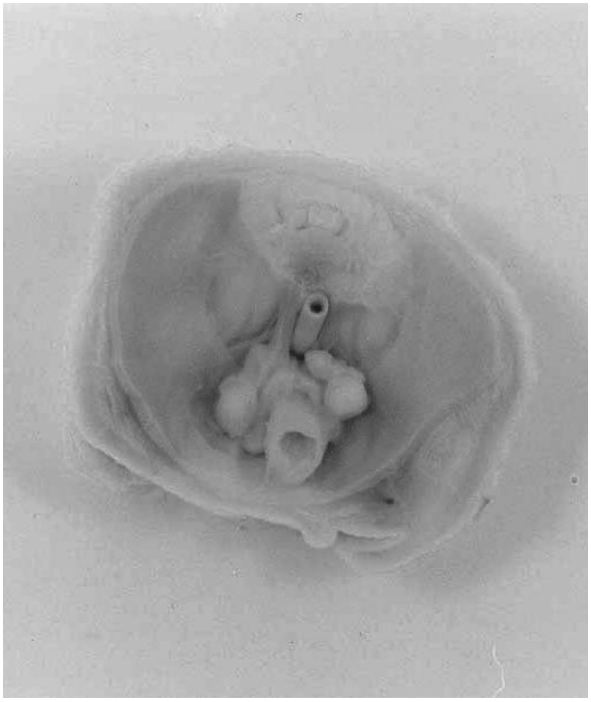
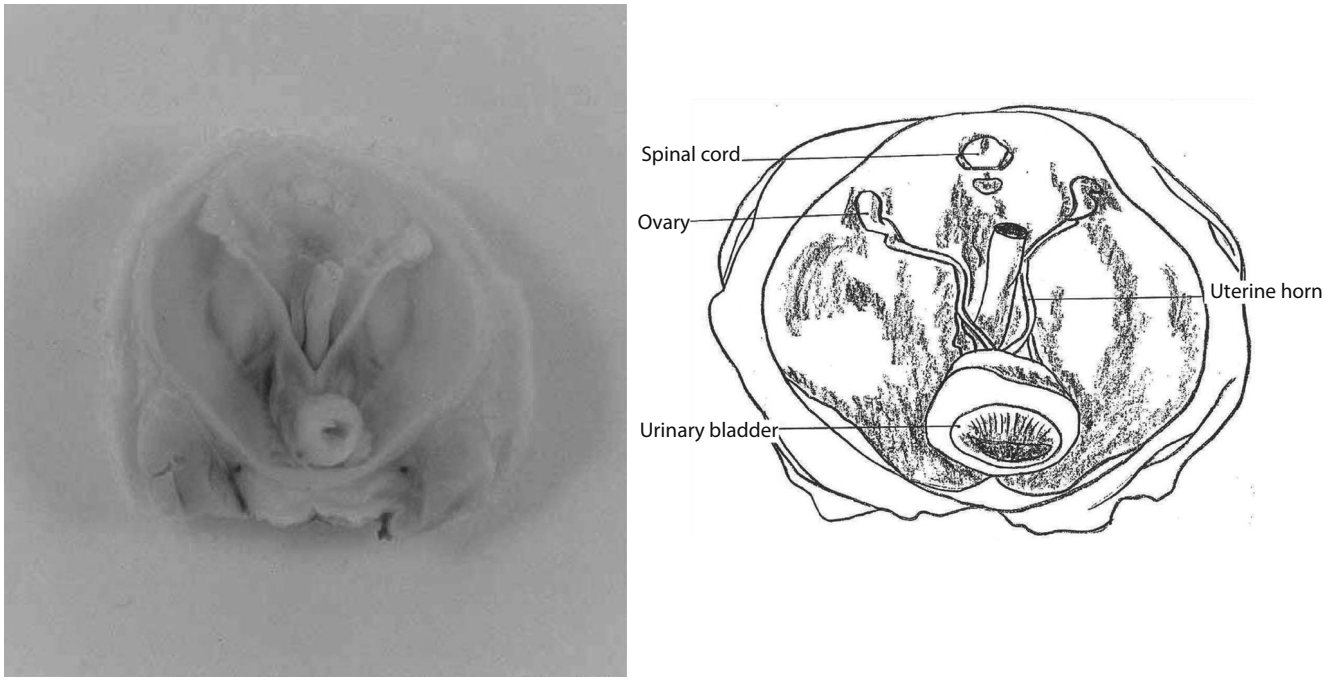


(H)

FIGURE 35.16 (continued) (G) Rat fetus—day 20 of gestation—Bouin's fixation—major organs around the heart. (H) Rat fetus—day 20 of gestation—Bouin's fixation—normal male reproductive organs.



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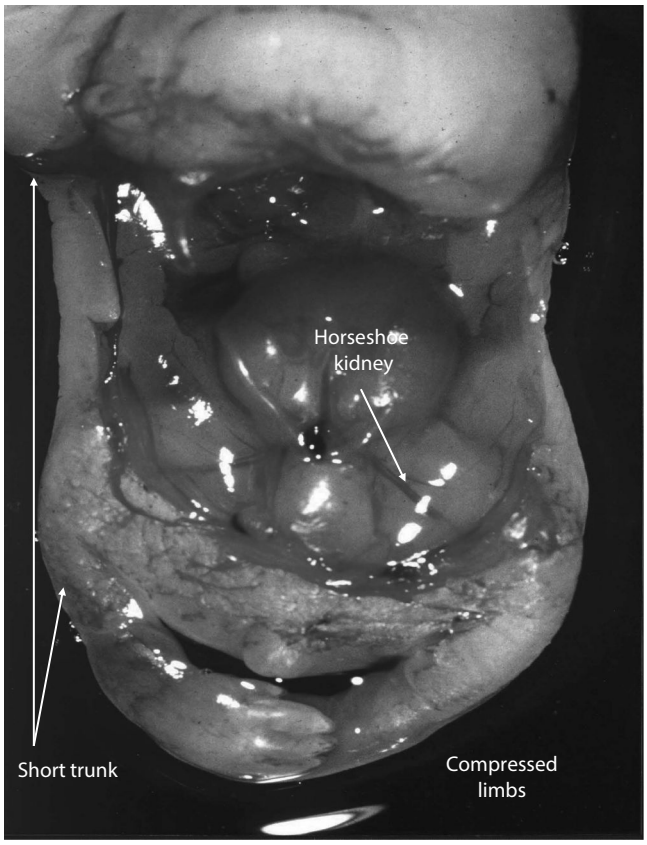


(J)

FIGURE 35.16 (continued) (I) Rat fetus—day 20 of gestation—Bouin's fixation—normal female reproductive organs. (J) Rat fetus—day 20 of gestation—dissected—short trunk, compressed limbs and horseshoe kidney.

artifacts associated with fixation and allowed processing of all fetuses for skeletal examination. The disadvantage of this method is increased personnel requirements (generally four technicians are required to simultaneously evaluate the fetuses, in order to complete all Caesarean-sectioning deliveries and evaluations within approximately 5 h, which greatly reduces inter-litter variability).

Wilson's Cross-Sectioning Technique

After the fetuses have been fixed in modified Davidson's or Bouin's solution long enough to harden soft tissues and decalcify the bones, but before sectioning, the fetus is reexamined for external abnormalities. Any previously noted abnormality is confirmed and any additional findings are recorded. The fetal identification tag and the limbs and tail of the fetus are then removed, and the fetus is cross-sectioned. The actual number of cross sections made is dependent on the size of the fetus when using the Wilson's cross-sectioning technique.[105] Keeping the sections moist with Bouin's solution, each section is examined with the aid of a stereo microscope at 7× to 40× magnification. Because so many diagrams and photographs of Wilson's procedure are available for brevity, only a few representative sections (Figure 35.16A through I) are provided here, along with a description of expected observations.

The initial sections are made through the nasal passages, which should be open and unblocked throughout. The roof of the mouth should be continuous (Figure 35.16A) and separate the oral cavity from the nasal passages. The nasal septum should completely divide the nasal passage in anterior sections, but loses its connection ventrally in more posterior sections. The cross section through the eyes should demonstrate the presence of two eyes of equal size (Figure 35.16B). The retina should be in one piece, without separation of the layers.

Cross sections through the cerebral hemispheres should demonstrate the presence of cranial nerves and minimal dilation of the lateral and third ventricles in the brain (Figure 35.16C through F). Both lobes of the pituitary should be identifiable, with minimal space between the lobes. The section through the inner ears should allow identification of the presence of the lumina of the cochlea, sacculus, and utriculus and absence of obstruction of the lumina of the semicircular canals.

Cross section through the upper neck should demonstrate that the esophagus is dorsal to the trachea, and that both are unobstructed. The thyroid should be present on each side of the trachea, with lobes of equivalent and appropriate sizes. The cross section through the lower neck should reveal the presence and size of the thymus, absence of obstruction of the esophagus and trachea, and presence of the trachea to the right of the esophagus. Subsequent cross sections through the upper thorax and great vessels should allow identification of the size and orientation of the great vessels, the size of the atria (they should not be enlarged), separation of the left and right atria (the atria are not separated at the level of the foramen ovale), absence of obstruction of the esophagus and bronchi, and the presence of four lobes in the right lung and one in the left lung. The three cusps of the aortic valve, separation of the left atrium and left ventricle by the mitral valve, and the tricuspid valve, separating the right atrium and right ventricle, should be identifiable. More posterior, the intact interventricular septum should be evident (Figure 35.16G).

The cross section at the level of the diaphragm should allow inspection of the surface of the diaphragm for any ruptures or herniations of abdominal viscera into the thoracic cavity. More posterior, the lobation of the liver should be evident, as well as the entry of the esophagus into the stomach. The mid-abdominal cross section should be evaluated for any unusual patches or raised areas in the mucosa of the stomach. More posterior, it should be noted that the right kidney is slightly anterior to the left kidney; the size and shape of the kidneys and each pelvis should be checked for enlargement and papillary development and the spleen evaluated for size, shape, and texture. Should the adrenal glands not be transected, they should be examined separately and sectioned; normal adrenal glands appear as a single solid mass of tissue.

The pelvic area should be examined for the ureters, located in the connective tissue of the lower back, and any deviations in the course or diameter noted. The sex of each fetus should be confirmed internally (Figure 35.16H and I), and any discrepancy in the externally identified sex noted. Bilateral testes and ovaries should be present, with the testes descended.

Staples' Dissection Technique

Unless also assigned to skeletal examination, the head of the rodent fetus is removed and stored in Bouin's solution for subsequent examination after cross sectioning, as described by Wilson[105,315] or Barrow and Taylor.[316] The fetus is placed on a board, and the limbs are restrained with elastic bands or dissection pins. Visceral examinations may be made using a binocular microscope or a magnifying lens and light, when necessary (for the larger nonrodent fetuses, a microscope is not generally required). A longitudinal cut is made, extending from below the umbilicus through the midline of the trunk. The diaphragm is examined for intactness, after which the cut is continued along one side of sternum exposing the thoracic viscera. The trachea is detached from the surrounding tissues, and the supporting ligaments are separated from viscera, after which the trachea, esophagus, and thymus are examined. The thymus is removed, and the heart and great vessels are examined for shape and position. The main arterial branches above the heart are examined, but subbranches of these major vessels are not explored, because of the extensive variability present in normal fetuses (Figure 35.17A). At the anterior portion of the heart, the semilunar values are present at the base of the pulmonary truncus. Also present are the ascending arch of the aorta, the innominate artery, the right carotid artery, the right subclavian artery, the left carotid artery, and the left subclavian artery. The heart is

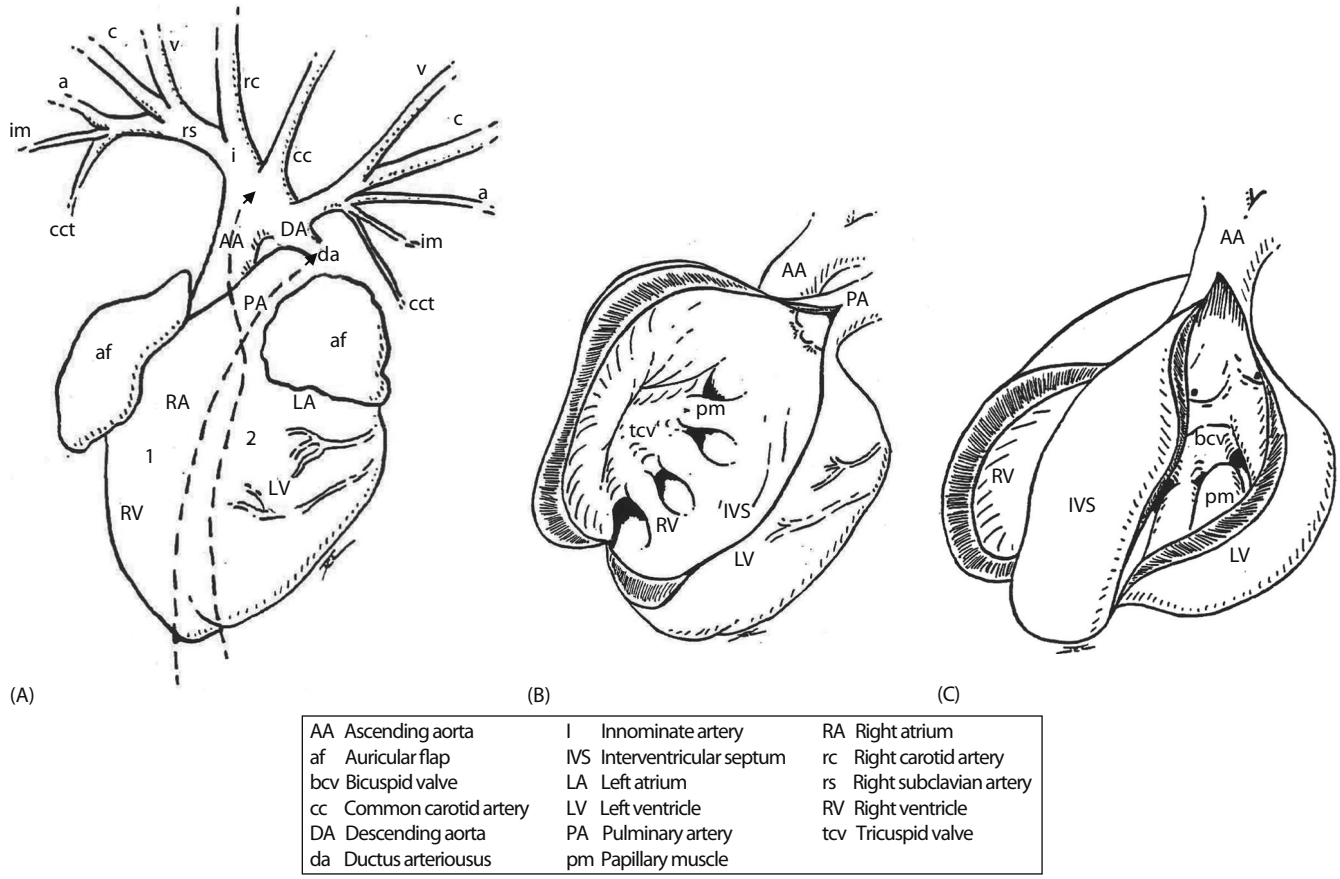


FIGURE 35.17 (A) Major arteries above the heart. (B) First heart cut. (C) Second heart cut.

then pulled over to the right, revealing the pulmonary arteries leading to the lungs and the ductus arteriosus.

The heart is cut and examined for internal alterations (Figure 35.17B and C), as follows. The first cut is made anteriorly from the apex of the heart, entering the right ventricle and exiting from the pulmonary truncus, without cutting the dorsal musculature of the heart. This cut reveals the papillary muscles, the tricuspid valve, and the three semilunar valves. In a beating heart preparation, a functional septal defect could be easily detected because blood would spurt through the septum. A second cut is made through the left ventricle, which will cross over the first incision and enter the ascending aorta. This cut will reveal the bicuspid valve, the papillary muscles, and the three semilunar valves.

The lungs, liver, stomach, pancreas, spleen, and gallbladder (when appropriate) are examined for color, size, shape, position, and appropriate lobation. The color, size, and position of adrenals, kidneys, ureters, intestines, bladder, and genitalia are then evaluated (the rectum should contain meconium) (Figure 35.16J). Particular attention is given to evaluation of the reproductive organs for gross integrity, size, shape, and position. During this procedure, gender (sex) is confirmed for rodent species and identified for rabbits (ferrets can be sexed either as fresh or fixed specimens). The ureters are observed for hydroureter, tortuousness, and obstruction, and it is noted whether urine is present in the urinary bladder. The kidneys are sectioned for a detailed examination of infrastructures (size of each renal pelvis, size and appearance of the renal papilla). The patency of the anus can be checked by the use of a hair.

When the head is examined for skeletal alterations, the skull and brain may be sectioned at the level of the frontal–parietal suture and the brain examined in situ. Alternatively, the head and/or brain may be retained in Bouin's solution for later sectioning, as previously described. The eyes are removed and examined for color, size, and shape (nonrodent species).

Evisceration of Fetuses

Fetuses are eviscerated to aid in clearing and staining of the skeleton. It is helpful to also remove the skin from nonrodent fetuses. Small scissors are used to make a longitudinal cut in rodent fetuses. This cut extends from below the umbilicus through the midline of the trunk and along one side of the sternum, severing costal cartilage, but not ribs, and avoiding the clavicle. Before further processing, the externally identified fetal sex is confirmed internally (evidence of testicles or uterine horns and ovaries). Similar procedures are then used to evaluate both rodent and nonrodent specimens, although small forceps are used for the rodent fetuses and

blunt forceps may be used for the larger nonrodent fetuses. Evisceration is performed by inserting the forceps into the thoracic cavity and pulling downward on the trachea and esophagus, removing these organs and the lungs and heart (thoracic viscera) as a unit. The diaphragm is gripped and pulled downward, and abdominal viscera (liver, kidneys, and intestines) are removed. Any remaining viscera in the abdomen or pelvis are then removed. Throughout this process, care must be taken to prevent damage to the skeleton.

For nonrodent fetuses, blunt forceps are used to remove as much of the skin and subcutaneous fat as possible, although skin can remain on the paws, tail, and snout, to prevent damage to the underlying bones. Any damage occurring during processing should be noted, to prevent the artifact from being potentially incorrectly identified as a skeletal anomaly. After checking that the fetal identification tag remains secure, that the eyes have been removed, and that the head was sectioned, the eviscerated, skinned fetus is returned to the holding tray, containing 95%–99% isopropyl alcohol or 70%–95% ethanol.

Clearing the Carcass

A 1% KOH solution is recommended for clearing rodent fetuses and a 1%–2% KOH solution for nonrodent fetuses. Although higher concentrations may be used, the speed of maceration is associated with fetal size, temperature and lighting conditions, and agitation; and small specimens can be easily and inadvertently dissolved. For optimal skeletal processing, minor changes should be made in any maceration/staining process, depending upon individual specimens. Inspection should be ongoing, and the speed of the process modified depending upon the observed degree of completion of the maceration and staining of the individual specimen. KOH is generally prepared or obtained in 1 gallon units, and alizarin red S is added (50.0 mg/1 gallon of 1% KOH solution) to stain the skeletons.

Several concentrations of glycerin are needed to clear and preserve the stained skeletal preparations during storage. As supplied by the manufacture, glycerol is 99.5% pure. This should be diluted to 20%, 40%, 60%, and finally 80% concentrations, so that for use as the macerated, stained fetuses can be gradually brought up to the 80% glycerin or 99.5% glycerol concentration used for storage. Too rapid an increase of the glycerin concentration will result in compression of the fetal skull.

Processing can be performed using compartmentalized polystyrene utility boxes, or alternate containers that do not react with the KOH or alizarin. Several commercial units are now available, although they are not recommended for use in staining very small fetuses, which should be observed during the processing, to prevent inadvertent damage.

The procedure described is a modification of the method of Staples and Schnell,[317] in which eviscerated, skinned (if appropriate) fetuses are fixed in plastic bottles filled with 99% isopropanol or 95% ethanol. Fixation of rodent fetuses generally requires at least 7 days, while nonrodent fetuses may require at least 14 days. After the alcohol fixation period, the fetuses are placed into individual compartmentalized plastic boxes and any remaining alcohol drained away. Each compartment is then filled with 1% (rodent) or 2% (nonrodent) KOH solution, and the fetuses are allowed to macerate for approximately 24 h. The KOH solution is then drained and replaced with 1% KOH solution containing the alizarin red S stain, and the fetuses are allowed to remain in the stain for approximately 24 h, after which this solution is drained and replaced with a fresh 1% KOH solution, in which the specimens remain for another 24 h. The KOH solution is again drained, and the rodent fetuses are cleared with progressively higher concentrations of glycerin (20%, 40%, 60%, and 80% for rodents; 20%, 40%, and 80% for nonrodents). After completion of this process, the fetuses are stored, ultimately for archiving, in plastic jars to which 80% glycerin and a few crystals of thymol, a preservative, have been added.

Staining of Fetal Skeletons

While there has been some emphasis on the use of double staining (alizarin red and alcian blue) to identify changes in cartilage in term fetuses during the last few decades, in practice, this procedure has not been widely utilized. In a double-stained skeleton, the ossified bone stains purple to red and the cartilage stains blue. The staining of the cartilage allows the examiner to ascertain the bone merely not yet ossified, while the cartilage anlage is present, or is the underlying cartilage absent. As stated by Tyl and Marr,[318] "…a cleft in the cartilage plate of the sternum explains bipartite ossification sites of the sternebrae, and a misalignment of the sternebrae may be seen by the abnormal fusion of the cartilage plate. The normalcy of the cervical vertebrae can only be determined in a double-stained specimen, as these are not normally ossified in the term fetal skeletal preparation. The visualization of the cartilaginous anlagen may allow distinction between a delay in a normal process (i.e., a variation; ossification is delayed, but the underlying cartilage is normal) versus a frank malformation (ossification may be delayed, abnormal or normal, but the underlying cartilaginous skeleton is abnormal)."

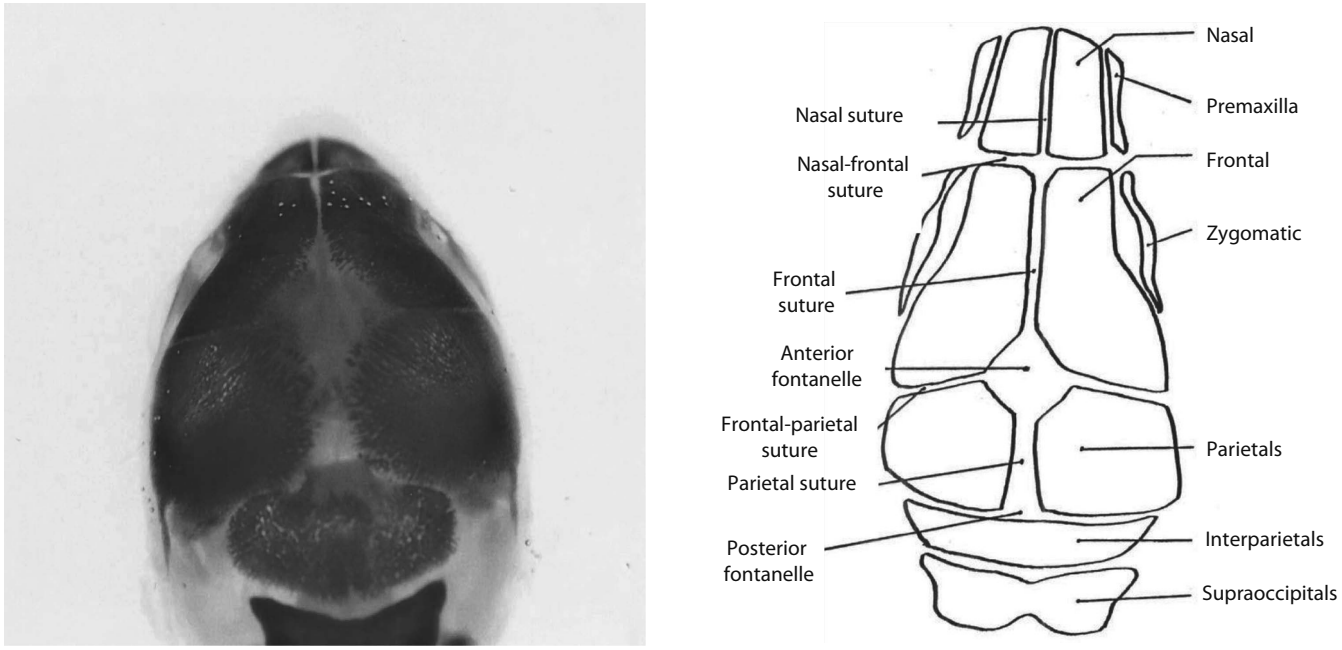
Technical training is critical. The technician must be familiar with the normal appearance of bone and cartilaginous structures in the fetus and with the degree of ossification that should be evident on different sacrifice dates. Double staining may be especially useful in pathogenesis studies of skeletal development. Although not described here, several references are available for double staining[319,320] and in an atlas by Menegola et al.[321,322] A description of age-related fetal observations in the rat skeleton can be found in Marr et al.[323]

Examination of Fetal Skeletons

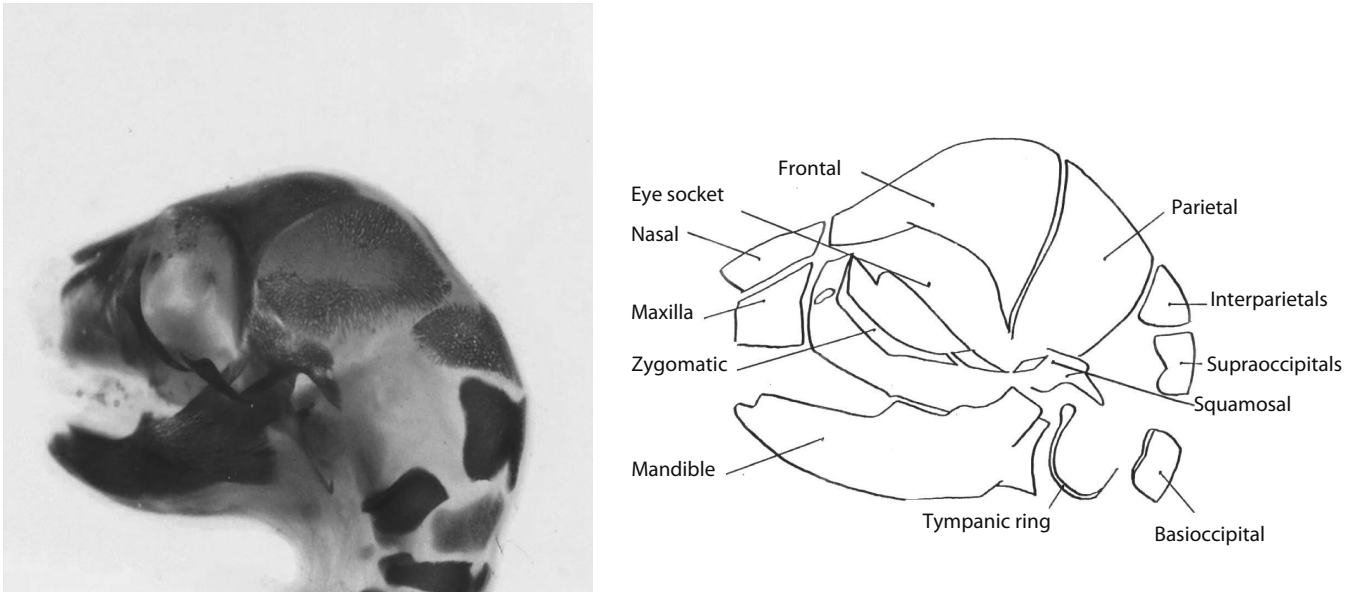
The skeletal system consists of axial and appendicular sections. The axial skeleton includes the skull, vertebral column, sternum, and ribs. The skull (Figure 35.18A through C) consists of the paired bones of the face and the upper jaw (premaxillae, maxillae, zygomatics, and squamosals). The zygomatic arch is formed by the zygomatic bone, squamosal

bone, and zygomatic process of the maxilla. The mandibles form the lower jaw. The exoccipital and supraoccipital bones form the cranial cavity's posterior wall, while the cranial roof's posterior portion formed by the interparietal bones and the anterior portion is formed by the parietal bones that also form the sides of the cranial cavity. The anterior portion of the cranial vault is formed by the paired frontal and nasal bones.

The vertebral column consists of the vertebrae, hyoid, ribs, and sternum. Each vertebra consists of three parts (the centrum and two arches). The vertebral column articulates anteriorly with the exoccipital bones of the skull. The vertebrae are divided into the following groups: cervical vertebrae (7; C1 through C7) (Figure 35.18D and E); thoracic vertebrae (rat, 13 or 14 [T1 through T13 or 14]; rabbit, 12 or 13 [T1 through T12 or 13]) (Figure 35.18F and G); lumbar vertebrae (rat, 5 or 6 [L1 through L5 or 6]; rabbit, 6 or 7 [L1 through L6 or 7]) (Figure 35.18H and I); sacral vertebrae (3 [S1 through S3; the vertebrae present between the



(A)



(B)

FIGURE 35.18 (A) Rat fetus—day 20 of gestation—normal skull, dorsal view. (B) Rat fetus—day 20 of gestation—normal skull, lateral view. (C) Rat fetus—day 20 of gestation—normal skull, ventral view.

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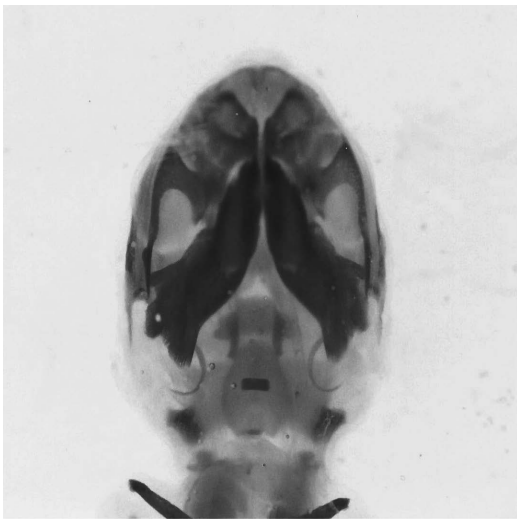

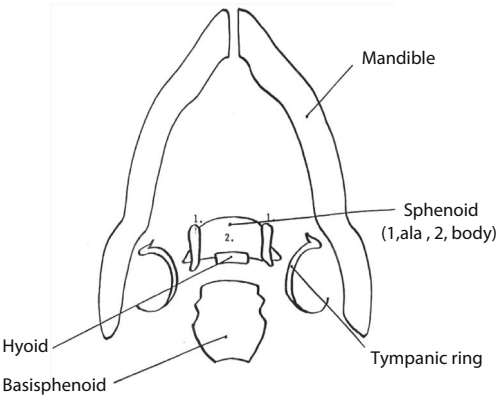

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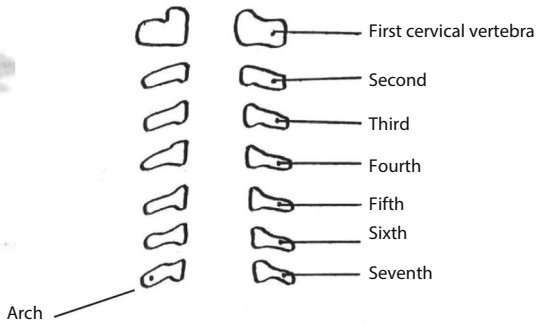

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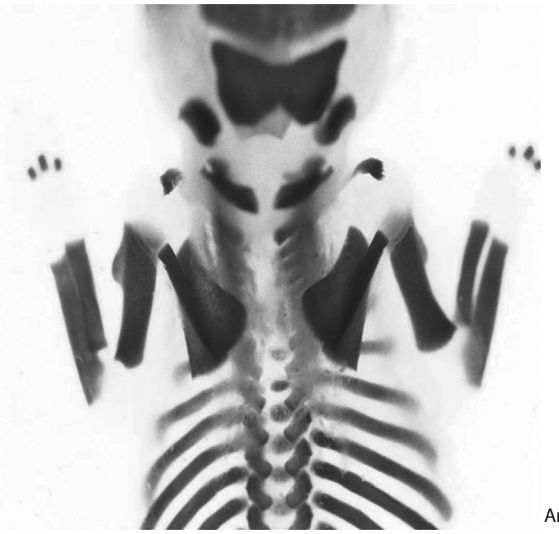
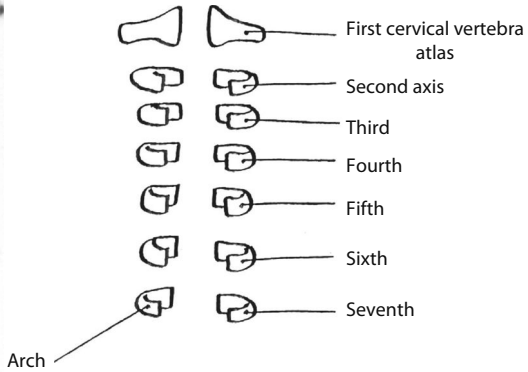
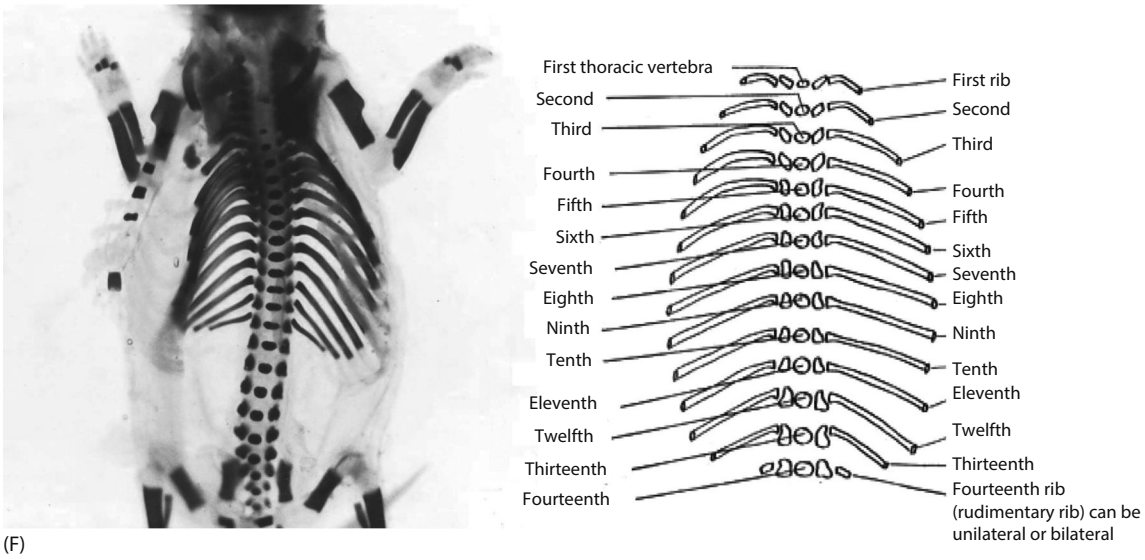




(E)

FIGURE 35.18 (continued) (C) Rat fetus—day 20 of gestation—normal skull, ventral view. (D) Rat fetus—day 20 of gestation—normal cervical vertebrae, ventral view. (E) Rat fetus—day 20 of gestation—normal cervical vertebrae, dorsal view.



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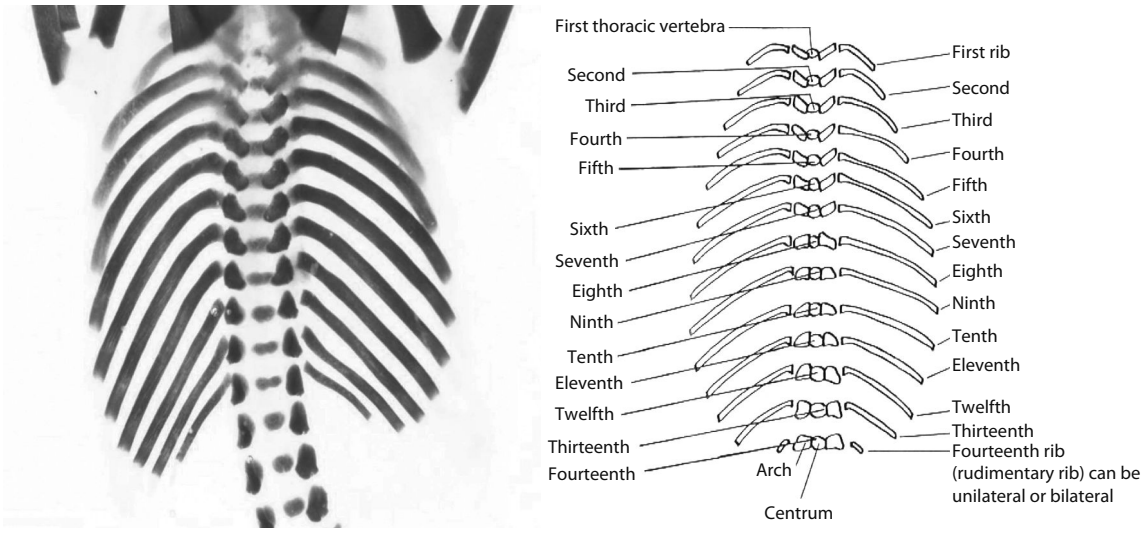

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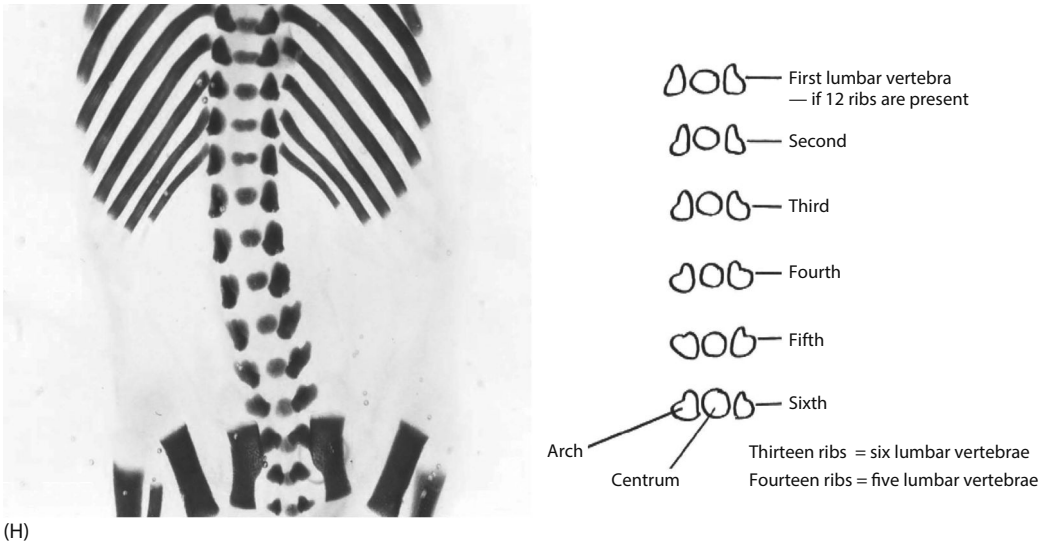

(H)

FIGURE 35.18 (continued) (F) Rat fetus—day 20 of gestation—normal thoracic vertebrae and ribs, ventral view. (G) Rat fetus—day 20 of gestation—normal thoracic vertebrae and ribs, dorsal view. (H) Rat fetus—day 20 of gestation—normal lumbar vertebrae, ventral view.

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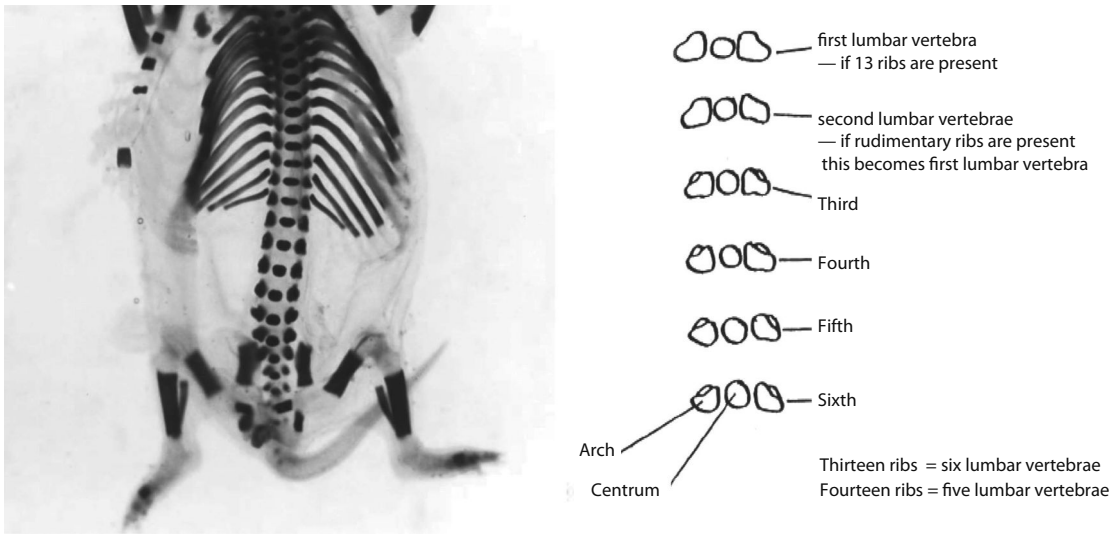

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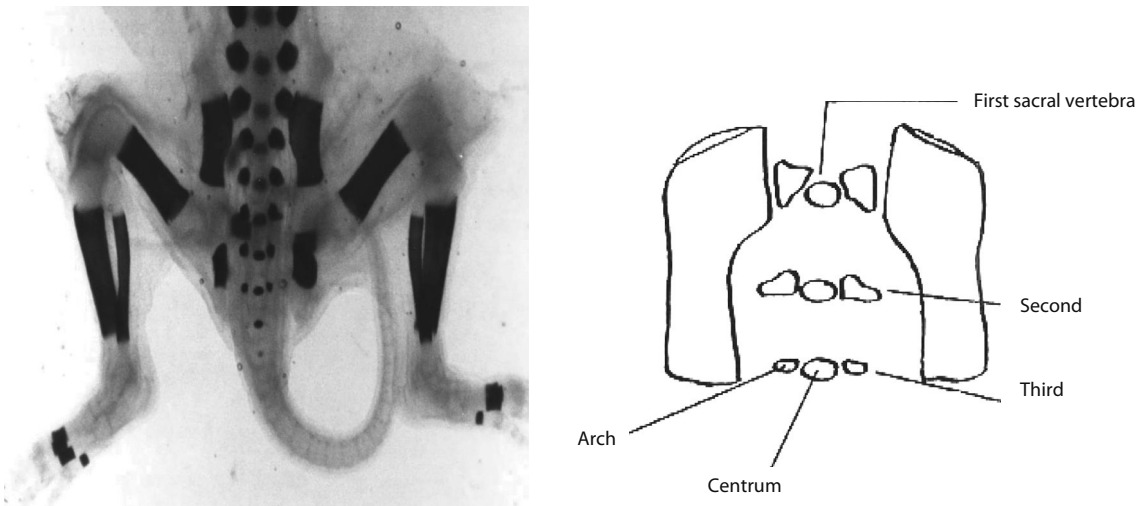

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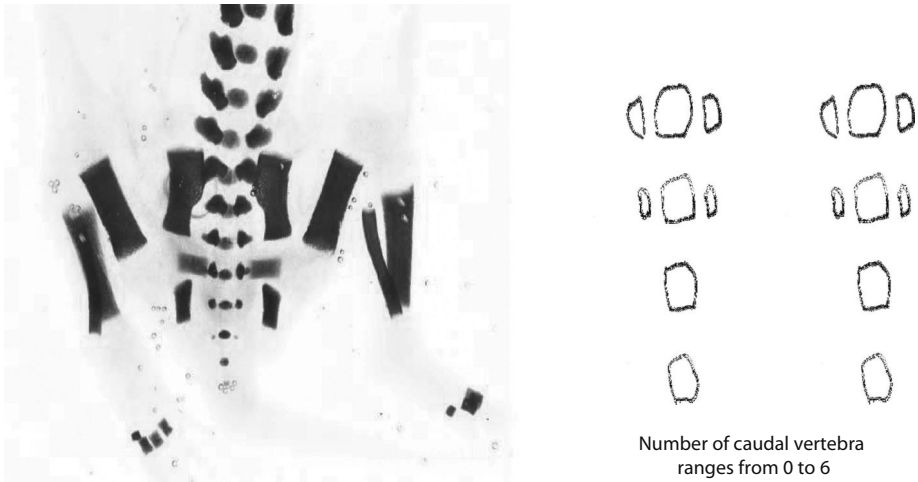
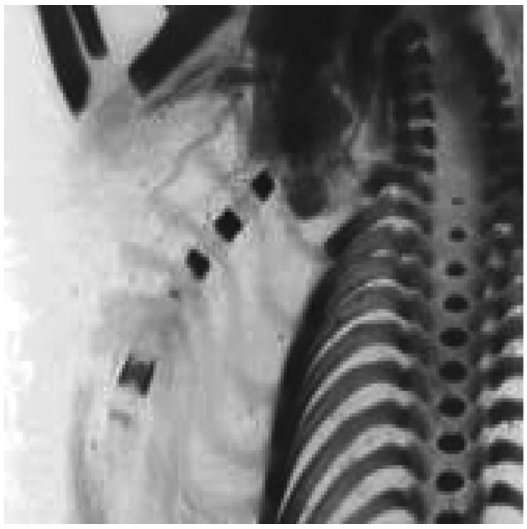


(K)

FIGURE 35.18 (continued) (I) Rat fetus—day 20 of gestation—normal lumbar vertebrae, dorsal view. (J) Rat fetus—day 20 of gestation—normal sacral vertebrae, dorsal view. (K) Rat fetus—day 20 of gestation—normal caudal vertebrae, dorsal view.



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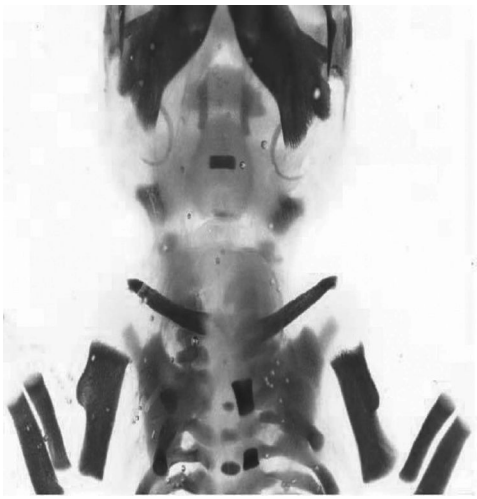

(M)

(N)

FIGURE 35.18 (continued) (L) Rat fetus—day 20 of gestation—normal sternebrae, ventral view. (M) Rat fetus—day 20 of gestation—normal claviculae, ventral view. (N) Rat fetus—day 20 of gestation—normal scapula, dorsal view.

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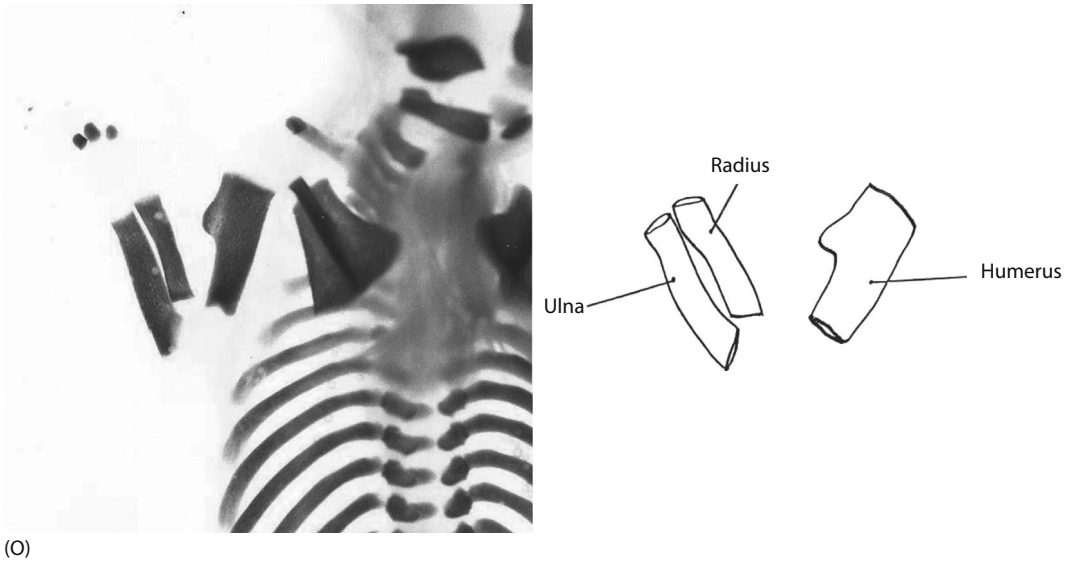

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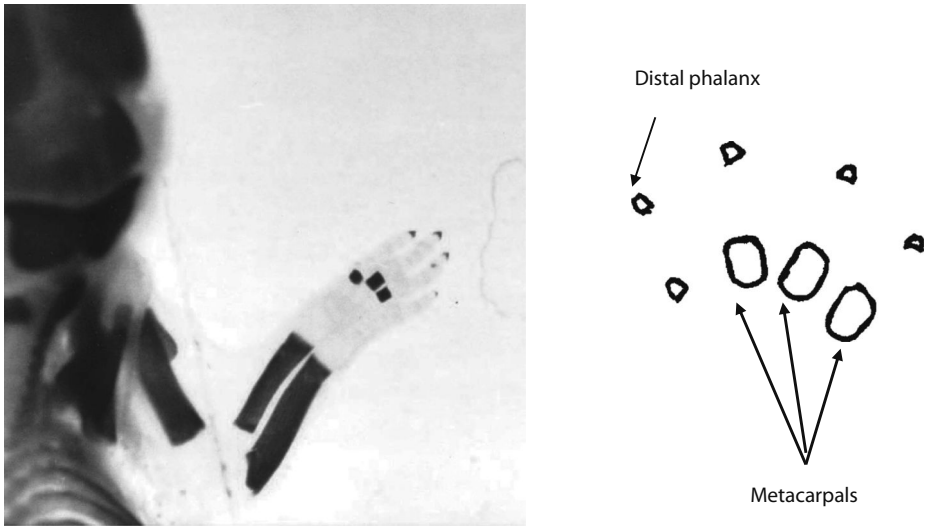

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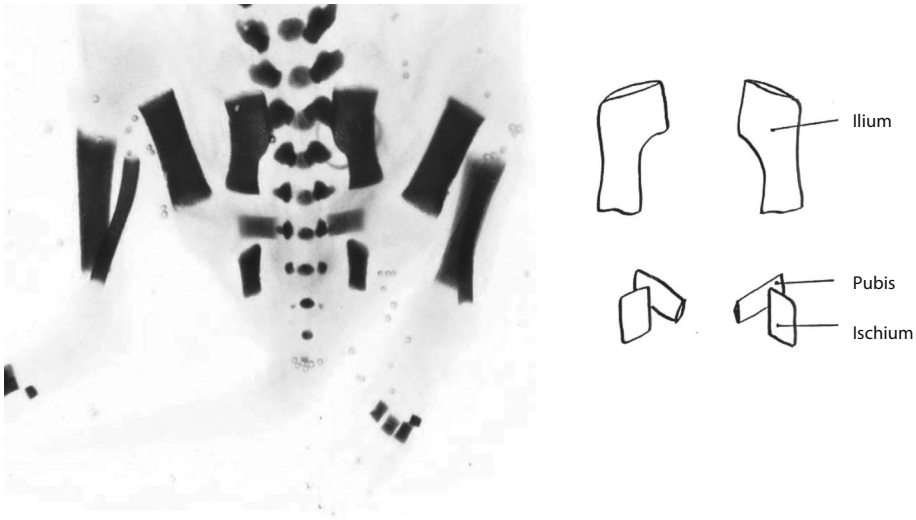

(Q)

FIGURE 35.18 (continued) (O) Rat fetus—day 20 of gestation—forelimb, dorsal view. (P) Rat fetus—day 20 of gestation—normal forepaw, dorsal view. (Q) Rat fetus—day 20 of gestation—normal pelvis, dorsal view.

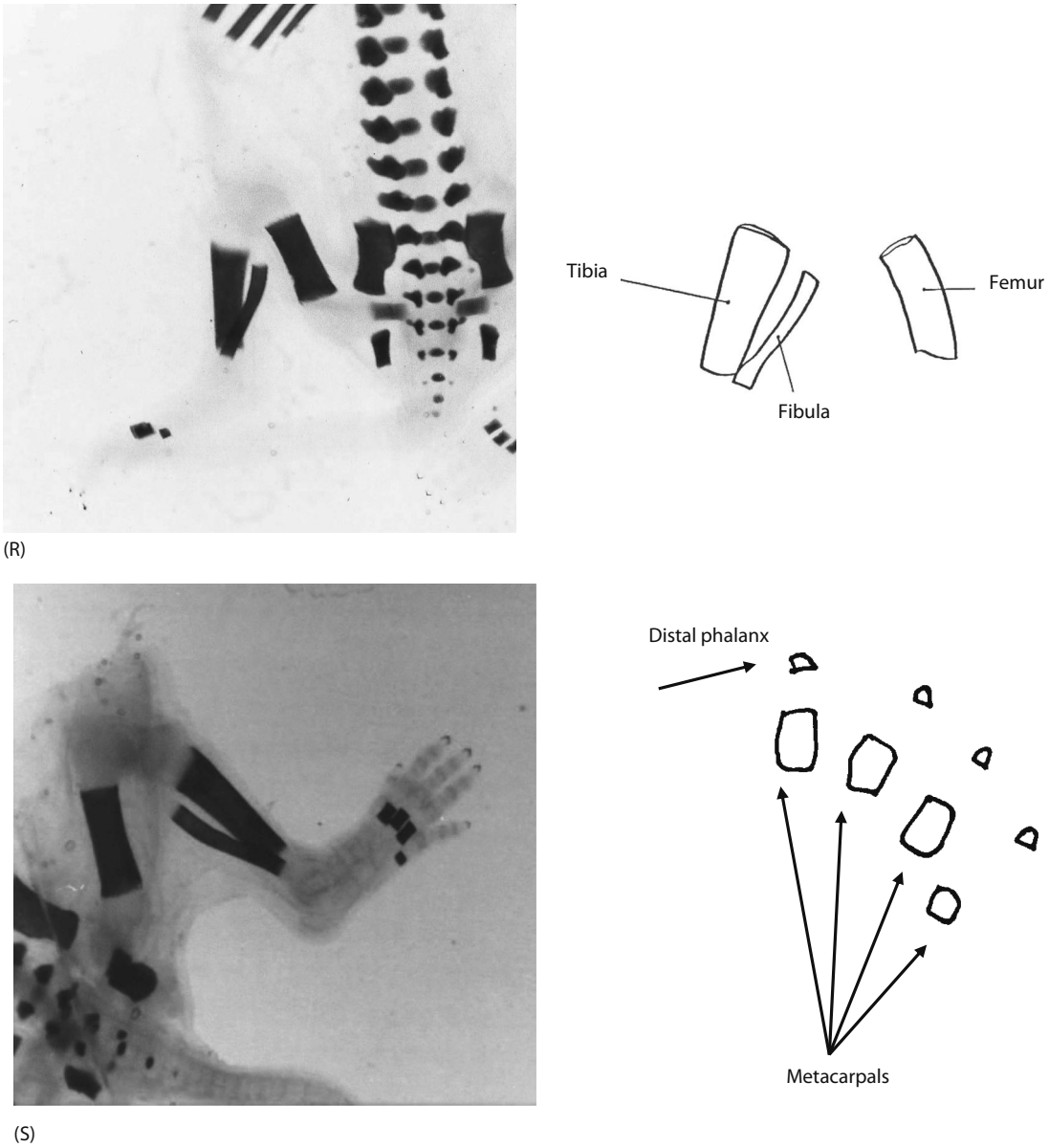

FIGURE 35.18 (continued) (R) Rat fetus—day 20 of gestation—normal hindlimb, dorsal view. (S) Rat fetus—day 20 of gestation—normal hindpaw, dorsal view.

limits of the ilia are counted]) (Figure 35.18J); and caudal vertebrae (the number varies [CA1 through CA*x*]) (Figure 35.18K). Each vertebra consists of three parts (the centrum and two arches). The centrum consists of an ossified center surrounded by cartilage (the caudal centra are entirely cartilage). The atlas (C1) and the axis (C2) are the two most anterior cervical vertebrae. Each thoracic vertebra articulates dorsally with a pair of ribs. Rodents usually have 13 pairs of ribs while rabbits usually have 12 pairs. Rat and rabbits often have a full rib, rudimentary rib, or an ossification site at L1. In rodents, this may be unilateral, but in rabbits, it is usually bilateral. Each rib normally consists of a cartilage tip proximal to the vertebral arch, an ossified middle portion, and a distal cartilaginous portion. The distal cartilages of ribs 1 through 7 are attached to the sternum, while ribs 8 through 11 curve upward with their tips in close proximity. Ribs 12 and 13 are free distally.

The hyoid bone (Figure 35.18C) is a U-shaped bone consisting of three named parts: the body and paired great and lesser horns. The hyoid bone is considered a lingual bone because of the tongue musculature attached to it. It also serves, however, as an attachment for the larynx, specifically the thyroid cartilage via the thyrohyoid membrane and the extrinsic muscles of the larynx. Embryologically, the hyoid bone is derived from mesodermal cells that form cartilaginous masses in the second and third branchial arches.

The sternum (Figure 35.18L) usually consists of six or seven ossification sites (the manubrium, the body or corpus consisting of four or five sternal centers, and the xiphoid). Sternal ossification is nonsequential, with centra 2 and 3

normally ossifying before centra 1. Incomplete ossification of the last (3rd, 4th, or 5th) centrum between the manubrium and xiphoid is considered normal and identified as a delay based on the number of ossified sites present (i.e., 2, 3, or 4).

The appendicular skeleton includes the pelvic and pectoral girdles and the appendages. The pectoral girdle is composed of paired scapulae and clavicles. The scapulae are flat, trapezoidal bones with anterior-dorsal projections; the clavicles are elongated, curving, slender bones that articulate laterally with the scapulae and medially with the sternum. The forelimb skeleton consists (from proximal to distal) of the humerus, the radius and ulna, and carpals, metacarpals, and phalanges of the forefoot. In GD 20 rodents, the only bones of the forefoot that have ossified are the metacarpals. By GD 21 in rodents, the phalanges have ossified. In term mice, usually only the second through fourth metacarpals are ossified, while in term rats, the second through fifth metacarpals are normally ossified. The pelvic girdle consists of three pairs of bones (ilium, ischium, and pubis). The hindlimb skeleton consists (from proximal to distal) of the femur (thigh) patella, the tibia, and fibula with hindfoot (tarsals, metatarsals, and phalanges). The five metatarsals ossify first. The hindfoot of the rabbit has only four digits.

When the sacrifice day is GD 21 (rats) or GD 18 (mice), there is less variability in the skeletal development; however, there is an increased risk of delivery prior to scheduled sacrifice.

Procedures

To perform a skeletal examination, each fetus is removed from the container holding the processed litter, checked for identification, and then placed in a petri dish and examined using a light source and magnification (5× to 10×). Rat, mouse, hamster, and ferret fetuses are examined using a binocular microscope. It is recommended that all nonrodent fetuses (except ferrets) be examined using a magnifying light.

Each skeletal examination proceeds systematically from head to tail. The skull is examined for size, shape, and extent of ossification; each skull bone is examined for ossification appropriate to the specimen's gestational age. The paired bones of the skull must be identified during the skeletal examination. When the fetus has a domed skull associated with marked or extreme dilation of the lateral ventricles in the brain or when the fetal skull is small and has retarded ossification, the anterior and posterior fontanelles are often enlarged and the parietals frequently contain holes. In such cases, the affected fontanelle and degree of dilation should be noted. When holes are present (Figure 35.20A), the number, location, and approximate size of each hole should be noted, as also should be intra- and interossification sites (Figure 35.20B).

The hyoid bone (Figure 35.18C) is observed to be present or not ossified. Rabbit fetuses frequently have angulation of the hyoid alae (Figure 35.20D); the hyoid is not ossified (cartilage is present) in rat fetuses at 20 or 21 days of gestation (Figure 35.18C). Appropriate alignment and closure of the upper and lower jaws (mandibles and maxillae) should be present (teeth should be present in rabbit fetuses). The size and shape of each eye socket should be checked and correlated with reported findings of microphthalmia or apparent anophthalmia.

The vertebral column is next examined (Figure 35.18D through K). The vertebral column articulates anteriorly with the exoccipital bones of the skull. As described earlier, each vertebra consists of three parts (the centrum and two arches) and should be evaluated to determine whether areas, other than those expected to have minor developmental alterations, demonstrate changes. The centrum consists of an ossified center surrounded by cartilage. The caudal centra are entirely cartilage. The examiner should record all unossified, asymmetric or small (Figure 35.19E and J), misaligned, bipartite (Figures 35.19D and 35.20I), dumbbell, or unilateral (Figures 35.19F, 35.20F and G) ossified centra and/or arch. In a normal GD 20 rat, all lumbar and sacral centra should be ossified, while thoracic centra T1 through T7 are normally unossified. The ossified portion of the centra may or may not be bipartite or dumbbell when the cartilage portion is bipartite or dumbbell. The centra must be examined for fusion of the cartilage between centra. A small arch(es), open arches, and fused centra and/or arches of vertebrae (Figures 35.19E, J and 35.20G) should be recorded. If the cartilage anlage is affected, it should be recorded along with any findings for the ossified portion. The vertebral arch is almost completely ossified except for a cartilage tip, which may or may not be present on the transverse process, depending on the relative maturity of the fetus. The spinous process is normally entirely cartilaginous and can be examined only from the dorsal side. The cartilage of the transverse processes of sacral centra (S1 through S3) is normally fused, providing extra support for the pelvis. The atlas (C1) and the axis (C2) are the two most anterior cervical vertebrae. Each thoracic vertebra articulates dorsally with a pair of ribs. Rodents usually have 13 pairs of ribs while rabbits usually have 12 pairs. Rats and rabbits often have a full rib, rudimentary rib, or an ossification site at lumbar 1. In rodents, this may be unilateral, but in rabbits, it is usually bilateral. Additional rib structures are typically classified as extra, full, or supernumerary ribs if they are at least one-half or greater than the length of rib 1 or 13. Ribs that are less than one-half the length of rib 1 or 13 are classified as rudimentary ribs. If the additional rib structures appear as small, round *dots*, they are classified as ossification sites. Extra ribs may also be found on seventh cervical arch (C7) and in rats are sometimes found only as cartilage (Figure 35.19C); this extra cartilage is sometimes found to be fused to rib 1 of the same side. Any alterations in rib ossification are noted, such as a thickened area of ossification (Figure 35.20E), waviness (Figure 35.19I), splitting (branching), fusion, or misalignment (Figures 35.19K, M, and 35.20H). To the extent possible, interrelated vertebral-rib malformations are identified and this malformation counted as one finding (Figure 35.20F and G). Abnormal rib numbers (unilaterally increased or decreased) associated with increased or absent ribs and the presence of a hemivertebra are excluded from the litter average. Altered ribs are noted by number (T1 through T13), affected area of ossification (proximal, beside the vertebral column; medial, middle of

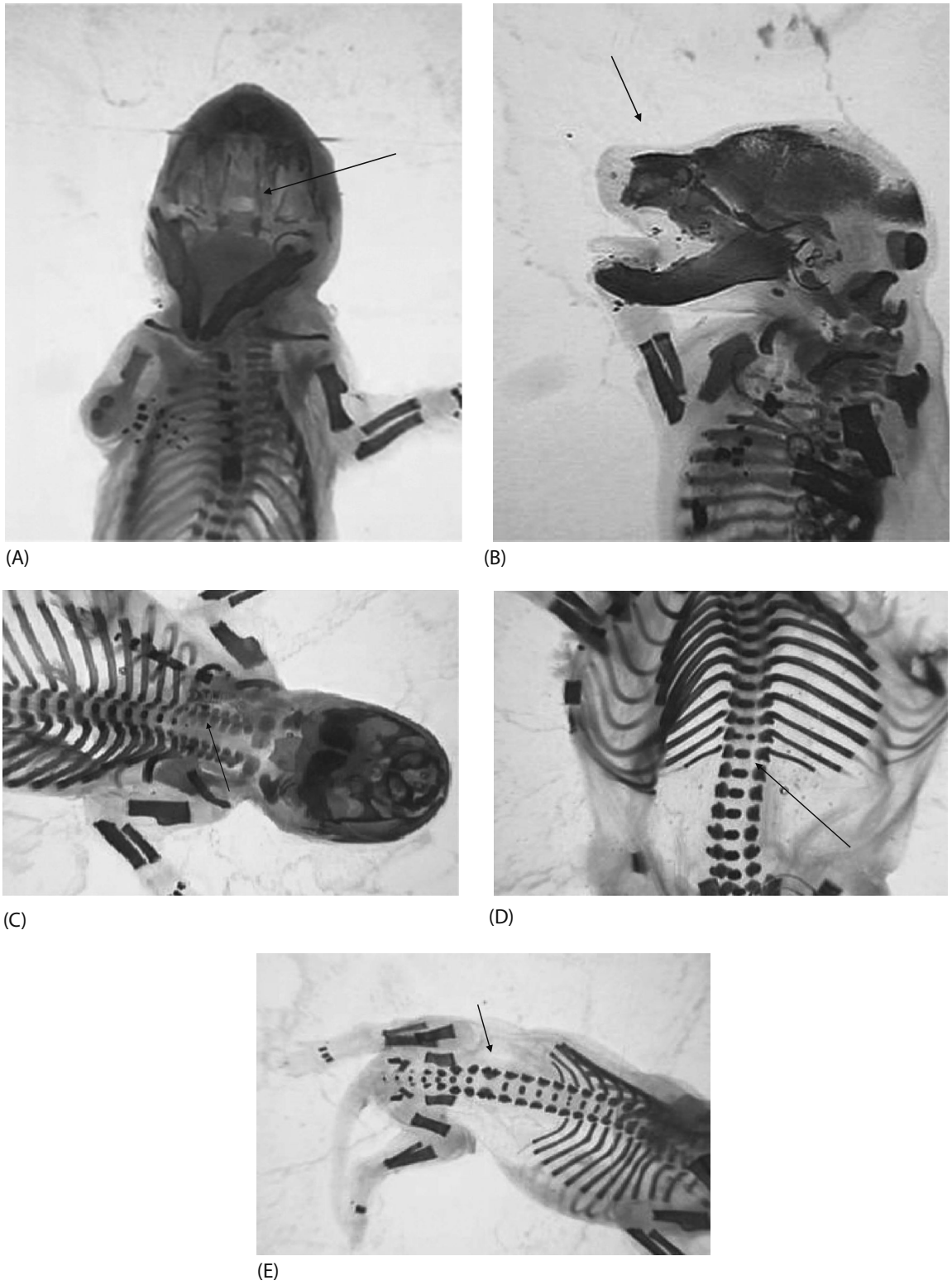

FIGURE 35.19 (A) Rat fetus—day 20 of gestation—incomplete ossification of palatine shelves. (B) Rat fetus—day 20 of gestation—short nasal bones. (C) Rat fetus—day 20 of gestation—cervical ribs present on C7. (D) Rat fetus—day 20 of gestation—bifid centrum, T12. (E) Rat fetus—day 20 of gestation—fused arches and absent centra of L4 and L5.

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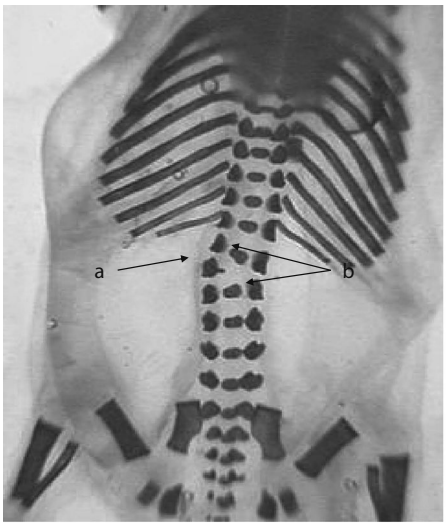
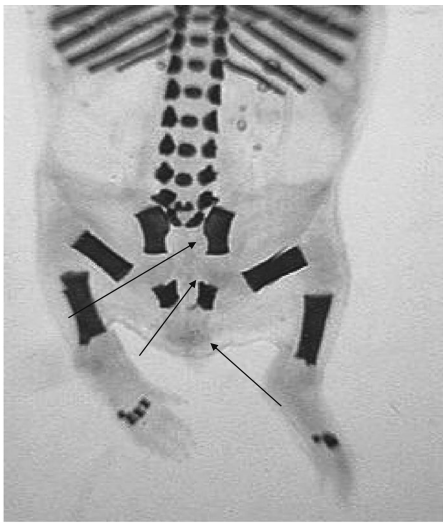
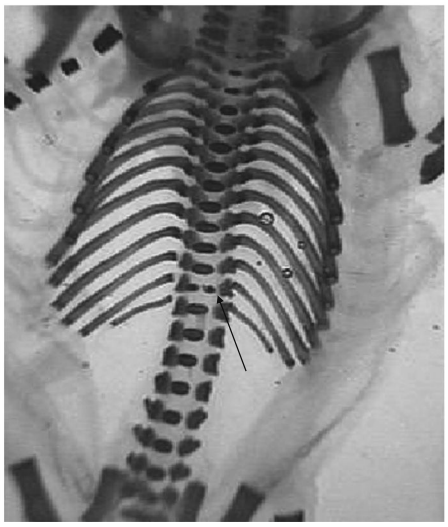


(F)



(G)



(H)

(I)

FIGURE 35.19 (continued) (F) Rat fetus—day 20 of gestation—interrelated malformations of lumbar vertebrae: (a) L2 present as a left hemivertebra with (b) asymmetry of centra in L1 and L3. (G) Rat fetus—day 20 of gestation—skeletal malformations associated with the absence of tail (absence of lumbar, sacral, and caudal vertebrae). (H) Rat fetus—day 20 of gestation—bifid vertebral centrum. (I) Rat fetus—day 20 of gestation—wavy, hypoplastic thoracic ribs.

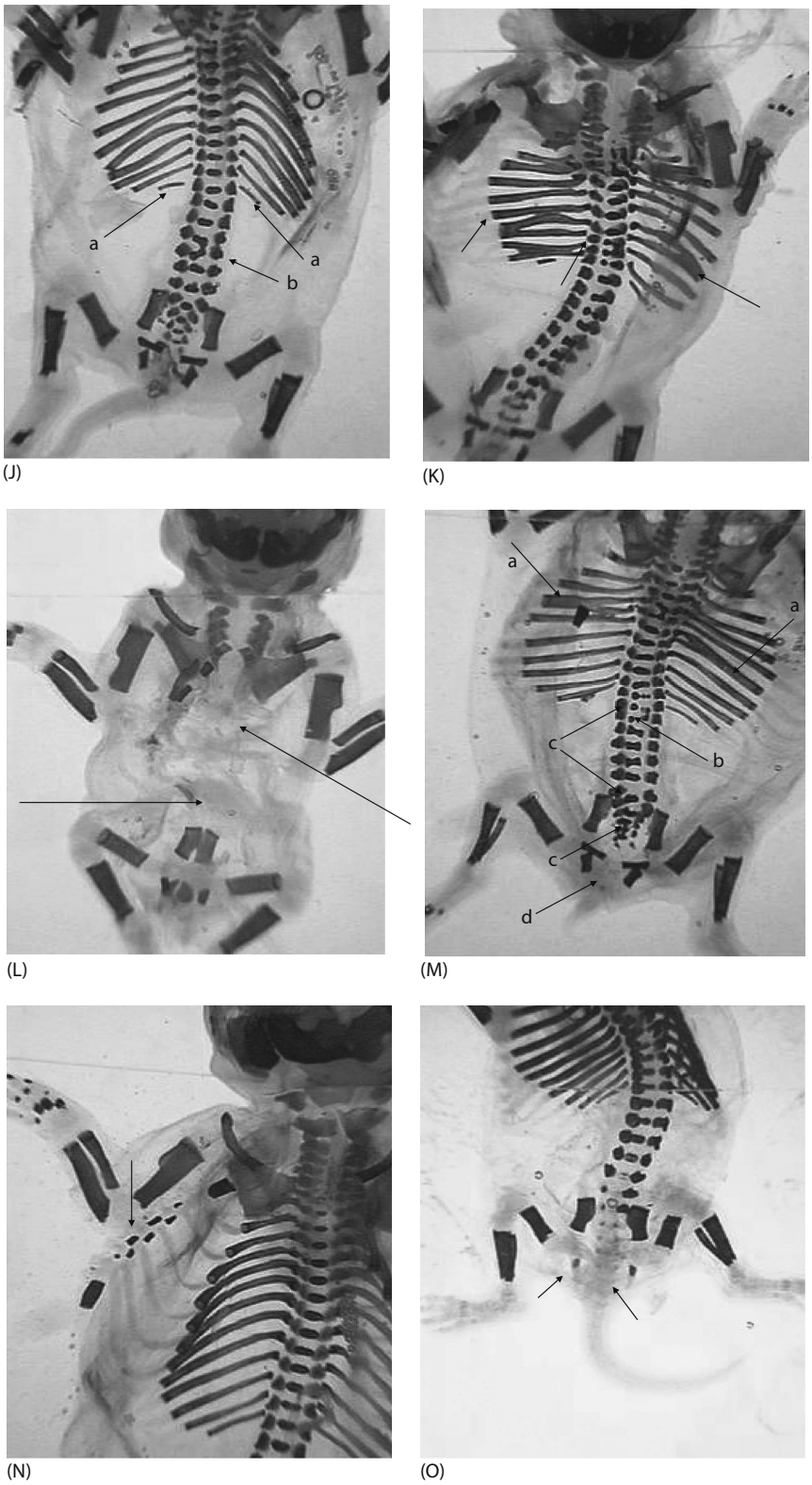


FIGURE 35.19 (continued) (J) Rat fetus—day 20 of gestation—(a) supernumerary thoracic ribs; (b) unilateral fusion of centra of L3 and L4. (K) Rat fetus—day 20 of gestation—multiple interrelated vertebral/rib fusions. (L) Rat fetus—day 20 of gestation—absence of vertebrae and ribs. (M) Rat fetus—day 20 of gestation—skeletal alterations associated with short trunk and short tail: (a) fused ribs; (b) bifid unilateral/incomplete ossification of vertebral centra; (c) fused vertebral arches and centra; and (d) absent caudal vertebrae. (N) Rat fetus—day 20 of gestation—asymmetric, incompletely ossified sternebrae. (O) Rat fetus—day 20 of gestation—incomplete ossification of pubes and absent ossification of ischia.

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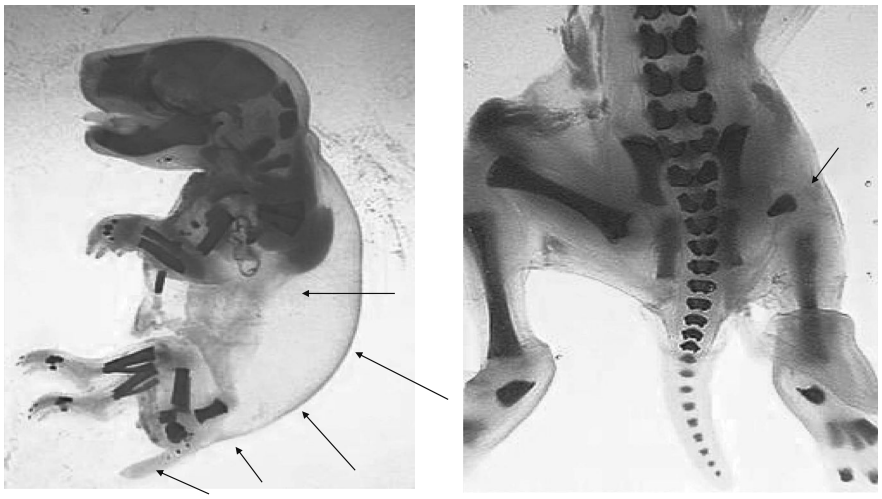
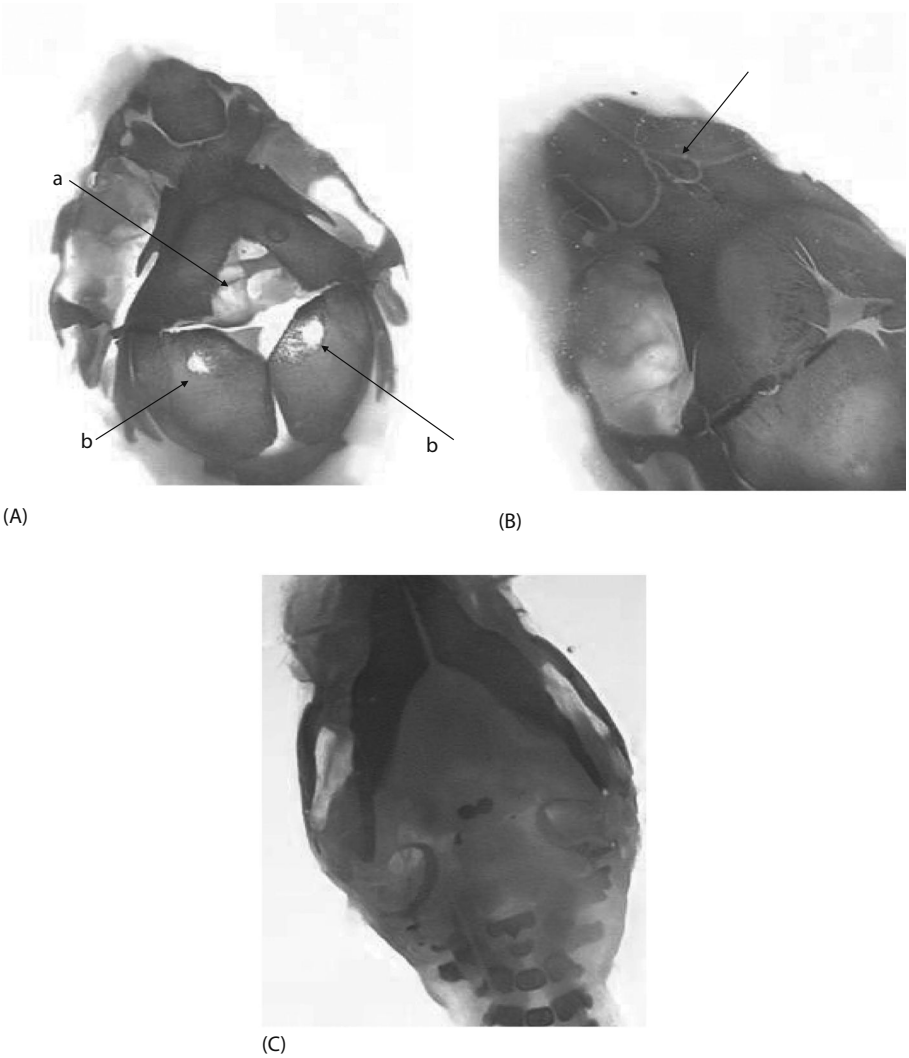


(P) (Q)

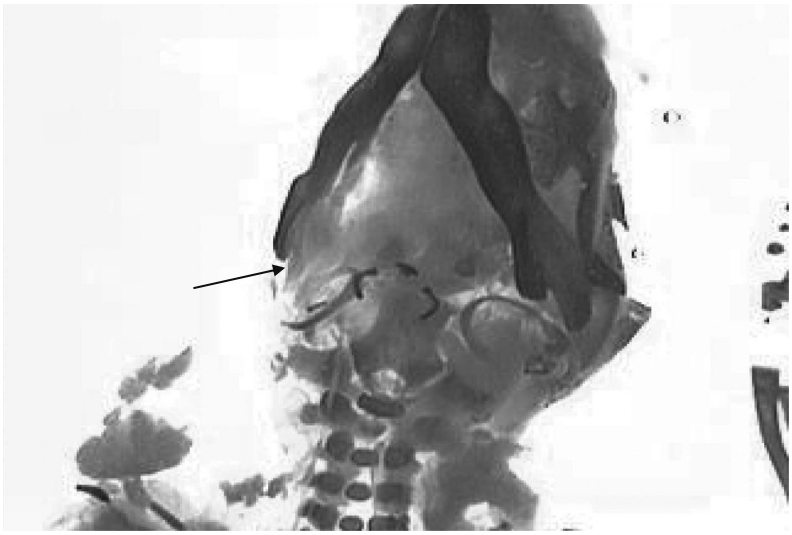
FIGURE 35.19 (continued) (P) Rat fetus—day 20 of gestation—absence of thoracic vertebrae and ribs, and absence of lumbar, sacral, and most caudal vertebrae. (Q) Rat fetus—day 20 of gestation—short femur.



(A) (B)

(C)

FIGURE 35.20 (A) Rabbit fetus—alterations associated with hydrocephaly and domed skull: (a) Large anterior fontanelle; (b) holes in parietals. (B) Rabbit fetus—intranasal ossification site. (C) Rabbit fetus—absent hyoid alae.



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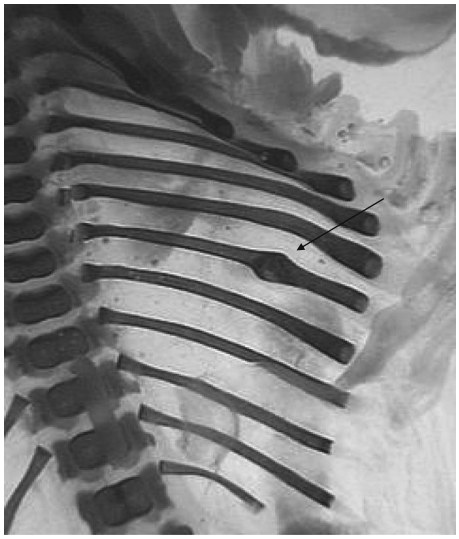

(E)

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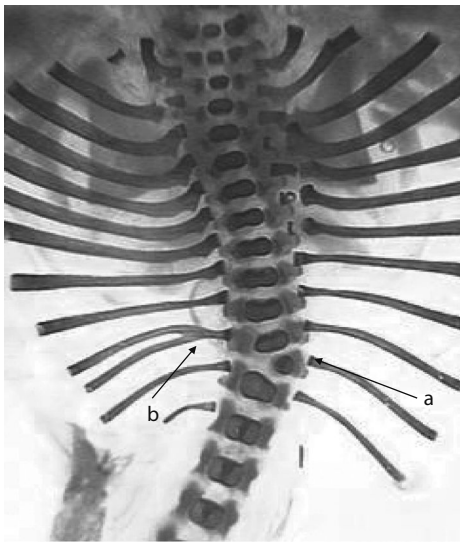

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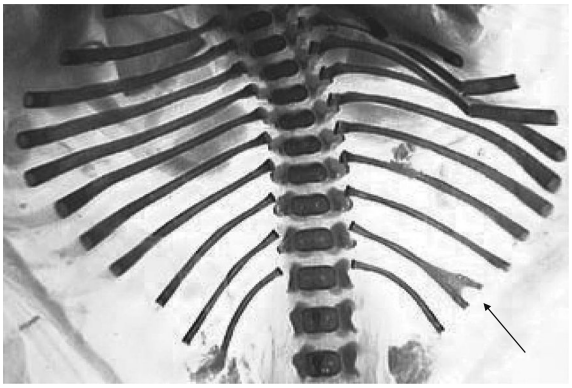

(H)

FIGURE 35.20 (continued) (D) Rabbit fetus—bent hyoid alae. (E) Rabbit fetus—thickened area of ossification in rib. (F) Rabbit fetus—interrelated vertebral/rib malformation: (a) T2 present as right hemivertebra with unilateral ossification of centrum and rib; (b) T3 asymmetric centrum; (c) unilateral extra rib present between right ribs 3 and 4. (G) Rabbit fetus—interrelated vertebral/rib malformations: (a) T12 present as left hemivertebra with unilateral centrum and rib; (b) forked right 10th ribs, compensating for the absence of 11th right rib. (H) Rabbit fetus—forked rib.

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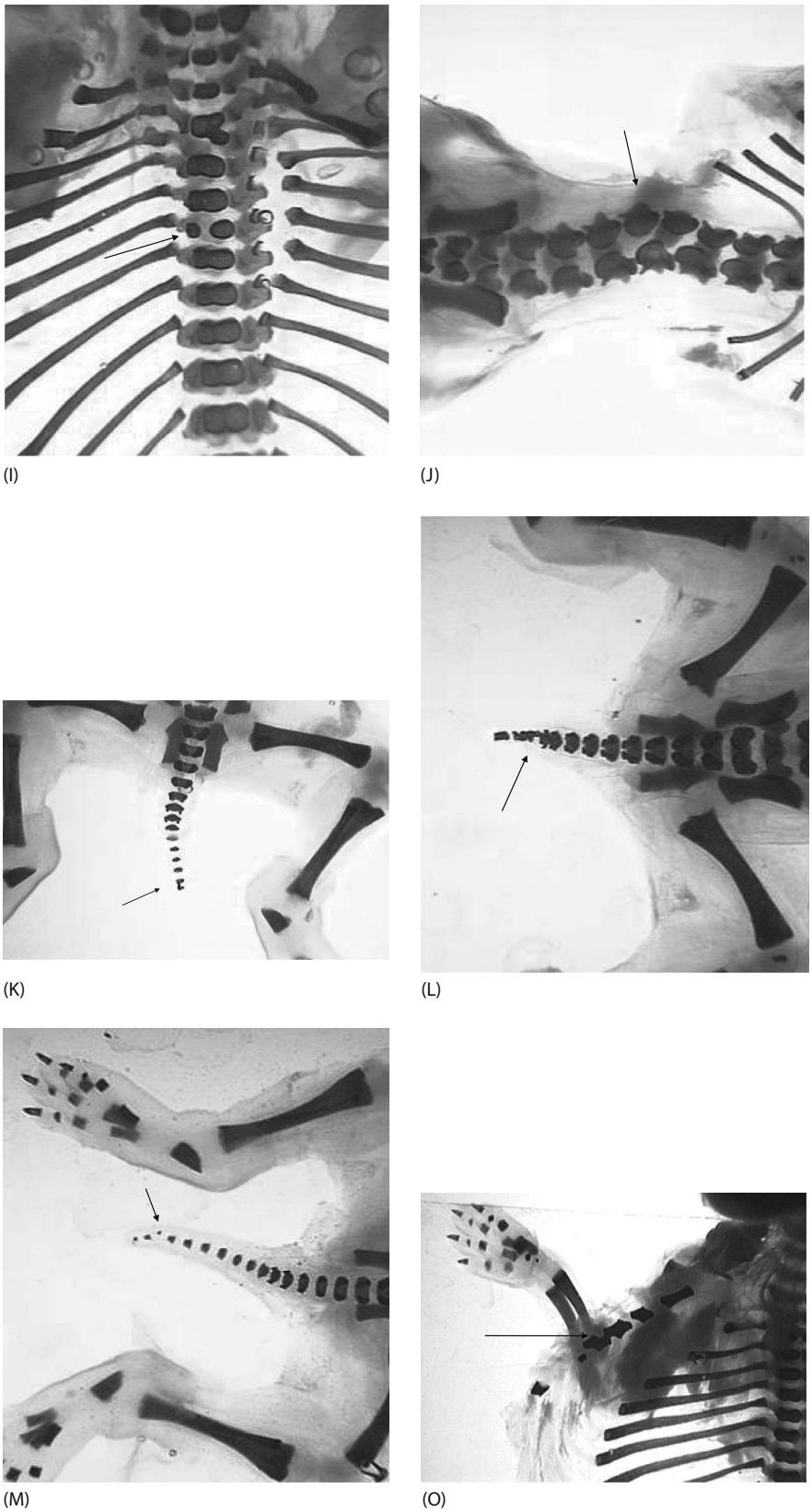
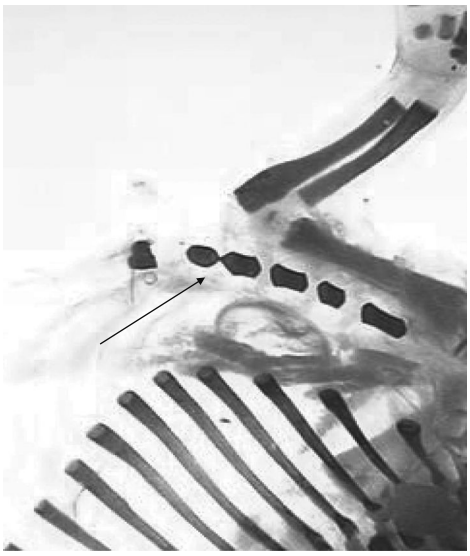


FIGURE 35.20 (continued) (I) Rabbit fetus—bifid centrum in T6. (J) Rabbit fetus—lumbar hemivertebra with associated scoliosis. (K) Rabbit fetus—fused caudal vertebrae. (L) Rabbit fetus—short tail with associated fusion of caudal vertebrae. (M) Rabbit fetus—caudal vertebra misaligned. (N) Rabbit fetus—asymmetric fused sternebrae. (O) Rabbit fetus—fused sternebrae 3 and 4.



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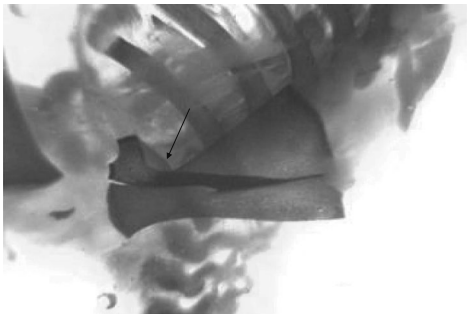

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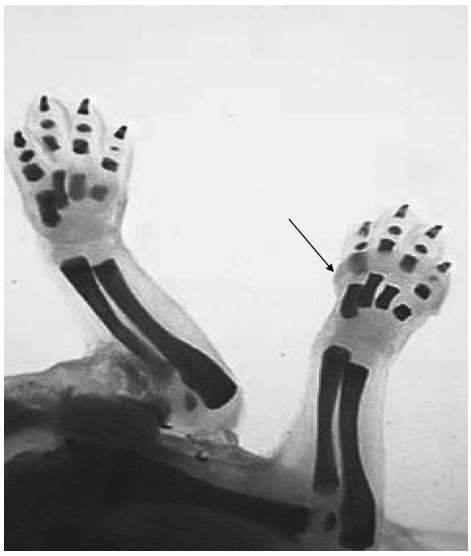

(R)

FIGURE 35.20 (continued) (P) Rabbit fetus—fused, asymmetric sternal centra. (Q) Rabbit fetus—bent scapular ala. (R) Rabbit fetus—absent pollex.

the ossified area; distal, near costochondral junction), and whether the alteration is unilateral (left or right) or bilateral.

The sternum is next to be evaluated (Figures 35.18L, 35.19N, 35.20N, and O). The adult sternum usually consists of six or seven ossification sites (manubrium, four or five sternal centers and xiphoid). For the fetal examination, the degree or lack of ossification of the manubrium and xiphoid is recorded followed by the intermediate sternal ossification sites, which may have degrees of delayed ossification (incompletely ossified or not ossified). Any alterations in sternal ossification are noted.

The pectoral girdle consists of two claviculae and two scapulae (Figure 35.18M and N). Any alterations in ossification, such as an irregular shape, waviness, bending, or small size, are noted. Each forelimb (Figure 35.18O and P) consists of long bones (humerus, radius, and ulna), the carpals, and the bones of the forepaws (metacarpals and phalanges). The carpals, metacarpals, and phalanges in each digit are evaluated for ossification. Each paw is evaluated separately and between-paw differences noted. The carpals are usually not ossified, while there are normally four or five metacarpals present in each forepaw. Because the preaxial metacarpal is often not ossified in fetuses, the presence of only four metacarpals indicates delayed ossification of the preaxial metacarpal, unless the finding is associated with the absence of the pollex (first preaxial digit), a relatively common finding in some rabbit strains. Each forepaw has five digits in most rodent and nonrodent species. The digit count begins at the preaxial digit, after which the phalanges in each digit are counted, including the distal phalanx (claw). Day 20 rat fetuses normally have 1, 1, 1, 1, and 1 ossified phalanges, and

day 29 rabbit fetuses normally have 2, 3, 3, 3, and 3 ossified phalanges in the five respective digits. Counts excluded from the ossification site averages are noted, for example, fused or absent digits and short, twisted, thick, bent, absent, or incompletely ossified long bones.

The pelvic girdle (pelvis) consists of two ilia, two ischia, and two pubes (Figure 35.18Q). Any alterations in ossification are recorded, including incomplete, absent, or abnormal ossification (Figure 35.19O). Each hindlimb (Figure 35.18R and S) consists of long bones (tibia and fibula) and the bones of the hindpaws (tarsals, metatarsals, and phalanges). Using the methods described to count the bones in the forepaws, the numbers of tarsals, metatarsals, phalanges in each digit are counted. Normally four metatarsals are observable. Rat fetuses have five hindpaw digits, and when four metatarsals are present, the finding indicates delayed ossification of the preaxial metacarpal. Rabbit fetuses have only four hindpaw digits and four metacarpal bones. Beginning with the preaxial digit, the hindpaw phalanges are counted across. Normal day 20 rat fetuses have 1, 1, 1, 1, and 1 phalanges, and normal day 29 rabbit fetuses have 3, 3, 3, and 3 phalanges, including the distal phalanx, or claw. Examples of reasons for exclusion of count values from averages include fused or absent digits, short, thick, and bent or absent long bones (Figure 35.19Q).

Some laboratories calculate the ossification site averages for each litter per group for the hyoid (body), vertebrae (cervical, thoracic, lumbar, sacral, and caudal), ribs (pairs), sternum (manubrium, xiphoid, and sternal centra between these sites), forepaws (carpals, metacarpals, phalanges), and hindpaws (tarsals, metatarsals, and phalanges) as a method for identifying delays in ossification. Separate averages for the manubrium, four or five sternebrae, and xiphoid better identify reduced ossification than citing retarded sternal ossification by site, to the exclusion of the interrelationship of the biological pattern of sternal development. Delays in ossification identified by using this method should be correlated with other evidence of retarded ossification that may be observed (e.g., a reduction in the average for the litter average number of ossified sternebrae per fetus should be compared with nonsequential sternal ossification). This method also allows association of increases in rib numbers (supernumerary ribs) with increases in thoracic vertebrae and reductions in lumbar vertebrae, thus eliminating identification of supernumerary thoracic ribs as rudimentary, full, unilateral, or bilateral, which often obscures an increase in supernumerary ribs because the counts are recorded as separate findings, rather than an overall response.

Interpretation of Fetal Findings

The interpretation of fetal findings requires a *weight of evidence* (WOE) approach. If there is a dose-related increase in the incidence of fetal malformations, then the finding(s) is considered adverse. Often, however, the incidence of fetal malformations is increased at the middle dose but not at the high dose. In this case, further examination of the data may reveal that there was increased in utero embryo–fetal mortality, which was greatest at the high dose. It is likely that the most affected conceptuses at the high dose had died and were not evaluated for malformations. In this case, the *lack of a dose–response* pattern is spurious. The *true dose–response* pattern will be indicated by evaluating the affected implants (nonlive plus malformed). When there are reduced fetal body weights, it is common to observe increased incidences of reduced ossification, especially in those skeletal areas that undergo ossification last. Reduced fetal body weights are also commonly associated with visceral growth retardation (e.g., dilatation of the renal pelvis (a delay in growth of the renal papilla), dilated (enlarged) lateral ventricles of the cerebrum without compression of the cerebral walls). Conversely, an increase in the incidence of an ossification site, partial or full rib on L1 (e.g., a 14th rib in rodents or a 13th rib in rabbits) may indicate an alteration in developmental patterning, genetic drift over time, and/or may predict possible greater effects at higher doses. When using the WOE approach, the biological plausibility must also be considered.

ICH Stage E—Birth to Weaning

E: adult female reproduction function, adaptation of the neonate to extrauterine life, including preweaning development and growth (postnatal age optimally based on postcoital age).

Observation and Timing of Parturition

Parturition, lactation, maternal–pup interaction, and pup growth and development until weaning are monitored during ICH stage E. Female rats should be housed to litter in boxes with bedding material no later than day 20 of presumed gestation (2 days prior to the day of expected delivery; earlier if required by protocol). The dams are observed periodically throughout the day (every few hours) for signs of parturition (e.g., stretching, visible uterine contractions, vaginal bleeding, and/or placentas in the nesting box). These signs are strong indicators of parturition onset and indicate that dams should be monitored closely for delivery of the first pup.

As soon as a pup is found, parturition is considered initiated. If required by protocol, the time should be recorded. To indicate that a delivery is *in progress*, a marker (e.g., a tape *flag*) may be placed on the outside of the nesting box. Once delivery has begun, the dams are checked periodically during the day for evidence of difficulty in labor or delivery (e.g., a pup only partially delivered, a dam cold to the touch and pale). Any apparent difficulty is recorded, and a supervisor should be notified if the difficulty seems extreme or unusual.

The calendar date on which delivery of a litter appears to be completed is defined as lactation day 0 (LD 0) for the dam and postnatal day 0 (PND 0) for the pup. The following maternal behaviors offer evidence that parturition is complete: (1) removal of amniotic sacs, placentas, and umbilical cords, and grooming of the pups by the dam; (2) self-grooming by the dam; (3) nesting behavior by the dam; and (4) nursing. However, parturition may be complete without all of these behaviors being observed. Individual pup observations (e.g., appearance and viability) should not be recorded until delivery is complete. Apparently, dead pups should be carefully removed from the nesting box during parturition to

preclude cannibalization by the dam. Care should be taken not to disturb the dam. The dead pups should be retained in a container, such as a weigh boat (labeled with the dam number), until pup statuses are recorded following the completion of parturition. Pups observed as delivered but not present at the end of parturition because of cannibalization should be recorded as *Undetermined Sex, Undetermined Viability.*

In order to preclude disruption of delivery and/or maternal care, dams in the process of delivering pups should not be administered the test substance. If a dam has completed parturition by the time the last additional animal in the normal sequence has been dosed, this dam can be administered the test substance. If the dam continues to actively deliver after daily test substance administration is completed, the dam's daily administration can be skipped for that day. A delayed or missed daily test substance administration and the reason for its occurrence should be recorded on the appropriate form.

The day on which delivery is completed is functionally defined as day 0 or 1 postpartum, based upon the laboratory's historical use. Because most deliveries occur overnight and the litter has been first observed at the morning viability check, the LD 0 was considered to have occurred the previous day, and the morning of discovery of the litter is designated as LD 1. If parturition begins overnight but is completed in the morning, that day is designated LD 0. To keep the data consistent, maternal and litter body weights are routinely recorded on LD 1 and PND 1, respectively. Appropriate maternal behavior is determined on the basis of examinations for maternal and pup nesting behavior. Criteria evaluated include pup appearance (clean and warm), presence of a nest in which the pups are grouped together, and evidence of nursing activity and/or milk in the pup stomach. When a dam with a litter dies before her scheduled sacrifice, the pups are sacrificed and necropsied. The sacrifice and necropsy observations are recorded. When all the pups in a litter die, the dam may remain on study until her scheduled sacrifice date, or the dam may be sacrificed at the discretion of the study director.

Occasionally, additional pups are born to a litter that has already been weighed and observed on PND 1. These pups are handled as follows:

- If delivery of a litter has been marked as completed, and it is within the same day, the additional pup(s) and dam are weighed on *PND 1 and LD 1* (e.g., a Monday).
- If an additional pup(s) is found when this litter was counted during the day after the litter was marked as completed (on PND 1 (e.g., Tuesday), the following comment (or a comment that conveys the same information) should be entered on the litter observation form for PND 1. "One additional (sex) pup present that was delivered after completion of weighing and recording of litter observations, for all pups delivered on PND 1." The additional pup(s) is not weighed until PND 4 (the next scheduled weighing interval for that litter). The pup(s) is tattooed if required by protocol.

Edits to the database will be required to change the number of pups in the litter. The date of the dam's DL 0 remains the same (it is not changed to the date that the additional pup was found). Pups are weighed on the days specified in the protocol. On those days when pup body weights are recorded, bedding is usually changed, and each litter is evaluated for maternal and pup nesting behavior. Each litter should be checked for viability and counted every day, with the number and status of the pups recorded.

Evaluation of Pups at Birth

The pups are further evaluated after removal from the nesting box. Gender is identified on the basis of observed anogenital distance (longer in male than in female pups). For studies in which endocrine perturbations are expected, these observations are made using calipers. Any gross physical alterations are identified and the viability and weight of each pup is identified. Dead pups are necropsied and a section of the lung placed in a container of water. Pups with lungs that float are assumed to have breathed and are considered liveborn. Pups with lungs that sink are identified as stillborn. Each pup is examined for the general shape of the head and features, bruises, lesions, number of digits, length and shape of the limbs and tail, presence of an anus, presence of milk in the stomach, and any injury inflicted by the dam.

Culling

Whether to cull is an area of debate.[324,325] Its appropriateness depends upon the purpose of the study and the degree of control required for evaluation of the endpoint in question. Although culling is a common practice, and required in some guidelines, this practice does increase the variability in litter values for viability and weight gain and has the potential to obscure late occurring effects because pups are removed from evaluation.

Cross-Fostering

Parturition observations begin three times daily on day 20 of gestation. If the beginning of parturition is observed, that is, the birth of the first pup, this is deemed postnatal/lactation day 0. If an animal delivers overnight and delivery is observed as complete at the morning parturition observation, then parturition is considered to have begun prior to midnight and the day of observed completion is deemed as postnatal/lactation day 1. When animals have completed delivering their litters, this information is recorded on the parturition observations sheet. After the morning parturition observation is completed and a list of litters to be cross-fostered on that day is finalized, the pups in each *birth* litter are separated by sex and counted. This information is entered on the deliveries sheet together with details of any pups that were excluded from the cross-fostering (e.g., runts, deformed pups), and the pups are returned to their mothers.

When the deliveries sheet has been completed, the cross-fostering plan is developed. The details of the birth litters

with the pups available for cross-fostering are entered into a spreadsheet entitled *Birth Litters*. This information is then verified. The *Birth Litters* information is then transferred to a spreadsheet entitled *Cross-Fostered Litters* where the pups are arranged into foster litters with no pup remaining with its birth mother and no same sex siblings in each litter. Ideally, no siblings at all will be in each litter. The *Cross-Fostered Litters* sheet is then verified and approved by the study director prior to use.

The physical cross-fostering is then performed. The litters are removed from their home cage and placed in containers by sex labeled with the birth dam's number and the sex of the pups. Using a partitioned box labeled with the foster dam's number and with each partition labeled with the pup number within the foster litter and using the Cross-Fostered Litters sheet, the required pups are removed from the birth litter containers and placed in the appropriate space in the foster litter box. Once the entire foster litter is filled, the pups are tattooed, weighed, sexed, and placed in their foster mother's cage. The placing of the foster litter in the foster dam's cage is achieved by removing the foster dam, placing the foster pups in the cage, rolling the pups in the bedding to acquire some of the scent of the foster dam/home cage, and then returning the foster dam to the cage. This process requires the ordering of approximately 20% more presumed pregnant females than litters required due to some of the dams being nonpregnant, or having litters with insufficient numbers and/or abnormal sex ratios. Cross-fostering of more than 30 litters per day is labor-intensive and difficult.

ICH Stages E (Postnatal Development to Weaning) and F (Postweaning Development of Reproductive Organs to Puberty)

E: adult female reproduction function, adaptation of the neonate to extrauterine life, including preweaning development and growth (postnatal age optimally based on postcoital age).

F: pediatric evaluation when treated; postweaning development and growth, adaptation to independent life, and attainment of full sexual development.

Anogenital Distance

With the event of enhanced concern regarding estrogenic agents, many investigators have incorporated determination of anogenital distance at Caesarean delivery or birth of fetuses and pups, respectively.[326] Anogenital distance is the length between the anus and the genital tubercle. In rodents and primates, the anogenital distance is greater for males than for females. For rodents, this differential begins on GD 17 and continues through PND 21. Mean anogenital distances at birth for males and females are approximately 3.5 and 1.4 mm, respectively. The increased growth of this region occurs in response to testosterone. The genders of rabbit fetuses or neonates cannot be reliably determined using anogenital distance but can be determined by examining the gonads during the visceral evaluation.

Anogenital distances for rat fetuses (GD 20 or 21) or pups on PNDs 0 to 3 are measured with a micrometer and a stereomicroscope. Measurements taken on pups on PND 4 or later should be done using a caliper. The fetus or the PNDs 0 to 3 pup is held in the technician's hand, and the tail is raised with the other hand to an 80°–90° angle from the horizontal, exposing the anus. (*Note*: Be careful not to pull the tail, as this stretches the anogenital distance.) The anogenital area is brought into focus with a calibrated stereomicroscope. The anogenital distance is measured from the cranial (or anterior) edge of the anus, which comes to a point, to the base (or posterior edge) of the genital tubercle. The base of the tubercle is not clearly differentiated as it slopes into the anogenital area. A base line between the distinct edges of the genital tubercle is visually estimated. It is very important that the anus and the base of the genital tubercle be kept in the same focal plane. The length from the base of the genital tubercle to the cranial edge of the anus is recorded.

Hold the PND 4 or older pup by its tail, keeping the tail at an 80°–90° angle from the horizontal. The arms of the caliper should be aligned as follows: for males, the anogenital distance is measured from the cranial (or anterior) edge of the anus to the base (or posterior edge) of the anogenital aperture; for females, the anogenital distance is measured from the cranial edge of the anus to the base of the urinary aperture (*not* the base of the vulva). The anogenital distance is recorded in millimeters.

The comparison of the anogenital distance between groups (especially for males) is confounded when the groups have different fetal or pup weights. The anogenital distance data can be normalized (when group body weight differences are not too severe) by using some other linear measurement (e.g., crown-rump) or by using the cube root of the body weight to simulate a linear measurement.

Balanopreputial (Male Rodents) and Vaginal (Female Rodents) Opening (Sexual Maturation)

Sexual maturation landmarks (preputial separation and vaginal patency) are required or recommended in perinatal/postnatal studies, multigenerational studies, and developmental neurotoxicity studies, where the offspring are raised to adulthood. The most appropriate way to perform these evaluations is to observe all animals daily until all animals (by sex) in the litter meet the appropriate criterion. Therefore, when differences occur on the basis of individual animal evaluations, litter analyses should be made. Evaluations are performed while the male or female rodent is held in a supine position.

Males: Evaluation of male rodents for balanopreputial separation is begun on day 22 (mouse), day 27 (hamster), or day 35–40 (usually 39) (rat) postpartum (Figure 35.21). In rats, balanopreputial separation is considered to result from balanopreputial membrane cornification, which leads to the detachment of the prepuce from the glans penis in the rat. Preputial separation occurs dorsolaterally and then ventrally on the penis and down the shaft of the penis. The prepuce remains attached to the glans penis on its ventral surface by the frenulum. (*Note*: The process of development of the prepuce in humans is different from that of the rat.) Published

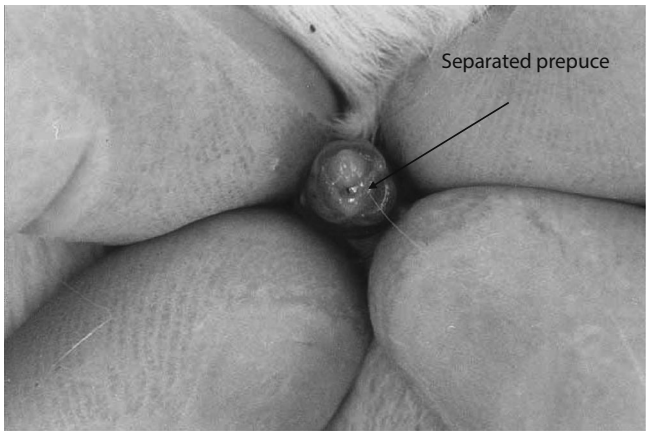

FIGURE 35.21 Rat pup—preputial separation.

preputial separation age range from PND 41 to 46,[327,328] depending on the observation criterion. Each male rodent is removed from its cage and held in a supine position. Because manipulation of the prepuce can accelerate the process of preputial separation, the males must be examined gently. Gentle digital pressure is applied to the sides of the prepuce, and the criterion is met when the prepuce completely retracts from the head of the penis (see previous discussions for alternate criterion). The foreskin can be attached along the shaft of the penis, but it cannot be attached to the opening of the urethra. Each male rodent is examined daily until acquisition or until PND 55, whichever is earlier. Body weight should be recorded on the day the criterion is met.

Females: Evaluation of female rodents for vaginal patency is begun on day 21 (mouse) or day 28 (rat) postpartum. The vaginal opening remains covered by a septum or membrane after canalization of the vagina has occurred in rats. The age when the septum is broken or no longer evident is described as the age of vaginal patency, and vaginal patency is the most readily determined marker for puberty in rats. Published Sprague-Dawley rat vaginal patency values range from PND 30.8 to 38.4.[320,329] Female rats are examined beginning a few days prior to the expected age of maturation (e.g., PNDs 25 to 28), continuing until the criterion for patency has been achieved or until PND 43, whichever comes first. The female is removed from the cage and held in a supine position, exposing the genital area. Pressure is gently applied to the side of the vaginal opening to see if the septum or membrane remains. When the membrane is present, the area has a slight *puckered* appearance; however, when the membrane has broken, the vaginal opening is about the size of a pinhead. The criterion has been met when the vagina is completely open. Body weight should be recorded on the day the criterion is met.

Additional Developmental Landmarks

Additional developmental landmarks can be assessed, including pinna detachment, hair growth (pilation), incisor eruption, eye opening (pups are born blind with eyelids closed), nipple development, and testis descent. The examinations must begin prior to the landmarks' historical day of onset and continue daily until each animal in the litter meets the criterion.[330] Data for each day's testing should be expressed as the number of pups that have achieved the criterion for each developmental landmark, divided by the total number of pups tested in the litter. Forelimb grip test and pupillary constriction tests are conducted on PND 21 only. Data for the forelimb grip test and pupillary constriction tests should be expressed as the number of pups that have achieved the criterion, divided by the total number of pups tested in the litter. The PNDs listed in Table 35.7 for each developmental landmark were compiled from several sources and are subject to variability between laboratories and subtle differences in assessment of the criteria.[330–332]

TABLE 35.7
Developmental Landmarks, Methodology, Initiation, and Acquisition Range

Developmental Landmark	Methodology	Evaluation Initiation	Acquisition Range	References
Day of first estrus	Daily vaginal smears to confirm estrus	Vaginal opening (PND 30)	PND 40 to 45	333
Pinna unfolding	Point of a pinna (ear flap) examined to determine detachment from a head	PND 1	PND 2 to 3	327,332
Hair growth	Pups examined until bristles appear on the dorsal surface of all pups in the litter	PND 1	PNDs 1 to 5	332
Incisor eruption	Eruption through the gum of either an upper or lower incisor	PND 7	PND 8 to 16, mean PND 11	327,332
Eye opening	Pups examined for break in the membrane connecting the upper and lower eyelids	PND 10	PNDs 11 to 18, mean PND 13 or 14	327,332
Nipple retention	Males in each litter examined for the presence of areolae and/or nipples by brushing the hair coat against the nap	PNDs 11	PNDs 12 to 13	327,332
Testes descent	Male examined for the presence of one or both testes in the scrotum	PND 19	PNDs 20 to 29, mean between PND 18 and 25	327,329,332,333

Behavioral or Reflex Ontogeny

Behavioral or reflex ontogeny is a component of developmental neurotoxicity testing that is often included in the preweaning period (e.g., air righting, surface righting, inclined plane (negative geotaxis), limb placing, cliff avoidance, and swimming ontogeny).

Surface-Righting Reflex

The surface-righting reflex is evaluated beginning on PND 1 for rat pups. Surface righting is regaining the normal position after the pup is placed on its back. This is a complex coordinated action requiring many different muscles in the neck, trunk, and limbs. Pups are placed on their backs on a flat surface and quickly released. The criterion is met when the pup regains its normal position on four paws on the floor within 5 s. This usually occurs between PNDs 1 and 9[330,331] in Sprague-Dawley rats, with the average acquisition around PND 6 or 7.[332]

Cliff Avoidance

Cliff avoidance is evaluated beginning on PND 1 for all pups. Cliff avoidance is the behavior of crawling away from an edge of a flat surface edge (cliff). Pups are placed on a table or platform, with their front paws over the edge. The criterion is met when the pup attempts to crawl away from the edge within a 10 s period. This usually occurs between PNDs 2[330] and 12[331] in Sprague-Dawley rats, with the average acquisition around PND 8 or 9.[332]

Forelimb Placing

Beginning on PND 7, rodent pups are tested for forelimb placing or lifting in response to tactile stimulus on the dorsal surface of the foot. The pup is suspended by holding the scruff of the neck so that one forelimb is in contact with a stainless-steel plate (e.g., cage tag holder) held horizontal to the working surface by a clamp and ring stand. The dorsum of the suspended foot is gently touched with a thin (approximately 2–3 mm in diameter) metal rod. The pup must immediately raise and place the suspended foot on the rod to meet the criterion. This usually occurs around PNDs 9 to 10.[323]

Negative Geotaxis Test

Beginning on PND 7, rodent pups are tested for the ability to change from a downward to an upward orientation on an inclined plane of approximately 30°. Each pup is placed facing *downhill* on a platform tilted at a 30° angle. The pup must turn 180° to face *uphill* within a 60 s interval to meet the criterion (a pup with its body positioned sideways and its head facing *uphill* does not meet the criterion). This usually occurs between PNDs 7 and 14[331] in Sprague-Dawley rats, with the average acquisition around PND 7 or 8.[332]

Pinna Reflex

The pinna reflex tests the somatomotor component of the seventh cranial nerve in rats. The presence of the pinna reflex is evaluated daily, beginning on PND 13. The inner surface (near the concha) of the pinna is lightly touched with a filament or the tip of an artist's brush. The criterion is met with the presence of any movement of the pinna (sudden twitch or flattening of the ear) made in response to the applied stimulus. If the first ear tested does not respond to the stimulus, the opposite ear is tested. This reflex usually occurs around PND 14[331] in Sprague-Dawley rats.

Auditory Startle Reflex

Beginning on PND 10, all pups are examined daily for the auditory startle reflex. The auditory startle reflex is noted as a sudden flinch or cessation of ongoing movement following the auditory stimulus. Each nesting box is removed from the study room and placed in a quiet room. Littermates remain outside the testing room in order to mitigate habituation to the auditory stimulus. The pup is placed into a container, such as a beaker, with approximately 600 mL of bedding material and taken into the testing room. A clicker is held directly above the beaker, but not touching it, and the clicker stimulus is delivered. Any observable whole body response (e.g., flinching, jumping, and freezing of activity) meets the criterion for the startle response. This usually occurs between PNDs 12 and 13[330,331] in Sprague-Dawley rats.

Hindlimb Placing

Beginning on PND 14, rodent pups are tested for hindlimb placing in response to a tactile stimulus on the dorsal surface of the foot. Each pup is held so that one hindlimb is in contact with the metal plate. The dorsum of the suspended foot is gently touched with a thin rod. The pup must immediately raise and place the suspended foot on the rod to meet the criterion. This usually occurs around PND 16.[331]

Air-Righting Reflex

The air-righting reflex is the ability of pups to land on all four paws when dropped from an inverted position. All pups in each litter are tested once each day beginning on PND 14 until all pups in the litter demonstrate the reflex. The pup is held in a supine position above a well-padded surface and then released. Rat and hamster pups are held approximately 38 cm above the surface; mouse pups are held 17–20 cm above the surface. The pups must land on all four limbs to meet the criterion. This usually occurs between PNDs 8[330,331] and 18 in Sprague-Dawley rats, with the average acquisition around PND 16 or 17.[332] The use of videotape to record the response will allow more information to be obtained, such as response speed, character, and progression.[334,335]

Forelimb Grip Test (Categorical)

Each pup is tested for forelimb grip on PND 21. A thin rod (approximately 2–3 mm in diameter) is supported by a ring stand suspended horizontally above the padded surface. The pup is held so it can grasp the thin rod with its forepaws and is then released. It must remain suspended for at least 1 s to meet the criterion. The number of pups that met the criterion, divided by the total number of pups tested, is recorded.

Pupil Constriction Reflex

All pups in each litter are examined once on PND 21 for direct and consensual pupillary constriction of both eyes in response to the beam from a penlight. This test evaluates the autonomic component of cranial nerve reflexes. Each nesting box is transferred from the study room to a quiet, isolated testing room, sufficiently dim to dilate the pupils of the eyes of the pups. Each pup is tested individually, with the following requirements to meet the criterion:

- The pup is removed from the nesting box, and the penlight is directed into one eye. Immediate constriction of the pupil of the eye being tested is the initial requirement.
- Without turning the light off, it is immediately directed into the contralateral eye, the pupil of which should already be constricted.

The light is turned off for a minimum of 5 s. This sequence is then repeated for the remaining eye. Reponses should be identical to meet the criterion.

OVERVIEW OF RISK ASSESSMENT

The National Research Council (NRC)[336] defined risk assessment as consisting of *hazard identification*, *dose–response assessment*, *exposure assessment*, and *risk characterization*. This general paradigm is used for risk assessment by many different agencies worldwide. These elements of the risk assessment paradigm are introduced briefly here, and then described in the following sections.

Hazard characterization is the qualitative evaluation of all available data, including laboratory animal data, in vitro studies, and human studies and case reports, to determine the nature of the effects observed and relevance to humans. In the context of the topic of this chapter, the focus is on whether and under what conditions reproductive toxicity and developmental toxicity occur.

Dose–response assessment is the quantitative evaluation between dose level and the response, with the aim of identifying a dose that would be safe for human exposure.

Exposure assessment involves determining who is exposed and the magnitude, duration, frequency, and route(s) of the actual or potential exposure.

Risk characterization integrates the result of the hazard characterization, quantitative dose–response analysis, and human exposure estimates to describe the risk to the exposed population under the scenario of interest, along with a characterization of uncertainty.

The recent NRC (2009) report on Science and Decisions: Advancing Risk Assessment[337] noted the importance of problem formulation and added it to the general risk assessment framework. Building on concepts from the ecological risk assessment field,[338] the process puts additional emphasis on the initial planning, on the *signal* that initiated the risk assessment, and on the options for managing the problem. The risk assessment might be initiated by *signals* such as a positive result for a chemical in an epidemiology study or animal bioassay, a finding of a disease cluster, evidence of high exposure, or the need to evaluate a drug before it enters the market. Note that exposure scenario often plays a key role in the problem formulation. The problem formulation determines the scope, nature, and depth of the risk assessment.

Hazard Characterization

The purpose of the hazard characterization is to conduct an overall WOE evaluation of all relevant available data in order to determine the effects caused by the agent of interest and relevance of those effects to humans. This evaluation requires consideration of both chemical-specific data and other data that may help to inform the WOE evaluation. For example, as described further in the following in the context of MOA evaluation, general biological information, and information on related chemicals may play key roles in the hazard characterization.

In conducting the hazard characterization, the assessor considers the quality and relevance of the available studies. For animal toxicology studies, important considerations include the appropriateness of route, dosing regimen, adequacy of sample size, and endpoints evaluated. Test guidelines for pharmaceuticals and industrial chemicals developed by the ICH[339] and the OECD,[340] respectively, aid in both the conduct of studies and the evaluation of study quality; EPA guidelines have been harmonized with those of the OECD.[89,235] In evaluating whether the data are indicative of an effect occurring in the animal study, a key consideration is whether a dose–response relationship is present.[341] However, one needs to evaluate the data holistically in considering the presence of a dose–response. For example, the incidence of malformations might decrease at high doses, due to the occurrence of a high incidence of resorptions or embryo lethality at those doses. In this case, there is insufficient survival for the malformations to develop.

Guidelines have been developed to aid in the interpretation of reproductive and developmental toxicity data in a risk assessment context and illustrate the critical thinking needed to integrate the data.[236,342] As discussed further later in this section, recent risk assessment approaches emphasize the use of chemical-specific and chemical-related data. However, when such data are not available, guidelines lay out some default assumptions to aid in addressing data gaps. As described by the EPA, the general assumptions used in the absence of data to the contrary include the following[236,342]:

- A chemical that causes an adverse reproductive or developmental effect in animal studies poses a potential adverse reproductive or developmental effect to human if sufficiently exposed at the critical stage.

- Effects of a chemical on male or female reproductive processes or developmental outcome are assumed to be predictive of similar effects in humans.
- With sufficient information, the most appropriate species for estimating risk to humans should be used for hazard characterization. In the absence of these data, the most sensitive species should be used.
- It is assumed that a chemical that affects the reproductive function or development of the offspring of one sex may also affect the reproductive function or development of the other sex.
- When the MOA and pharmacokinetics data are available, they should be used for low-dose extrapolation. In the absence of these data, a threshold is assumed for chemicals that produce reproductive or developmental toxicity.[343]
- Effects of xenobiotics on male and female reproductive processes are assumed to be generally similar unless demonstrated otherwise.
- All four types of developmental toxicity effects (death, structural abnormalities, growth alterations, functional deficits) are of potential concern.
- Developmental effects seen in animals are not necessarily the same as those that may occur in humans.

As noted, these default assumptions apply when data are not otherwise available, but it is the risk assessor's responsibility to consider all of the available data before resorting to defaults. These data include not only the toxicology data on the chemical and any available human data. Other information can come from in vitro studies or knowledge of molecular structure–activity relationships.[344] These latter types of data sources can be particularly important in contributing to an understanding of a chemical's MOA. The MOA can be thought of as a general description of how the chemical causes toxicity[345] and is distinguished from the detailed description at the molecular level that comprises a description of a chemical's MOA. The MOA is defined in terms of *key events*, empirically observable precursor steps (or markers for such steps) that are necessary for the development of the toxic endpoint of interest. Both chemical-specific information and general understanding of the relevant biology can be used in defining an MOA.

An important shift in the thinking about risk assessment is reflected in the transition from hazard identification (determining effects occurring in animal studies) to hazard characterization. Hazard characterization is a holistic WOE evaluation of the data, including evaluation of the relevance of the observed effects to the exposure scenario of interest and relevance to humans, based on such factors as the dose at which the effect occurred, the chemical's MOA, and interspecies differences. In conducting this integrative evaluation, the risk assessor considers any differences in patterns of effects, any differences seen between studies, and the potential reasons for the observed differences. These reasons could include first-pass metabolism, and differences between rat strains in endocrine regulation, or differences in study design. Rather than simply adding positive and negative results, the WOE evaluation includes a critical evaluation of all of these.

MOA is a key concept that plays several roles in the hazard characterization. It can aid in the development of a full description and an integrated understanding of the effects caused by the chemical. MOA is also used in evaluation of the human relevance of effects observed in animals and in prioritization and screening applications, where structural similarities and an understanding of the chemical and physical properties are used to reach some general predictions of a chemical's expected effects and relative potency. Some key MOAs relevant to developmental and reproductive effects include cytotoxicity, changes in cell fate regulation, and hormonal perturbations, which can result from the chemical acting as a hormone mimic, changes in hormone metabolism, effects on hormone-producing or regulatory tissue, or other mechanisms.

The data regarding a hypothesized MOA are evaluated using the modified Hill criteria.[346] These criteria include the following:

- Dose–response relationship
- Temporal relationship
- Consistency—reproducibility across database, related endpoints
- Coherence—biological plausibility of MOA
- Strength—magnitude of association
- Specificity—apical effect seen only following the occurrence of key event

Of these criteria, evaluating data on the dose–response relationship and temporal relationship between key events and the effect of interest is particularly important. If the key event is part of the MOA, the key event should occur at lower (or comparable) doses and earlier (or comparable) times compared with the endpoint of interest.

Once the MOA has been established in the experimental animal species, the data can be used to evaluate the human relevance of that MOA. This is done using the framework developed by the Internationale Programme on Chemical Safety (IPCS) and International Life Sciences Institute (ILSI).[345,346] This approach is presented in Figure 35.22. If the MOA in animals cannot be determined, the endpoint is assumed to be relevant to humans, and the dose–response implications of toxicokinetic and toxicodynamic differences are evaluated. If the MOA is established in the experimental animal species, the human relevance framework goes on to ask whether (1) human relevance of the MOA can be reasonably excluded on the basis of fundamental, qualitative differences in key events between animals and humans and (2) human relevance of the MOA can be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between animals and humans. If the answer to either of these questions is yes, then the MOA is determined to not be relevant to humans. If the answer to both questions is no, implications of toxicokinetic

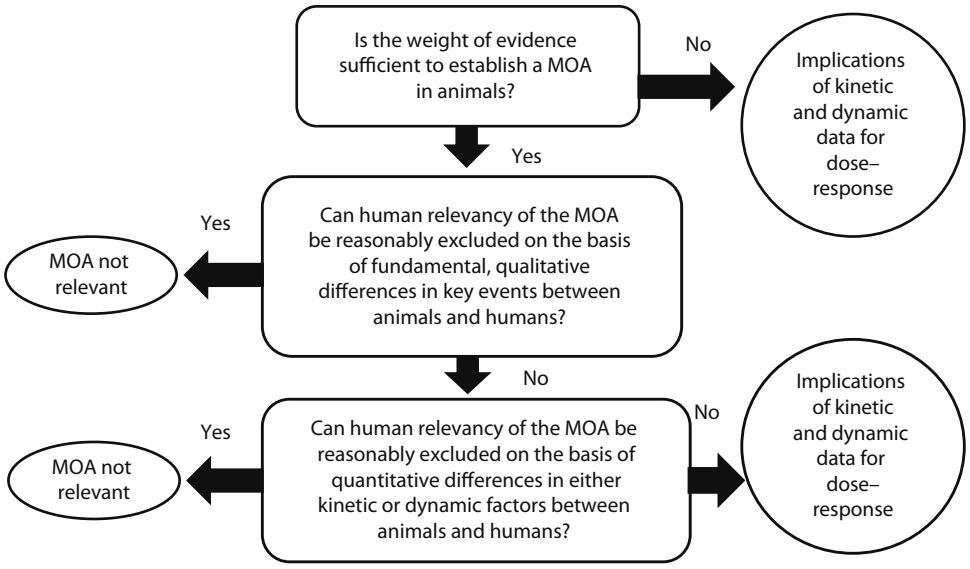

FIGURE 35.22 IPCS/ILSI mode of action framework.

and toxicodynamic differences on the dose–response are evaluated. Both chemical-specific information and a general understanding of biology and physiology are used in addressing the questions.

The result of the hazard characterization is a determination of which endpoints should be carried forward into the dose–response assessment. As discussed in the following, the hazard characterization for pharmaceuticals also includes a risk–benefit evaluation, weighing the risk of adverse effects against the benefit from the intended use of the drug.

Dose–Response Assessment

Dose–response assessment is the quantitative evaluation between dose level and the response, with the aim of identifying a dose that would be safe for human exposure. The same general approach is used for multiple regulatory agencies, including agencies that regulate industrial chemicals, food additives, and pesticides. Dose–response assessment for pharmaceuticals typically follows a comparative approach, comparing the doses at which the pharmaceutical would be administered with those projected to cause adverse reproductive or developmental side effects. Fewer extrapolations tend to be needed for the assessment of pharmaceuticals, although reproductive or developmental data are usually not available in humans.

General Quantitative Approach

Many different regulatory organizations use similar approaches for calculating safe doses, or health-based guidance values (HBGVs) although the name used to describe that value varies. These include International Programme on Chemical Safety's Tolerable Intake (TI),[347] U.S. EPA's Reference Dose (RfD)[348] or Reference Concentration (RfC),[349] or the WHO's Acceptable Daily Intake (ADI), which is also used by WHO agencies for food additives and pesticides. The general safe dose approach is designed to protect sensitive populations from lifetime exposure to the chemical of interest. HBGVs are calculated by dividing a point of departure, such as a NOAEL, lowest-observed-adverse-effect level (LOAEL), or NOAEL surrogate, such as a benchmark dose/concentration (BMD/BMC), by uncertainty factors that account for areas of uncertainty and extrapolations needed to calculate the HBGV from the available data. The goal for the point of departure is to identify the dose boundary for the onset of the effect from the most relevant species, or the most sensitive species, if the most relevant is not known.

The approaches used by different organizations in determining uncertainty factors (UFs) share many commonalities, although there are some differences. All organizations include UFs for extrapolation from animal data to humans (interspecies factor) and for considering human variability and protection of sensitive populations (intraspecies factor). These factors reflect variability that is inherent to the population; additional data can characterize that variability, but not remove it. For these areas of uncertainty, the state of the science is to develop a *chemical-specific* (also called *data derived*) adjustment factor to quantify the interspecies differences or human variability, if data are available. If such data are not available, then a default uncertainty factor of 10 is generally used. IPCS has developed guidelines for the data needed to develop chemical-specific adjustment factors (CSAFs) based on chemical-specific or chemical-related data as the basis for adjustments to the point of departure instead of default uncertainty factors.[350]

The U.S. EPA is perhaps the most explicit in considering the areas of uncertainty, an approach that is followed by some other agencies. The EPA considers five areas of uncertainty in the development of its HBGV (the RfD/RfC). In the absence of data, a default factor of 10 is used for each

area; rules for combining uncertainty factors reflect the overlap that exists between factors.[351] In addition to the two uncertainty factors noted earlier (addressing interspecies differences and intraspecies variability), EPA uses three uncertainty factors to address database deficiencies. These factors address true uncertainty and can be replaced by data. These areas of uncertainty are for LOAEL to NOAEL extrapolation, subchronic to chronic extrapolation, and (other) deficiencies in the database.

Two of the uncertainty factors are of particular importance for issues related to DART endpoints, including potential systemic and developmental effects in children. The first is the factor addressing human variability, including variability resulting from such factors as genetic differences and age-related differences. Increased sensitivity in children could result, for example, because many enzyme systems have low activity during the first year of life, resulting in increased sensitivity if the parent chemical is the active form, but decreased sensitivity if the metabolite is the active form. Other physiological differences, including the potential for increased sensitivity in the growing organism, may also make children more sensitive than adults. Several risk assessment frameworks or guidance documents are available on issues to consider in conducting risk assessment for different life stages or developmental stages.[352–354]

The second uncertainty factor of particular importance to DART endpoints is the factor addressing deficiencies in the database. This uncertainty factor addresses the question of whether the correct critical effect has been identified, or whether a different critical effect at a lower dose would be identified if an additional study were conducted. In the absence of adequate human data, U.S. EPA considers that the minimum database for development of a high-confidence RfD or RfC, sometimes referred to as a *complete* database for calculating an RfD/RfC, includes the following studies[349,351]:

- Two adequate mammalian chronic toxicity studies by the appropriate route in different species
- One adequate mammalian multigenerational reproductive toxicity study by an appropriate route
- Two adequate mammalian developmental toxicity studies by an appropriate route in different species

Because the minimal database for calculating an RfD/RfC is a subchronic systemic toxicity study, the data gaps often include reproductive and/or developmental toxicity studies. In an analysis of pesticide data (for which all five study types were available), Dourson et al.[355] found that the systemic toxicity and reproductive toxicity studies were most likely to identify the critical effect. For most chemicals, developmental effects occurred at higher doses.

For pesticides, the Food Quality Protection Act (FQPA) factor addresses specific concerns about the potential for greater sensitivity of children. However, EPA has stated that an additional factor addressing hazard considerations for children is not needed if "appropriate care has been taken in accounting for all deficiencies and uncertainties in the database using the currently available uncertainty/variability factors."[356] The FQPA factor also incorporates exposure considerations that result in age-dependent sensitivity and are not addressed with the standard uncertainty factors.

EPA guidelines for developmental toxicity risk assessment[236] also describe the approach for setting RfDs specific for developmental toxicity (RfD_{DT}s). The approach is similar to that used for general RfDs, except that the point of departure is based on the most sensitive *developmental* effect. The interspecies and intraspecies uncertainty factors and the factor for LOAEL to NOAEL extrapolation would be applied as for general RfDs. However, the factors for subchronic to chronic extrapolation and (other) deficiencies in the database would generally not be applied in developing an RfD_{DT}. Although the RfD_{DT} is noted in EPA guidance, relatively few have been developed; the RfD for lifetime exposure is intended to protect from developmental effects and may be lower than the RfD_{DT}.

State of the Science Methods

The BMD or its lower confidence limit (the BMDL) can be used as a point of departure instead of an NOAEL or LOAEL.[357–360] The BMD is determined by fitting a flexible mathematical model to the data and determining a dose corresponding to a defined response level. BMD modeling has several advantages over the NOAEL method: (1) the BMD is not limited to the tested doses, (2) a BMD can be calculated even when the study does not identify an NOAEL, and (3) unlike the NOAEL approach, the BMD approach accounts for the statistical power of the study. The EPA has developed user-friendly software for conducting benchmark dose modeling (BMDS)[361]; functionally similar software is available from Netherlands National Institute for Public Health and the Environment (RIVM).[362] Of particular interest in the context of this chapter are the specialized BMD modules for modeling the nested data available for developmental toxicity studies when data are available at the level of the individual pup within each litter. These models can account for the fact that measurements are made on pups, but the litter is the unit of measurement, due to the impact of the mother's biology and toxicokinetics. The models can include both intralitter correlations and litter-specific covariates, such as litter size. Based on the results of a series of studies comparing BMDLs associated with different definitions of the benchmark response (BMR) and NOAELs,[363–366] the standard BMR used as the point of departure for developmental toxicity studies when quantal nested data are available is 0.05.[364] Modeling of reproductive endpoints is conducted in the same manner as for systemic endpoints, using the standard BMDS models. The default BMR for reproductive endpoints is 0.1 for quantal endpoints, or a 1 standard deviation change in the mean for continuous endpoints, if information on the degree of change that is considered adverse is not available.[360]

Physiologically based pharmacokinetic (PBPK) models can be used to improve the extrapolations needed in developing an HBGV. PBPK models describe the flow and transformation of the chemical by the body (toxicokinetics) using

species-relevant organ and tissue volumes, blood flows, and kinetic transformation parameters (reviewed by Clewell[367]). This allows the estimation of the biologically important dose delivered to the target organ(s), based on the relevant dose metric. When selecting the PBPK models compartments to be represented for reproductive and developmental endpoints, consideration must be given to the target site (e.g., fetus, sperm) and portal of entry (e.g., lung, placenta). A challenge unique to PBPK models for developmental toxicity is the need to capture the rapidly changing growth dynamics of the maternal and fetal tissue.[368] Since the metabolism in reproductive organs is often limited in terms of their overall impact, these organs are not routinely represented as separate compartments and are frequently lumped together. PBPK models have been used to replace the default UF for interspecies differences based on a characterization of the relationship between exposure and tissue dose.[369]

A key concept for both interpreting developmental toxicity studies and applying the results in a risk assessment is the idea of a window of susceptibility. It is assumed that a single exposure at a sensitive developmental stage can produce an adverse effect. Thus, even though standard developmental toxicity studies involve exposures for all of the gestational period, or during major organogenesis, it is assumed that a single exposure could cause an effect if it happened during the window of susceptibility to the effect. The relevant window should be considered in interpreting the results of toxicity studies, particularly if exposure did not include the entire gestational period. Similarly, human exposures that are compared with an HBGV should not be adjusted for the duration of exposure or pattern of exposure.

A particularly challenging issue in interpreting developmental toxicity data is the interpretation of developmental effects that occur in the presence of maternal toxicity, and determining whether the effects reflect true developmental toxicity or are secondary to the maternal toxicity. Fetal endpoints commonly associated with maternal toxicity include rib malformations, cleft palate, decreased fetal body weight, and fetal death.[370] This issue has been addressed in several review articles and guidance documents,[370–372] which provide information on approaches to use in considering the issue. The relative severity and dose–response for the maternal and fetal effects are important for this evaluation. For example, the observation of mild fetal effects (e.g., mild decrease in body weight) only in the presence of frank maternal toxicity is consistent with the effect being secondary to maternal toxicity, while fetal effects seen only in the presence of more mild maternal toxicity should be considered as evidence of potential developmental toxicity. Mechanistic studies and pair-feeding studies can also aid in determining whether the developmental effects reflect a direct effect of the chemical or are secondary to maternal toxicity.

Exposure Assessment

The exposure assessment includes characterizing the exposed population, and the magnitude, duration, frequency, and route(s) of the actual or potential exposure. Several considerations are of particular interest in the context of developmental and reproductive toxicity. Exposure of the fetus or neonate can occur via placental transfer or breast milk, and so these exposures are dependent on maternal absorption, distribution, metabolism, and excretion. Direct exposure of neonates and children may also occur via the environment (water, air, soil) and therefore may require estimates of exposure from multiple sources. Duration and timing of exposure must be related to the stage of development (first, second, or third trimester; infancy; childhood; or adolescence).

EPA's Guidelines for Exposure Assessment[373] discuss various approaches for monitoring actual exposures or modeling various exposure scenarios. Exposure factors have been published for adults and children of various ages to aid in estimating exposure from a variety of sources, including food and water consumption, respiration, activity patterns, body weight, soil ingestion in children, and respiration rates in children and adults.[374,375] These data can be used to estimate exposure from various sources, or as input into exposure modeling.

Risk Characterization

The risk characterization integrates the result of the hazard characterization, quantitative dose–response analysis, and human exposure estimates to describe the risk to the exposed population under the scenario of interest, along with a characterization of uncertainty. The characterization includes the strengths and weaknesses of each component of the risk assessment, as well as major assumptions, relevancy to humans, scientific judgments, level of confidence, and when possible, qualitative descriptions and quantitative estimates of uncertainties. Key risk characterization principles are transparency, clarity, consistency, and resonableness.[376] Key aspects specific to reproductive and developmental toxicity include ensuring that the exposure assessment and dose–response assessment were consistent in terms of the problem formulation, particularly the population exposed and the exposure scenario.

Drug Safety Studies

There are two key differences between risk assessment for chemicals and for pharmaceuticals. The first is that pharmaceuticals are used to treat specific diseases, and so risk is considered on a risk-benefit basis; that is, the beneficial uses of the agent are compared with the risk of its use. The second is that pharmaceuticals are provided to a targeted population, and so developmental and reproductive risks can be managed by not administering the drugs to certain populations (e.g., pregnant women).

These issues are addressed in the labeling of the pharmaceutical, published, for example, as the official FDA-approved package insert of a drug or biologic. This system was established by law in 1979 to assist physicians prescribing drugs to pregnant women and categorizes risk by assigning a pregnancy label category to drugs and biologics (Table 35.8). Category A is the safest, having proven safety

TABLE 35.8
FDA Labeling Requirements: Pregnancy Categories

Category	Effects	Criteria
A	Animal studies and well-controlled studies in pregnant women failed to demonstrate a risk to the fetus.	Most stringent criteria
B	Animal studies have failed to demonstrate risk to fetus; no adequate and well-controlled studies in pregnant women.	Less stringent criteria
C	Animal studies showed adverse effect on fetus; no well-controlled human studies.	Assumes risk without data
D	Positive evidence of human fetal risk based upon human data, but potential drug benefit outweighs risk.	Expect effects on humans
X	Studies show fetal abnormalities in animals and humans; drug is contraindicated in pregnant women.	Indicates high adverse risk

Source: Adapted from Collins, T.F.X. et al. Principles of risk assessment—FDA perspective, In: *Developmental and Reproductive Toxicology—A Practical Approach*, Hood, R.D., ed., 2nd edn., CRC Press, Boca Raton, FL, 2006, pp. 877–909.

in humans. Category X drugs are the most hazardous, having proven adverse developmental or reproductive effects in humans or animals and thus are contraindicated during pregnancy. Precautionary statements, regardless of animal study findings, are often included in product labels, since there are limitations in predicting human risk from animal data. An example of such a statement is as follows: "There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction and developmental studies are not always predictive of human response, 'the drug' should be used during pregnancy only if clearly needed."

The categories may mislead healthcare providers (and the women they counsel) to believe that risk increases from category A to B to C to D to X. In fact, that is not the case, because Categories C, D, and X are based not just on risk, but reflect risk weighed against benefit. That means that a drug in categories C or D may pose risks similar to a drug in Category X.

FDA Guidance[372] provides an approach for integrating nonclinical reproductive and developmental toxicity data, in order to determine the appropriate wording for labeling. The overall approach is conceptually consistent with the concepts discussed earlier for hazard characterization. According to this approach, *positive signals* are broadly classified as reproductive or developmental toxicities. Reproductive toxicity is broken into three subclasses: male fertility, female fertility, parturition, and lactation; these endpoints include both structural and functional changes affecting the reproductive competence of male and/or female animals in the parental (F_0) generation. Developmental toxicity has four subclasses: developmental mortality, dysmorphogenesis, alterations to growth, and functional toxicity. The decision process described in Figures 35.23 through 35.25 applies to any reproductive or developmental endpoint. Human data are considered separately from nonclinical findings but carry considerable weight in the overall evaluation.

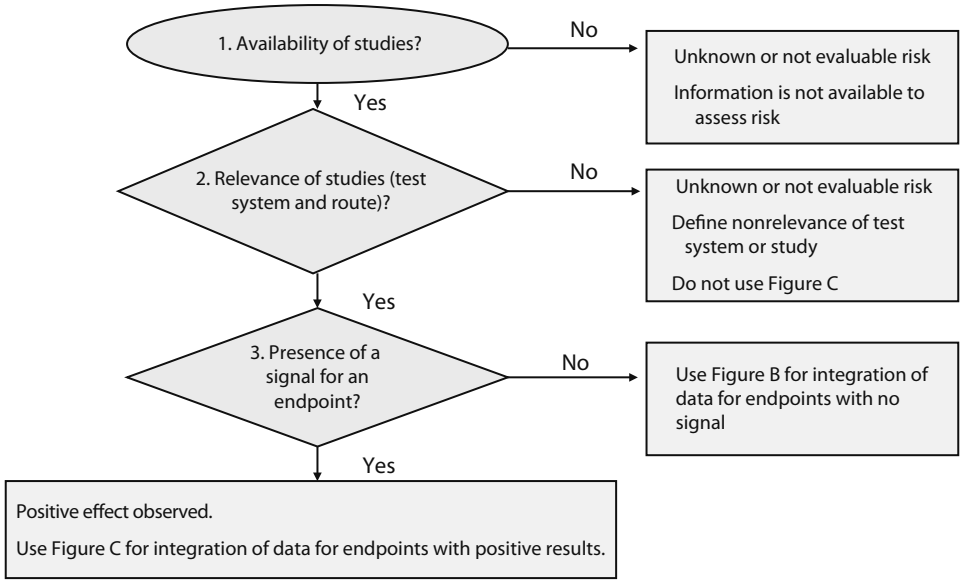

FIGURE 35.23 FDA Flowchart A: Overall decision tree for evaluation of reproductive/developmental toxicities.

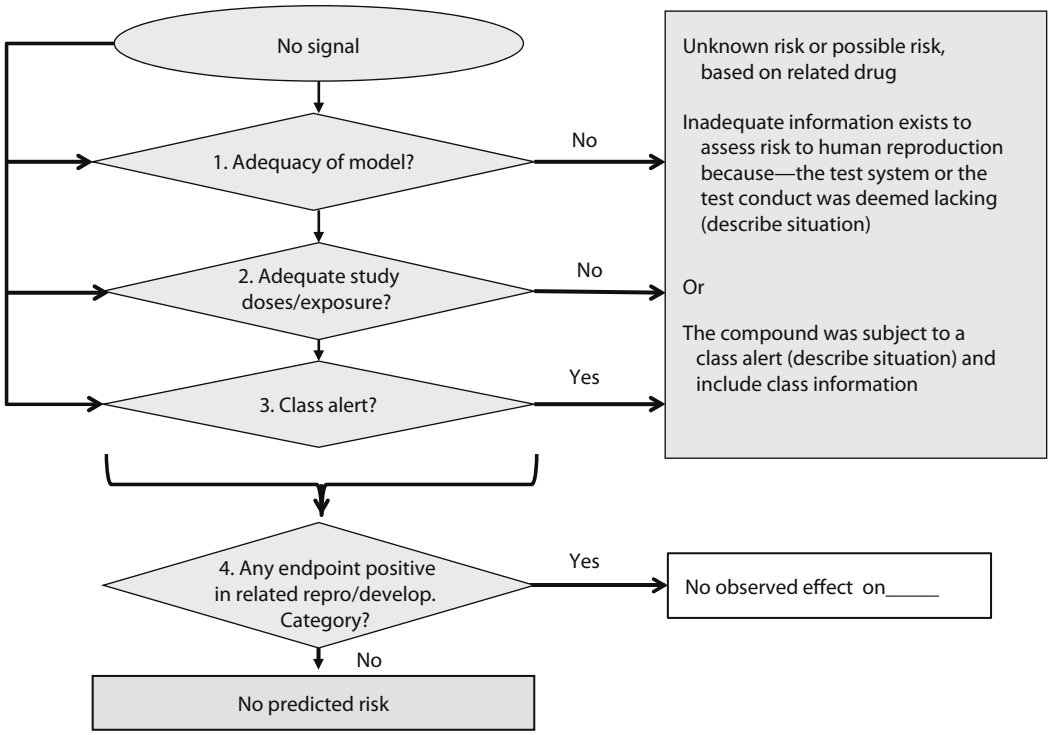

FIGURE 35.24 FDA Flowchart B: Decision tree for endpoints with no signal.

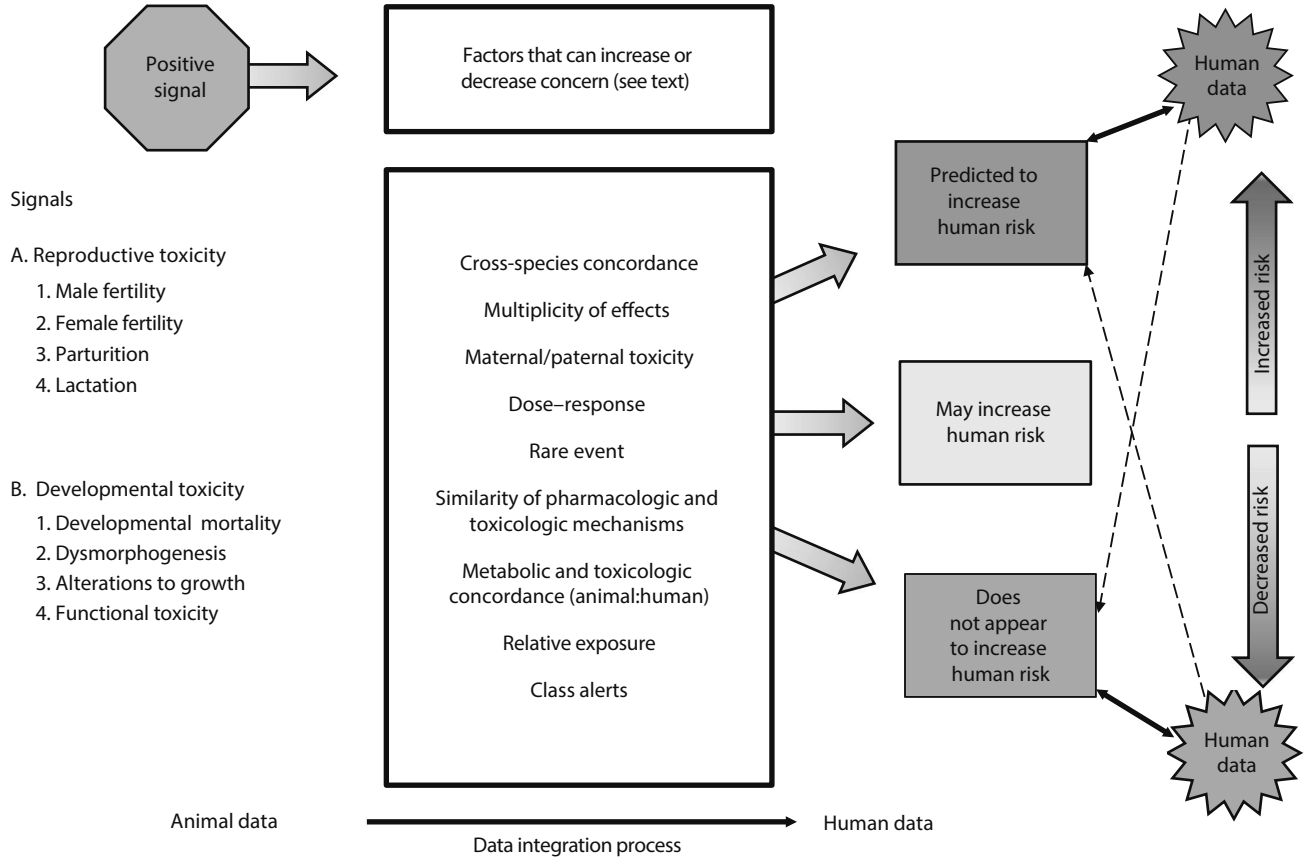

FIGURE 35.25 FDA Flowchart C: Integration of reproductive or developmental toxicities with a positive signal.

Flowchart A (Figure 35.23) is used to evaluate the available studies and determine the next steps in the evaluation. If relevant studies conducted via a relevant route are available, the presence of a positive signal for an endpoint is evaluated. If there are no positive signals in any of the studies addressing a particular reproductive or developmental endpoint, the evaluation continues with Flowchart B (Figure 35.24). This flowchart includes further evaluation of the test adequacy and consideration of other potential reasons for concern, such as findings for related chemicals or for related endpoints. When positive signals exist for reproductive or developmental endpoints, factors that can increase or decrease the level of concern are evaluated for class of toxicity (see Flowchart C, Figure 35.25).

Flowchart C illustrates a number of key factors that contribute to the overall evaluation by increasing or decreasing the level of concern for adverse effects in humans. Scientific judgment is critical in integrating the data for a final conclusion; other factors may also contribute. The factors in the flowchart are as follows:

- Cross-species concordance—observation of the same *or related* types of toxicity in multiple species increases the concern
- Multiplicity of effects—observation of two or more positive signals in a single species within the general categories (reproductive or developmental toxicity) increases concern
- Maternal or paternal (in fertility studies) toxicity—signal in the absence of maternal toxicity increases concern; signal only in the presence of frank maternal toxicity may decrease concern if attributed to maternal toxicity
- Dose–response—concern is increased when the incidence or severity of effect increases with dose
- Rare event—an increased incidence of rare events increases the concern, even though small increases may not be statistically significant
- Similarity of pharmacologic and toxicologic mechanisms—concern is increased if the observed effect is related to the drug's intended pharmacologic MOA
- Metabolic and toxicologic concordance between animals and humans—concern is increased by this concordance and decreased by lack of concordance, as illustrated in the MOA discussion earlier
- Relative exposure—the appropriate dose metric (e.g., area under the [concentration × time] curve [AUC], Cmax) is identified and used to evaluate relative exposure in animals versus humans, with ratios <10 increasing concern and ratios >25 decreasing concern
- Class alerts—concern is increased when the drug belongs to a class known to produce adverse reproductive/developmental effects in humans and animals and is decreased only where a class of compounds has produced no adverse effects on human reproduction, despite adverse effects in animals

Based on these factors, a WOE approach is used to reach an overall conclusion regarding the drug's potential for reproductive or developmental toxicity in humans.

Timing of Preclinical Developmental and Female Reproductive Toxicity Studies

The timing of the conduct of preclinical reproductive toxicity studies is based on the population in which the clinical trials are going to be performed and on the ICH countries that the investigational new drug and/new drug application (IND/NDA) will be submitted.[377,378]

Women Not of Childbearing Potential

Women who do not have childbearing potential (i.e., permanently sterilized, postmenopausal) can be included in clinical trials without reproductive toxicity studies if the relevant repeated-dose toxicity studies (which include an evaluation of the female reproductive organs as described earlier) have been conducted.

Women of Childbearing Potential

A high level of concern exists for the unintentional exposure of an embryo or fetus of the women of childbearing potential (WOCBP). When WOCBP are included in clinical trials, the risk to the embryo or fetus should be minimized. Minimizing the risk can be achieved by conducting preclinical reproductive toxicity studies to understand the inherent risk of a drug. Alternatively or in addition, the risk can be limited by taking precautions to prevent pregnancy during clinical trials. Informed consent should be based on any known pertinent information related to reproductive toxicity, such as a general assessment of the potential toxicity of pharmaceuticals with related structures or pharmacological effects. If no relevant reproductive information is available, the potential for risks should be communicated.

Timing of Clinical Trials[379]

The recommendations on timing of reproductive and developmental toxicity studies to support the inclusion of WOCBP in clinical trials are similar in all ICH regions (the United States, EU, and Japan). WOCBP can be included in clinical trials without nonclinical developmental toxicity studies (e.g., embryotoxicity studies) in certain circumstances. WOCBP can be enrolled in clinical trials of short duration (such as 2 weeks) with intensive control of pregnancy risk.[380] WOCBP can also be enrolled in longer duration clinical trials when the objectives of the clinical trial cannot be effectively met without their inclusion, when the disease occurs predominantly in women, and when pregnancy risk can be adequately controlled. WOCBP can also be considered for inclusion in early clinical studies without the nonclinical developmental toxicity studies, based on the knowledge of the type of pharmaceutical agent (e.g., an antibody), its MOA, its absorption, distribution, metabolism, and excretion (ADME) characteristics (such as half-life, AUC, Cmax), and difficulty of conducting developmental toxicity studies in an appropriate animal model.

Generally, where appropriate preliminary developmental toxicity data are available from two species but the definitive reproductive toxicity testing has not been completed, and where adequate birth control methods are used, WOCBP can be included in clinical trials for a relatively short duration (up to 3 months), and a relatively small size (consisting of 150 subjects) can be used. An appropriate preliminary embryo–fetal study is defined as a study with adequate dose levels with a minimum of six dams per group that are treated over the period of organogenesis: assessment of fetal survival, body weight, and external and visceral examinations. The timing of the conduct of the reproductive and developmental toxicity studies varies by region:

- In the United States, assessment of embryo–fetal development can be deferred until prior to phase III for WOCBP using highly effective contraceptive methods.
- In the EU and Japan, definitive nonclinical developmental toxicity studies should be completed prior to exposure to WOCBP.
- In all ICH regions, nonclinical studies that specifically address female fertility should be completed to support inclusion of WOCBP in phase III trials.
- In all ICH regions, the pre- and postnatal development study should be submitted for marketing approval or earlier if there is a cause of concern.

Pregnant Women

All of the reproductive toxicity studies and the standard battery of genotoxicity tests should be conducted prior to the inclusion of pregnant women in clinical trials. In addition, safety data from previous human exposure should be evaluated.

Pediatric Populations

Reproductive toxicity studies relevant to the age and gender of the pediatric patient populations under study can also be important to provide information on direct toxic or developmental risks (e.g., fertility and prenatal and postnatal developmental studies). Embryotoxicity studies are not critical to support clinical studies for males or prepubescent females. Results from repeated-dose toxicity studies of appropriate duration in adult animals, the core safety pharmacology package, and the standard battery of genotoxicity tests should be available prior to the initiation of trials in pediatric populations. Juvenile animal toxicity studies are designed on a case-by-case basis.[225]

Labeling Approaches

Proposition 65 (California)

As described on the California EPA website,[381] Proposition 65 was an initiative approved by California voters in 1986 to address their growing concerns about exposure to toxic chemicals. That initiative became the Safe Drinking Water and Toxic Enforcement Act of 1986, better known by its original name of Proposition 65. Proposition 65 requires the State of California to publish a list of chemicals known to cause cancer or birth defects or other reproductive harm. This list has grown to include approximately 800 chemicals since it was first published in 1987. Proposition 65 also requires businesses to notify Californians about significant amounts of chemicals in the products they purchase, in their homes or workplaces, or that are released into the environment. For example, labeling of products is required if exposure to a chemical listed based on reproductive or developmental toxicity is greater than the no observable effect level (NOEL) divided by 1000. As illustrated by this program, the ability to assess and characterize the reproductive and developmental health of a community is often limited by a lack of data and a lack of consensus as to the adverse effects of a specific chemical. Evidence cited by Mattison[382] indicates that of the estimated 90,000 chemicals in commerce in the United States, only 4000 have been tested in animals for reproductive or developmental toxicity, and up to one third of the tested substances may be reproductive or developmental toxicants. A study by the NRC[383] also concluded that only a small fraction of the chemicals tested contained enough toxicity information for reproductive and developmental hazard identification. The growing field of high-throughput screening, alternative test systems, and in silico (computer analysis) evaluation of structure–activity relationships is likely to help address this data gap, although numerous challenges remain.[384–386] These approaches, combined with a thoughtful approach to consideration of MOA, can help focus resources on the chemicals most likely to have reproductive or developmental effects.

Globally Harmonized System (GHS); REACH

Labeling of developmental and reproductive toxicants is also required under both the GHS and REACH. GHS is a harmonized system of classification and labeling of chemicals under the UN.[387] REACH is an EU regulation addressing the production and use of chemicals and their effect on human health and the environment. Both labeling systems follow IPCS guidelines for chemical evaluation and are based on WOE approaches.

CONCLUSION

The goal of this chapter is to familiarize the reader with the basic anatomy and physiology of the female reproduction system and embryo/fetal/neonatal development, methods used to assess the impact of chemical exposure on these endpoints, and how to apply those concepts in a risk assessment context. The importance of these topics is reflected in the reports of barren women and malformations in babies dating back to ancient times. Exposure to biotoxins, chemical toxicants, radiation, malnutrition, disease, hyperthermia, or stress in the environment, home, or workplace can lead to infertility, adverse pregnancy outcome, and poor childhood development. Appreciating how these exposures can adversely affect reproduction and development requires understanding of the sequence of events involved. The continuous biological changes that occur in the mother, placenta, fetus, and neonate follow a tightly controlled series of events, complicating the evaluation of the effects of chemical exposures.

Even with current experimental paradigms and international harmonization of DART testing guidelines, animal

studies will still provide the vast majority of information needed for regulatory risk assessment for humans for at least the immediate future. The future of DART testing will likely focus on three major areas: (1) advances in new real-time technologies to examine the fetus in utero, such as ultrasound, positron emission tomography (PET) imaging, and microcomputer tomography (micro-CT); (2) continued application of receptor-mediated developmental toxicity and intercellular signaling pathway analyses; and (3) refinement of current in vitro and in silico models to better identify and predict potential reproductive hazards and risks, including the use of human ESCs and induced pluripotent stem cells (iPSCs) that provide an unprecedented window to examine reproductive and developmental toxicants. Genomic technologies are developing new reproductive and developmental screening strategies, biomarkers for toxicity, mechanisms of cellular toxicity and molecular perturbations, and monitoring alterations in key biochemical pathways.[388] Together, these new developments will help improve the speed and predictivity of nonanimal testing methods, leading to cheaper, faster, and more accurate assessments.

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KEYWORDS

Developmental and reproductive toxicology, Gametogenesis, Fertilization, Ovarian and placental toxicity, Estrous, Implantation, Parturition, Lactation, Birth defects, Perinatal mortality, Behavior, Testing guidelines, Risk assessment

QUESTIONS

35.1 A new carbamate (AChE inhibitor) insecticide needs approval. What developmental and reproductive testing needs to be conducted?

35.2 A new biologic (polypeptide) drug for diabetes is being developed. Would going straight to the nonhuman primate to conduct the developmental toxicity testing instead of the standard rat and rabbit developmental toxicity studies be appropriate? When would using transgenic animal models be better for this testing?

35.3 A new drug is being developed for breast cancer. What maternal, fetal, and developmental landmark endpoints may be of particular interest and why?

35.4 What in vitro or in silico tests have been developed to screen for potential female reproductive toxicity?

35.5 How would you use MOA information to address the implications of data gaps for the choice of uncertainty factors for an industrial chemical?

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36 Hormone Assays and Endocrine Function

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CONTENTS

INTRODUCTION

All physiological responses are essentially controlled by two primary monitoring/control systems: (1) the nervous system, which is electrical in nature and controls processes requiring immediate attention, such as breathing, skeletal muscular movements, and heartbeat, and (2) the endocrine system, which is chemical in nature and controls slower processes, such as cell growth and development, metabolism, and sexual function. Both systems are critical to the body's function, and they frequently work together to allow the body to function at its most optimal level.

The purpose of this chapter is to examine the endocrine system of physiological control. The endocrine system basically consists of ductless glands or cells that secrete chemical messengers that are released into the bloodstream or local cellular fluid. Hormone receptors strategically placed throughout the body recognize and ultimately react to the presence of the hormone and affect a biological change. Hormones are usually very specific in nature, binding to a specific receptor in much the same way a key fits into a lock. There may be many keys present at the location of the locks, but only one fits and creates a hormonally activated receptor; therefore, even though hormones reach all parts of the body, only target cells with compatible receptors are equipped to fully respond. When a receptor and a hormone bind, the receptor carries out the instructions of the hormone by either altering the existing proteins of the cell or building new proteins. These actions create hormonally mediated chemical reactions throughout the body.

In conjunction with the nervous system, the endocrine system integrates many different processes that allow multicellular organisms to maintain homeostasis. It is estimated that there may be at least 50 hormones in mammals.[1] Several drugs and chemicals are known to affect the endocrine system.[2] Perturbation of endocrine homeostasis often produces consequences that can lead to metabolic derangements, developmental abnormalities, and sexual or reproductive dysfunction. Endocrine disruptors, including environmental estrogens, is a contemporary term used to describe such effects. Endocrine toxicology involves the study of chemicals and drugs that disrupt endocrine processes, leading to either augmentation or inhibition of a physiologic response.[3] In a broad sense, endocrine toxicology encompasses reproductive toxicology (see Chapters 33 and 34).[4] In addition, endocrine toxicology can also include metabolic derangements that result from toxic injury to nonendocrine systems; for example, renal or hepatic toxicity may alter the rate of hormone catabolism. This chapter reviews endocrine physiology, morphology, and endocrine function as it relates to toxicology. Another area of interest is endocrine pharmacology, which involves the therapeutic and diagnostic use of hormones and other nonhormonally related agents. It can also encompass endocrine toxicology, as many nonhormonal medicinal products possess side effects that can alter the biochemical activity of the organs of internal secretion.[5] Importantly, molecular biology continues to provide a number of techniques that are very useful in endocrinology.[6]

Although some endocrine organs appear to be more sensitive or vulnerable to toxicologic agents than others, hormone–target organ interrelations often lead to a substance causing multiple disruptions in the hormonal balance of the organism. It is not uncommon to witness chemically induced changes in gonadal function along with alterations in thyroid gland activity. In some species, chemically induced changes in sex steroids can affect pancreatic secretion of insulin. Chemically induced stress leading to increased secretion of glucocorticoids (GCs) can also affect insulin secretion but more importantly can affect adrenocorticotropin hormone (ACTH) levels and hence alter the pituitary–adrenal axis.

The thalidomide tragedy of the 1960s led to the formulation of toxicologic testing guidelines for the field of teratology beginning as early as 1962. No such guidelines have been invoked for the endocrine system. Pesticide-induced sterility (e.g., dibromochloropropane [DBCP]) first reported in 1977[7] eventually led to concern about the deleterious actions of such substances in both male and female reproductive systems.[8,9] The inherent estrogenicity of *o,p′*-dichlorodi phenyldichloroethane (*o,p*-DDD) can affect the reproductive

system, and concern has been expressed about the possible relation between diethylstilbestrol (DES) and the incidence of vaginal and cervical cancers[10]; thus, numerous examples of chemically induced changes in the endocrine system have been reported. Because many of these agents represent reproductive hazards in the workplace, the endocrine system falls prey to many such agents. Polyhalogenated biphenyls, dibenzodioxins, and dibenzofurans can interfere with thyroid hormone metabolism. Certain insecticides and pesticides can be toxic to the pancreatic β cell.

Agents toxic to the endocrine system reach a relatively small number of individuals at the site of manufacture, which can be controlled, but they gain access to entire communities when, for example, toxic wastes are released into the environment. Large geographic regions are exposed to natural goitrogens, such as resorcinol, that are derived from regional coal and shale deposits. Likewise, radioisotopes of iodine released during atmospheric nuclear weapons testing or reactor accidents can be disseminated over very large areas in detectable, if not clearly toxic, concentrations throughout the world.[11]

Conceptually, the endocrine system is vulnerable to chemical toxicity at multiple points. Most, if not all, tissues are target organs of one or more hormones and are influenced in some fashion by endocrine substances. A vast number of critical biological processes, for example, are regulated by the endocrine system, including brain and nervous system development, development of the reproductive system and secondary sex characteristics at puberty, metabolism, and blood sugar maintenance. In fact, major physiological events from conception and infancy through adolescence to old age are generally mediated by large numbers of various hormones working together with the nervous system to achieve this immensely complex task. Because of structural similarity to certain hormones, some toxic substances interfere with hormone metabolism or subsequent actions at the receptor site. Still other toxic agents interfere directly with the glands that synthesize and secrete hormones. Compounding the chemical vulnerability of the endocrine system are the tier and feedback systems of many hormones.

The effects of thyroid hormone on a target organ are dependent on the quantity of thyroid hormone secreted by the thyroid. Thyroid secretion is regulated by the serum concentration of the tropic hormone, thyroid-stimulating hormone (TSH), which is secreted by the pituitary thyrotroph cell. The function of the thyrotroph cell is likewise influenced by another tropic hormone, thyrotropin-releasing hormone (TRH). Multilevel regulatory systems for thyroid and other hormones are counterregulated in a negative system by the serum concentration of the hormone. A transient excess of circulating thyroid hormone would, for example, feed back upon the hypothalamus and pituitary thyrotroph to bring about a corrective reduction in levels of TRH and TSH secretion.

In effect, the glands producing the regulatory hormones are also target organs of the primary hormone; hence, every cell in the endocrine system is chemically linked to every other cell within the sphere of influence. Given this complexity of the endocrine system, it is not difficult to understand how the similar endocrine toxicities of relatively diverse compounds may, in fact, represent common manifestations of interventions of the compound at very different points in a hormone's pathways of production, regulation, and action. The effects can be widespread and can be life-threatening if they do not function in synchrony.

Interestingly, chemically induced changes in the endocrine system are not always considered undesirable. Indeed, with what is now a very common type of birth control, synthetic steroids are therapeutically directed to inhibit pituitary gonadotropins, thereby providing a chemical method of birth control for millions of women. Chemical (or drug) suppression of target organ hormone secretion can also be therapeutically useful in such organs as the thyroid gland and the adrenal gland.

HISTORICAL PERSPECTIVE

Claude Bernard first broached the subject of internal secretions as early as 1855 in his research on the pancreas. His concept of a stable *milieu intérieur*, or the internal environment, in spite of the constantly changing exterior environment was critical in the understanding of maintenance of body functions.[12] Ivan Pavlov's early experiments on classical conditioning in dogs provided additional groundwork for the concept of internal control of bodily functions; however, the digestive processes described by Pavlov were thought to be controlled solely by the nervous system. In 1902, William Bayliss and Ernest Starling were able to identify secretin in experiments in which they were able to chemically increase pancreatic secretions in dogs. They were first to use the term *hormone* (from the Greek word *horman*, meaning "to set in motion") to describe secretin as a chemical messenger.[13] Their experiments revealed that secretin could cause changes in physiology at a distant site from the site of entry after being transported through the circulatory system.

In 1915, Walter Cannon revealed the connection between the endocrine glands and emotions such as fear, pain, hunger, and rage.[14] Later that year, Edward Kendall isolated thyroxin, which is the active component of the thyroid and for which he was awarded the Nobel Prize in 1950.[15] Frederick Banting, J.J. McLeod, and Charles Best were awarded the Nobel Prize in 1923 for their work in 1921 on isolating insulin in the control of carbohydrate metabolism.[16] In 1953, Vincent du Vigneaud synthesized oxytocin and other posterior pituitary hormones for which he was awarded the Nobel Prize in 1955. [17–19] In 1959, Rosalyn Yalow and Solomon Berson first published a method to detect minute amounts of hormones in humans with the radioimmunoassay (RIA).[20] They were awarded the Nobel Prize in 1977 for their work on the development and application of RIAs for peptide hormones.[19] Köhler and Milstein published work in 1975[21] on theories concerning the specificity in development and control of the immune system and the discovery of the principle for the production of monoclonal antibodies (mAbs) and were awarded the Nobel Prize for this groundbreaking work in 1984.[19] The nervous and chemical control of bodily functions works

intimately in concert with the immune system to maintain a delicate internal balance that is collectively termed *homeostasis*. Momentous events in scientific history have led to our current understanding of this field. The importance of these findings to mankind cannot be minimized in light of this worldwide recognition of their importance to the health and welfare of humans. A focus of this chapter will be to review testing assays in an effort to understand the chemical control exerted by the endocrine system.

GENERAL PRINCIPLES

The basic foundations of the endocrine system include glands, the hormones they produce, and the receptors at the site of action. The fundamental concept of the endocrine system is that endocrine cells release a hormone that is transported to a receptor site in the target tissue, where the hormone binds and subsequently exerts its biological effect. In a traditional sense, endocrine systems have been portrayed as encompassing those tissues that release a hormone that is transported through the bloodstream to a target tissue, resulting in an effect at some point distant from the original tissue. This has been found to be a simplistic definition, as many variations have been discovered. New techniques in tissue cell culture and molecular biology have permitted the identification of several intercellular signaling pathways that do not export hormones in the traditional way via the general circulation to target tissue; for example, paracrine effects are produced when an effector cell releases a hormone that acts on adjacent target cells to produce a local effect. Many examples of paracrine systems can be found among various growth factors and inflammatory mediators (such as arachadonic acid metabolites, complement factors, cytokines, clotting factors, and somatostatin). Autocrine systems occur when a particular cell type releases a hormone that can act on the same cell to augment a particular response. Examples of autocrine systems include the cytokine interleukin-1 in monocytes and peripheral membrane protein in T-cells.

Several classification schemes have been developed for hormones. One classifies hormones according to their source, another classifies them according to their solubility characteristics in water or fat, and yet another classifies them according to their chemical composition.[22,23] Examining hormones based on their chemical composition also leads to several sets of data. One grouping that is routinely utilized in the endocrine literature divides hormones into one of six general chemical categories (Table 36.1). These include biogenic amine hormones, polypeptide hormones, protein hormones, steroid/sterol hormones, thyroid hormones, and fatty-acid-derived hormones.

Amine hormones include catecholamines (notably, epinephrine, norepinephrine, and dopamine) as well as serotonin and derivatives of tryptophan, tyrosine, or glutamic acid. They are hydrophilic in nature and are related to compounds found in the nervous system that can exert local effects in tissues such as the gastrointestinal tract. Biogenic amines are small, biologically active, modified, single amino acids that are stored in vesicles prior to their release into the circulation. They act primarily as neurotransmitters and are capable of affecting mental functioning. In contrast to most polypeptide hormones, stores of biogenic amines can be quickly restored by rapid synthesis.

TABLE 36.1
Categories of Hormones

Biogenic amines (e.g., epinephrine)
Polypeptides (e.g., thyroid-releasing hormone)
Proteins (e.g., insulin, growth hormone)
Steroids (e.g., estrogens, androgens)
Thyroid hormones (e.g., thyroxine)
Fatty acids (e.g., prostaglandins)

Source: Adapted from Gardner, D.G. and Nissenson, R.A., in *Basic and Clinical Endocrinology*, 7th edn., Greenspan, F.S. and Gardner, D.G., eds., McGraw-Hill, New York, pp. 61–84, 2004; Porterfield, S.P., ed., *Endocrine Physiology*, 2nd edn., Mosby, St. Louis, MO, 2001.

Polypeptide hormones are composed of short amino acid chains; protein hormones are composed of many amino acids and polypeptides together in a single molecule and are generally hydrophilic in nature. These types of hormones can range in length from three amino acids (as in the case of TRH) to several hundred amino acids (e.g., growth hormone [GH]). They can be composed of two or more subunits (e.g., gonadotropins) or may be linked by disulfide bonds (e.g., insulin). These hormones are synthesized in the endoplasmic reticulum and subsequently transferred to the Golgi apparatus for inclusion in secretory vesicles. Other post-translational modifications, such as glycosylation, may occur that can affect biological activity. Following synthesis in an endocrine cell, hormones may be stored in vesicles prior to stimulation of the endocrine system. Patterns of peptide and protein hormone secretion can be either pulsatile (regulated) or basal (constitutive).

Steroid and sterol hormones are derived from cholesterol, contain a cyclopentanoperhydrophenanthrene ring (four ring) structure, and are hydrophobic in nature. Steroid hormones are generally synthesized in the adrenal cortex, placenta, and gonads and are further characterized by being immediately secreted by these glands. Because they are fat soluble, these hormones can readily cross cell membranes and have intracellular receptors. When they have entered the cytoplasm, these steroid hormones bind to a specific receptor (e.g., alpha (α) and beta (β) estrogen receptors [ERs]), a large metalloprotein. Further binding occurs that permits the steroid to enter the nucleus. In the nucleus, the steroid–receptor ligand complex binds to specific DNA sequences and induces transcription of its target genes. Examples of steroid hormones include the sex steroid hormones (e.g., testosterone, estrogen, and progesterone), corticosteroids, mineralocorticoids, vitamin D, and retinoic acid. Recent evidence suggests that megalin, a member of the low-density lipoprotein

receptor superfamily of endocytic proteins, is an important facilitator of steroid entry into cells.[25]

Thyroid hormones are an intermediate-molecular-sized group of compounds that fall in between biogenic amines and short polypeptides. Thyroxine is produced by attaching iodine atoms to the ring structures of tyrosine molecules. Thyroxine contains four iodine atoms. They are composed of two iodinated tyrosine residues, which are converted to triiodothyronine by deiodinases to enhance biological activity.

Fatty acid hormones or eicosanoids are generally derived from polyunsaturated fatty acids such as linoleic acid and phospholipids, with the most prominent of these being arachadonic acid. They are synthesized throughout the body, and their primary effects are paracrine and autocrine in nature through both surface and nuclear receptors.[23] These hormones are effective at minute concentrations and are typically active for 5–10 s. These hormones include prostaglandins, prostacyclin, leukotrienes and thromboxanes, lipoxins, isoprostanoids, and hydroxylated fatty acids.

Transport of hormones from endocrine tissues to target tissues frequently involves carrier plasma proteins (e.g., sex-hormone-binding protein [SHBP]) that bind the hormones with high affinity and specificity while the hormones are transported within the circulatory system. Although many of the carrier proteins possess high specificity and affinity, they often possess low capacity, so the availability of binding sites is limited. Other nonspecific carrier proteins, such as albumin, can bind hormones with a high capacity but possess low specificity and characteristics unlike biogenic amines, steroid, thyroid, and some polypeptide hormones, which have specific binding proteins. Some hormones such as protein hormones are transported free in the blood, while steroid and thyroid hormones are transported bound to plasma proteins.

After a hormone is transported to the target tissue, it must then interact with a receptor within the target tissue to exert a biological effect. An important concept of hormone action is that only the free, unbound hormone can interact with its receptor to exert a biological effect in the particular target tissue; therefore, the hormone must become unbound from any carrier protein before it can interact with a receptor. Hormonal signal transduction occurs when the hormone interacts with the target tissue receptor, which in turn produces an intracellular change in the target tissue. Mechanisms of signal transduction depend on whether the target tissue receptor is bound to the cell membrane surface or is present in the cytoplasm.

To permit the exposure of the required amount of hormone, a delicate equilibrium exists among the bound hormone (*B*), the plasma protein (*P*), and unbound or free hormone (*F*), which is expressed in the following equation[23]:

$$F \times P = \left[B\right] = \frac{\left(\left[F\right] \times \left[P\right]\right)}{\left[B\right]}.$$

Not only is the free hormone the critical moiety for activation, but it is also critical for deactivation in feedback control. Signal transduction by cell-membrane-bound receptors is usually either via intracellular second messengers (e.g., cyclic adenosine monophosphate [cAMP], calcium, or phosphatidylinositol metabolites) or through mechanisms such as phosphorylation of serine, threonine, or tyrosine residues of intracellular kinases and other enzymes.[26] Most polypeptide hormones interact with cell membrane surface receptors, although there is some evidence to suggest that hormonal receptor internalization may play a role in some processes. In contrast, steroid and thyroid hormones interact with intracellular receptors. Transport into the cell may be aided by cell membrane transporters (e.g., megalin), and once inside the cell, the steroid or thyroid hormone receptor complex is transported to the nucleus, where it may modulate gene expression by binding to certain DNA regulatory sequences.

CONTROL OF ENDOCRINE ACTIVITY

Regulation of hormonal activity is critical to all biological systems in their quest for biological homeostasis. Overall, the endocrine system utilizes two major control systems to respond to physiological changes: (1) automatic internal controls from the various concentrations of the hormones themselves and (2) nervous system override of the self-regulating chemical system. The primary function of the nervous system override is to respond to stimuli (i.e., fight or flight situations) and produce a hormonal readjustment. The automatic and continuous self-regulation is a complex physiologic hierarchy of chemically mediated controls that modulates the homeostasis of the endocrine system through a series of feedback loops.

The maintenance of biological systems involves negative feedback loops where the output of a hormone controls the input in a simple inverse relationship. As the concentration of the hormone increases in the system, the amount of input into the system is decreased, in much the same way as a thermostat regulates room temperature. As the temperature increases, the thermostat turns off the heating unit until the temperature drops to a predetermined point, whereupon the signal is sent to the heating unit to increase heat once again. Such would be an example of simple hormonal regulation where the involvement includes a single endocrine gland. Complex hormonal regulation is another level of control involving the anterior pituitary (adenohypophysis) as well as the target receptors. The anterior pituitary is frequently referred to as the *master gland* because of its control over a number of other glands and target cells. In this case, one endocrine gland controls the hormone release of another, which ultimately regulates another distant target. An example of this type of control is the mechanism involving the pituitary gland, the adrenal cortex, and the gonads. The anterior pituitary releases hormones that stimulate the adrenal cortex, which, in turn, releases hormones that effect a change at the gonads. The secretory releases of both the adrenal cortex and the gonads feed back to decrease the hormonal secretion of the anterior pituitary. The overall effect of negative feedback loops is that hormone

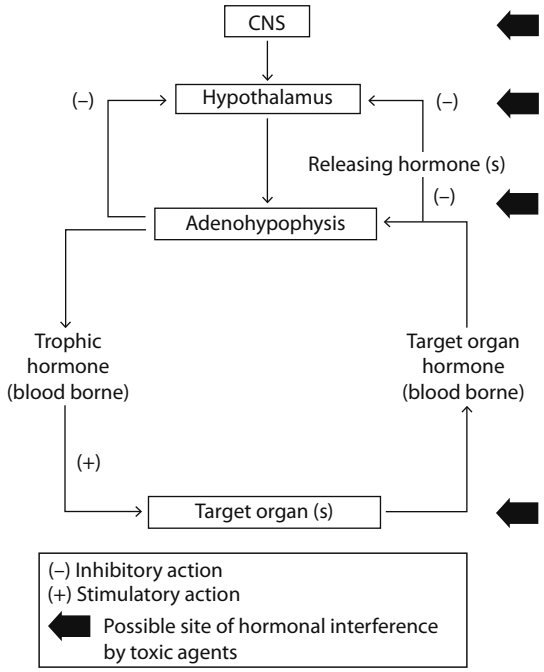

FIGURE 36.1 Relationship between adenohypophyseal–hypothalmic axis and hormone target organs.

levels are maintained within the narrow range the body needs to maintain normal homeostasis. A negative feedback loop inhibits an endocrine pathway, whereas a positive feedback loop enhances or augments an endocrine response (Figure 36.1). Positive feedback relationships, where hormone levels are actually increased by elevating levels of a hormone (i.e., ovulation), are also operants.

The concentration of a hormone at the target cells is generally determined by three factors: (1) the rate of hormone production, (2) the rate of hormone delivery, and (3) the rate of hormone elimination. The rate of hormone production is affected by many factors and is primarily controlled by feedback loops. Feedback loops can be further modulated by other endocrine systems that are pulsatile, cyclical, or stimulated through other mechanisms. The integration of various feedback loops gives rise to a complex cascade of endocrine responses to particular stimuli. The rate of hormone delivery is affected by the rate of blood flow to the target receptor. The rate of elimination is affected by two factors: (1) the body's ability to metabolize and excrete the hormone from the bloodstream and through renal elimination and (2) the physiological rate of degradation of the hormone. Some hormones have short biological half-lives, and others have long biological half-lives. Protein binding can affect a hormone residence time in the blood.

ENDOCRINE DISRUPTION

Several drugs and chemicals are known to affect the endocrine system.[2,27] Perturbation of endocrine homeostasis often produces consequences that can lead to metabolic derangements, developmental abnormalities, and sexual or reproductive dysfunction. The U.S. Environmental Protection Agency (EPA) has defined an endocrine disruptor as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes."[28,29] Endocrine disruption has become a subject of intense scientific research, in large part due to the publication of *Our Stolen Future* in 1996.[30] The book and its findings became sensationalized and politicized, which may or may not have been warranted; however, with the recognition of the effects of estrogen-like environmental contaminants, logically, the concern extends to many types of contaminants that might in some way effect unwanted changes to the endocrine system. Such exposures might result in ill-timed maturation, development, growth, or regulation, resulting in the reduced ability of an organism to cope with external changes or birth defects if the changes occurred during gestation. Indeed, the EPA has indicated that at least four categories of adverse effects might be linked to endocrine disruptors, including cancer, immunological effects, neurological effects, and reproductive and developmental effects involving the adrenals, thyroid, pituitary, and gonads.[31]

Evidence indicates that a number of chemicals can perturb the endocrine systems of animals in a laboratory setting, where one can precisely measure the changes in a controlled environment. There is also substantial evidence that birds,[32] dolphins,[33] alligators,[34] and other wildlife[35–37] exposed to high levels of various environmental contaminants manifest adverse developmental and reproductive effects. Although the logical extension of this concept is that it could produce similar effects in humans, the data are not clear and are scientifically controversial.[28,38]

Endocrine Disruptor Screening Program

Based on this and other evidence, Congress passed the Food Quality Protection Act and the Safe Drinking Water Act (SDWA) Amendments in 1996 requiring that EPA screen pesticide chemicals for their potential to produce effects similar to those produced by the female hormones (estrogen) in humans and giving EPA the authority to screen certain other chemicals and to include other endocrine effects. Based on recommendations from an Advisory Committee, EPA has expanded the Endocrine Disruptor Screening Program (EDSP) to include male hormones (androgens) and the thyroid system and to include effects on fish and wildlife.

An Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was charged with developing "a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect as the administrator may designate."[29,38,39]

The EPA announced the initial list of chemicals to be screened for their potential effects on the endocrine system (or Tier I testing) on April 15, 2009, and the first test orders were issued on October 29, 2009. Test orders are requests for data. Testing will eventually be expanded to cover all pesticide chemicals, as well as substances that may occur in the sources of drinking water to which a substantial population may be exposed. Now that screening is underway, EPA is reviewing test order responses and making available the status or test order responses and/or any decisions regarding testing requirements.

EPA has developed a second list of chemicals for screening and draft policies and procedures that the agency will use to require testing of chemicals for Tier 1 screening. EPA has also developed the EDSP Comprehensive Management Plan[40] that provides strategic guidance for agency personnel and outlines the critical activities that are planned for the EDSP over the next 5 years. The plan describes the technical review processes that will be used in implementing this program and how the agency intends to factor technology advancements into the program. The plan is flexible to allow opportunities to streamline and promote efficient processes. This EDSP Comprehensive Management Plan was developed as an internal EPA document; however, it is currently being made publically available consistent with the EPA's transparency objectives.

Developed in response to recommendations from the Office of Inspector General, "The EDSP Universe of Chemicals and General Validation Principles" document[41] provides an overview of the general validation principles that will be used to evaluate computational toxicological methods for chemical prioritization. EPA has also presented the "List of EDSP Universe of Chemicals"[42] as a separate document, which will be updated as new information becomes available. The List of the EDSP Universe of Chemicals contains approximately 10,000 chemicals as defined under the Federal Food, Drug, and Cosmetic Act (FD&C Act)[43] and SDWA[44] 1996 amendments, although not all of the chemicals are expected to undergo EDSP Tier 1 screening. The agency plans to prioritize these chemicals for screening by considering physical chemical properties, exposure, and an effect-based approach using advanced computational toxicological methods. These methods and tools will be evaluated using Organization for Economic Cooperation and Development (OECD) validation principles for their utilization in chemical prioritization, and this topic will be the focus of the upcoming planned Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Science Advisory Panel review on January 29, 2013.

Series 890—Endocrine Disruptor Screening Program Test Guidelines

The purpose of the EDSP Tier 1 battery of screening assays is to identify chemicals that have the potential to interact with the estrogen, androgen, or thyroid (E, A, or T) hormonal pathways. Currently, the battery consists of 11 assays that have been developed and validated through a collaborative effort involving EPA program and research offices and published as harmonized test guidelines by the Office of Chemical Safety Pollution and Prevention. EPA intends to evaluate the results of the Tier 1 screening assays using a weight-of-evidence (WoE) approach to determine whether or not a chemical has the potential to interact with E, A, or T hormonal pathways and to assess the need for Tier 2 testing.

The purpose of Tier 2 testing is to further characterize the effects on E, A, or T identified through Tier 1 screening by using Tier 2 in vivo studies that establish dose–response relationships for any potential adverse effects for risk assessment. EPA refers to the WoE approach as "a collective evaluation of all pertinent information so that the full impact of biological plausibility and coherence is adequately considered" (U.S. EPA, 1999). In its recommendations to EPA, the EDSTAC referred to the WoE approach as "a process by which trained professionals judge the strengths and weaknesses of a collection of information to render an overall conclusion that may not be evident from consideration of the individual data" (EDSTAC, 1998).

EPA's EDSP uses a tiered approach for determining whether a substance may have an effect in humans that is similar to an effect produced by naturally occurring estrogen, androgen, or thyroid hormones. The core elements of the EDSP are setting priorities for chemicals to be screened and tested: Tier 1 screening, Tier 2 testing, and hazard assessment. The final guidelines are part of a series of test guidelines that have been developed by the Office of Chemical Safety and Pollution Prevention (OCSPP) for use in the testing of pesticides and toxic substances, and the development of test data for submission to the agency. Various factors contributed to selecting the Tier 1 screening that generally included the following:

- The potential of the assays to evaluate E, A, or T hormonal pathway effects in different taxa
- Estrogen- and androgen-mediated effects via receptor binding (agonism and antagonism)
- Estrogen-mediated gene transactivation
- Enzyme inhibition involving the reproductive steroidogenesis pathway
- Interactions with gonadal estrogen and androgen production that may alter feedback mechanisms involving the hypothalamic–pituitary–gonadal (HPG) axis
- Androgen- and estrogen-influenced endpoints within an assay that are complementary among the assays
- Interactions with thyroid hormone production or function and associated alterations in feedback relationships involving the hypothalamic–pituitary–thyroid (HPT) axis

Assays Included in the Tier 1 Screening Battery

The Tier 1 battery's suite of in vitro and in vivo screening assays covers several modes of action (Table 36.2). The Tier 1

TABLE 36.2
Endocrine Disruption Screening Program—Tier 1 Screening Battery

	Modes of Action							
	Receptor Binding				Steroido-genesis		HPG Axis	HPT Axis
Screening Assays	**E**	**Anti-E**	**A**	**Anti-A**	**E**	**A**		
In vitro								
ER binding (890.1250)	●	●						
ERα transcriptional activation (890.1300)	●							
AR binding (890.1150)			●	●				
Steroidogenesis H295R (890.1550)					●	●		
Aromatase recombinant (890.1200)					●			
In vivo								
Uterotrophic (890.1600)	●							
Hershberger (890.1400)			●	●		●		
Pubertal male (890.1500)			●	●		●	●	●
Pubertal female (890.1450)	●	●			●		●	●
Amphibian metamorphosis (890.1100)								●
Fish short-term repro (890.1350)	●	●	●	●	●	●	●	●

battery's suite of in vitro and in vivo screening assays (Table 36.2) includes the following:

In vitro

OPPTS 890.1150 Androgen receptor (AR) binding—rat prostate cytosol
OPPTS 890.1200 Aromatase—human recombinant microsomes
OPPTS 890.1250 ER binding—rat uterine cytosol
OPPTS 890.1300 ER—(hERα) transcriptional activation—human cell line (HeLa-9903)
OPPTS 890.1550 Steroidogenesis—human cell line (H295R)

OPPTS 890.1150 Androgen Receptor Binding (Rat Prostate Cytosol)

Purpose: The AR binding assay is a sensitive in vitro test to detect chemicals that may affect the endocrine system by binding to the AR isolated from the rat prostate. It will give added confidence that positive results seen in the Hershberger assay are truly due to an AR binding mechanism.

Design: Cytosol isolated from the rat prostate provides the source of the AR. Test chemical and R1881, a strong ligand, compete for binding with the AR when incubated together overnight. The assay measures the binding of [^{3}H]-R1881 in the presence of eight test chemical concentrations. Unlabeled R1881 serves as a strong positive control producing the standard curve. Dexamethosone is run with each block of test chemicals as a weak positive control. Solvent is run as the negative control.

Endpoints: The disintegrations per minute (DPM) of [^{3}H]-R1881 are measured by liquid scintillation counter. Data for the standard curve and each test chemical will be plotted as the percent [^{3}H] R1881 bound versus the molar concentration through the use of a four-parameter nonlinear regression program.

Interpretation: Performance criteria have been set for the top, bottom, and slope for R1881 and the weak positive, dexamethasone.

- If the binding curve crosses 50% (competes to displace the standard ligand by 50%), the test chemical is considered to be a binder.
- Chemicals for which the binding curve crosses 75%, but not 50%, are considered to be equivocal.
- Chemicals that do not fit the model or for which the binding curve does not cross 75% are considered to be nonbinders.

Strengths:

- Sensitive, rapid, and inexpensive
- Uses relatively few animals
- Specific for identifying an interaction with the AR receptor providing mechanistic information

Weaknesses:

- It cannot distinguish the consequences of binding (*i.e., functional response*).
- It does not account for potential metabolic activation or deactivation of test chemical.
- Other steroids that are not natural ligands for the AR may bind at high concentrations.
- Chemicals that denature the receptor may be identified as false positives.

OPPTS 890.1200 Aromatase (Human Recombinant)

Purpose: The aromatase assay detects chemicals that inhibit aromatase activity. Aromatase is the enzyme that metabolizes androgens such as testosterone to estrogens.

Design: Androstenedione (AND) and [1β-^{3}H]-androstenedione (ASDN) serve as substrate for human recombinant microsomal aromatase. Full activity control (ASDN in medium, no inhibitor), background activity control (no nicotinamide adenine dinucleotide phosphate [NADPH]), positive control (4-hydroxyandrostenedione at eight concentrations), and test chemical (eight concentrations) are run in the reaction for 15 min, and the reaction products produced are measured and plotted as percent enzyme activity (inhibition curve) through the use of a nonlinear regression program.

Endpoints: The formation of ^3H_2O, one of the coreaction products along with estrone, is measured by liquid scintillation counter per unit reaction time.

Interpretation: Chemicals that reduce enzyme activity levels by 50% or more (as determined by the inhibition curve calculated by a four-parameter nonlinear regression program) are considered to be inhibitors of aromatase. Chemicals that fit the inhibition curve but allow 50%–75% activity, that is, reduce activity by 25%–50%, are considered equivocal. Chemicals that do not fit the model or that fit the model but reduce inhibition by 25% are considered to be noninhibitors of aromatase.

Strengths:

- Highly specific to inhibition of aromatase activity providing mechanistic information
- More sensitive than typical in vivo assays
- Rapid and inexpensive
- Capable of high throughput (HTP)
- Provides useful information for the interpretation of in vivo assays

Weaknesses:

- Cannot detect chemicals that induce aromatase activity.
- False positives could result from chemicals that denature the enzyme.
- Limited/no ability to metabolize xenobiotics.

OPPTS 890.1250 Estrogen Receptor Binding Assay Using Rat Uterine Cytosol

Purpose: The purpose of the ER binding assay is to identify test chemicals that can bind to the ER isolated from the rat uterus.

Design: A saturation radioligand binding experiment is conducted to demonstrate that the ER binding assay is working under optimal conditions within a given laboratory. This assay is conducted by measuring the equilibrium binding of increasing concentrations of ^{3}H-estradiol to rat cytosolic or human recombinant ERα. Nonlinear regression analysis of the data provides estimates of the affinity of the receptor for 17β-estradiol (K_d) and the concentration of receptors (B_{max}).

A competitive ER binding assay is conducted by measuring the equilibrium binding of a single concentration of ^{3}H-17β-estradiol at various concentrations (over a range of at least six orders of magnitude) of a test chemical in rat cytosolic or human recombinant ER. After equilibration, the amount of radioactivity bound to the ER is measured as an indicator of how much was displaced by the test compound at each concentration. Data analysis provides an estimate of the potency of the test chemical for binding to the ER relative to 17β-estradiol. 17β-estradiol is run as a reference standard with each run, as are a weak positive and a nonbinder.

In each portion of the study, three replicate data points are collected at each concentration in one run, and three independent runs are performed to constitute one assay.

Endpoint: Binding curve fit to a four-parameter Hill equation, where the parameters are top, bottom, slope, and log(IC_{50}) (i.e., base-10 log of the molar concentration of test chemical that inhibits 50% of binding by the radioligand).

Interpretation: Performance criteria have been set for the top, bottom, and slope for 17β-estradiol and the weak positive, norethynodrel, for the competitive ER binding portion of the assay. Within-run variability is also subject to a performance criterion.

Classification of test chemicals:

- *Positive:* A log(IC_{50}) value can be obtained from an unconstrained curve fitted to the Hill equation that has a slope of approximately −1.0.
- *Equivocal:* Acceptable binding curve reaches 25% displacement of radioligand but not 50% at the highest concentration. Also applied if slope is unusually steep or shallow.
- *Negative:* Acceptable binding curve does not reach 25% displacement of the radioligand, or curve cannot be fit and no data point shows displacement of more than 25%.

Strengths:

- Quick (2 days)
- Uses relatively few animals
- Specific for identifying an interaction with ER (i.e., provides mechanism-related information)

Weaknesses:

- It cannot distinguish the consequences of binding (i.e., functional response).
- Does not account for potential metabolic activation or deactivation of test chemical.

OPPTS 890.1300 Estrogen Receptor Transcriptional Activation (Human Cell Line [HeLa-9903])

Purpose: In vitro transcriptional activation (TA) assays are based upon the production of a reporter gene product induced by a chemical, following the binding of the chemical to a specific receptor and subsequent downstream TA. TA assays using activation of reporter genes are screening assays that have long been used to evaluate the specific gene expression

regulated by specific nuclear receptors, such as the ERs.[45–48] They have been proposed for the detection of estrogenic transactivation regulated by the ER.[47,49,50] The nuclear ERs exist as at least two subtypes, termed α and β encoded by distinct genes and with different tissue distribution, relative ligand binding affinities, and biological functions. Nuclear ERα mediates the classic estrogenic response; therefore, models currently being developed to measure ER activation mainly relate to ERα. The aim of this TA assay is to evaluate the ability of a chemical to function as an ERα ligand and activate an agonist response, for screening and prioritization purposes, but can also provide mechanistic information that can be used in a WoE approach.

Initial considerations and limitations: Estrogen agonists act as ligands for ERs and may activate the transcription of estrogen responsive genes. This interaction may have the potential to trigger adverse health effects by disrupting estrogen-regulated systems. This Test Guideline describes an assay that evaluates TA mediated by the hERα. This process is considered to be one of the key mechanisms of possible endocrine disruption–related health hazards, although there are also other important endocrine disruption mechanisms. These include the following:

- Actions mediated via other nuclear receptors linked to the endocrine system and interactions with steroidogenic enzymes
- Metabolic activation or deactivation of hormones
- Distribution of hormones to target tissues
- Clearance of hormones from the body

This Test Guideline exclusively addresses TA of an estrogen-regulated reporter gene by agonist binding to the hERα, and therefore, it should not be directly extrapolated to the complex in vivo situation of estrogen regulation of cellular processes. Furthermore, this Test Guideline does not address antagonist interaction with the hERβ and subsequent effect on transcription.

This test method is specifically designed to detect hERα-mediated TA by measuring chemiluminescence as the endpoint. However, nonreceptor-mediated luminescence signals have been reported at phytoestrogen concentrations higher than 1 μM due to the overactivation of the luciferase reporter gene.[51,52] While the dose–response curve indicates that true activation of the ER system occurs at lower concentrations, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like overactivation of the luciferase reporter gene needs to be examined carefully in stably transfected ER TA assay systems.

Principle of the test: The TA assay using a reporter gene technique is an in vitro tool that provides mechanistic data. The assay is used to signal binding of the ER with a ligand. Following ligand binding, the receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer. Luciferase activity can be evaluated quickly and inexpensively with a number of commercially available test kits.

The test system provided in this guideline utilizes the hERα-HeLa-9903 cell line, which is derived from a human cervical tumor, with two stably inserted constructs:

- The hERα expression construct (encoding the full-length human receptor)
- A firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin estrogen-responsive element (ERE) driven by a mouse metallothionein (MT) promoter TATA element

The mouse MT TATA gene construct has been shown to have the best performance, and so is commonly used. Consequently, this hERα-HeLa-9903 cell line can measure the ability of a test chemical to induce hERα-mediated transactivation of luciferase gene expression. Investigators must use the stably transfected hERα-HeLa-9903 cell line for the assay. The cell line can be obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank.[53] Investigators must use only cells characterized as mycoplasma-free in testing. Real-time polymerase chain reaction (RT PCR) is the method of choice for a sensitive detection of mycoplasm infection.[54–56]

Data interpretation: Based upon whether the maximum response level induced by a test chemical equals or exceeds an agonist response equal to 10% of that induced by a maximally inducing (1 nM) concentration of the positive control (PC) 17β-estradiol (E2) (i.e., the PC_{10}).

Strengths:

- Specific for identifying an interaction with the ER receptor providing mechanistic information
- Sensitive, rapid, and inexpensive
- Confirms a functional response (TA) of ER activation

Weaknesses:

- Does not account for potential metabolic activation or deactivation of test chemical

OPPTS 890.1550 Steroidogenesis (Human Cell Line—H295R)

Purpose: The H295R steroidogenesis assay is intended to identify xenobiotics that affect the steroidogenic pathway beginning with the sequence of reactions occurring after the gonatotropin hormone receptors (follicle-stimulating hormone receptor [FSHR] and leutinizing hormone receptor [LHR]) through the production of testosterone and estradiol/estrone. The steroidogenic assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamus or pituitary gland.

Principle of the test: H295R cells (ATCC CLR-2128) are plated in a 24-well plate setup and are exposed to test

chemicals. The guideline provides detailed information regarding the handling of samples including the extraction of hormones from medium, storage of samples and extracts, and references having detailed standard operating procedures (SOPs) or protocols for the analysis of testosterone (T) and 17 β-estradiol (E2). The protocol also describes the procedure for the Live/Death® cytotoxicity test to evaluate any possible effects of the test chemicals on cell viability.

The Guideline also provides a complete list of required reagents and solvents and describes the preparation of all solutions and reagents used during the exposure experiments with the cells as well as during the subsequent extraction, hormone analyses, and cell viability measurements. It also provides descriptions of quality assurance/quality control (QA/QC) procedures that must be performed prior to initiating chemical testing and the analysis of positive controls with each run to verify proper performance of the cells. Cell culturing procedures (including initiating cell cultures from frozen stock, cultivation and splitting of the growing cells, the freezing of cells for storage in liquid nitrogen, and the maintenance of a proper cell culture diagram to track the progress of a cell line) are also described.

Performance of hormone measurement system: Each laboratory may use a hormone measurement system of its choice for the analysis of the production of T and E2 by H295R cells. Prior to the initiation of cell culture and any subsequent test runs, it is expected that each laboratory demonstrates the conformance of its hormone measurement system (e.g., enzyme-linked immunosorbent assay [ELISA], RIA, liquid chromatography–mass spectrometry [LC–MS]) with the QC criteria by analyzing supplemented medium spiked with an internal hormone control. Due to the cross-reactivity of some of the antibody-based hormone ELISAs and RIAs with hormone metabolites/conjugates produced by the H295R cells, an extraction of the medium is required prior to the measurement of hormones if an assay is used that employs antibodies.

Chemical hormone assay interference test: If antibody-based hormone measurement assays are to be used, prior to initiation of testing, it is recommended that each chemical be tested for potential interference with the hormone measurement system being utilized. It has been previously shown that some chemicals can interfere with antibody-based assays such as ELISAs and RIAs.[57] This *chemical interference test* will be conducted as described for the analysis of medium samples within the Guideline (Subsection (h)(5)).

Test results: The following results must provide raw hormone concentration data for each well for control and test substances (each replicate measure in the form of the original data provided by the instrument utilized to measure hormone production [e.g., optical density (OD), fluorescence units, counts per minute (CPM), and so on]); validation of normality or explanation of data transformation; and mean responses ±1 SD for each well measured.

Interpretation: A chemical is judged to be positive if the fold induction is statistically different from the solvent control at doses that fall within the increasing or decreasing portion of the dose–response curve. Statistically significant increases in fold induction indicate the chemical is an inducer of one or more enzymes in the steroid synthesis pathway. Statistically significant decreases in fold induction indicate the chemical is an inhibitor of one or more enzymes in the steroid synthesis pathway. Statistically significant differences at concentrations that do not follow a dose–response curve may be due to random effects; such results are considered to be equivocal. Results exceeding the limits of solubility or at cytotoxic concentrations are not included in interpreting results.

Strengths:

- Only in vitro assay that can evaluate effects on the entire steroidogenesis pathway—cells have all of the enzymes necessary for steroidogenesis
- Rapid and inexpensive
- Detects chemicals that inhibit and induce steroidogenesis
- Can effectively distinguish strong, moderate, and weak inducers and inhibitors

Weaknesses:

- Limited account for potential metabolic activation or deactivation of test chemical

In vivo

OPPTS 890.1100 Amphibian metamorphosis (frog)
OPPTS 890.1350 Fish short-term reproduction
OPPTS 890.1400 Hershberger (rat)
OPPTS 890.1450 Pubertal female (rat)
OPPTS 890.1500 Pubertal male (rat)
OPPTS 890.1600 Uterotrophic (rat)

OPPTS 890.1100 Amphibian Metamorphosis (Frog) [AMA]
Purpose: The amphibian metamorphosis assay (AMA) is a screening assay intended to empirically identify substances that may interfere with the normal function of the HPT axis. The AMA represents a generalized vertebrate model to the extent that it is based on the conserved structure and functions of thyroid systems. It is not intended to quantify or confirm endocrine disruption, or to provide a quantitative assessment of risk, but only to provide evidence that thyroid-regulated processes may be sufficiently perturbed to warrant more definitive testing.

Design: The general experimental design entails exposing *Xenopus laevis* tadpoles at NF stage 51 to a minimum of three different aqueous concentrations of a test chemical and a dilution water control for 21 days. There are four replicate tanks at each test substance concentration or treatment. Larval density at test initiation is 20 tadpoles per test tank for all treatment groups.

Endpoints: Daily mortality.

Morphological endpoints: Whole-body length/snout–vent length (d 7 and 21); hind limb length (d 7 and 21); wet weight (d 7 and 21); developmental stage (d 7 and 21); histology thyroid gland (d 21).

Interpretation: Results are evaluated for evidence of interaction of the test chemical with the HPT axis as follows.

If necessary, test concentrations with overt toxicities are removed from the data set. Significant histological findings in thyroid tissue deem the assay positive. If no thyroid gland histopathology is observed, then developmental landmarks are evaluated. If development is accelerated or asynchronous, the test is deemed positive. The assay is considered negative if no effects are detected in thyroid gland histology or morphological landmarks of development.

Strengths:

- Intact in vivo system on an animal undergoing morphological development.
- Effects on metamorphic development are relatively specific indicators of HPT axis perturbation.
- HPT axis is conserved for other vertebrate classes.
- Redundant endpoints, maximizing chance for detection while minimizing false negatives.
- Provides toxicological data in a taxon (amphibians) underrepresented.
- Well-established relationship between endpoints and endocrine system.
- Endpoints easy to measure.

Weaknesses:

- Inherent difficulties in testing some substances not amenable to aquatic systems.
- Sensitivity of the assay has not been fully characterized.
- Nonthyroidal toxicities have the potential to affect some of the morphological endpoints of the assay.

OPPTS 890.1350 Fish Short-Term Reproduction

Purpose: The fish short-term reproduction assay (FSTRA) is a screening assay intended to identify changes in morphology, histopathology, spawning, and specific biochemical endpoints, which may reflect interference with the normal function of the HPG axis. It is not intended to quantify or confirm endocrine disruption, or to provide a quantitative assessment of risk, but rather to provide suggestive evidence that endocrine-regulated processes may be sufficiently perturbed to warrant more definitive testing.

Design: The FSTRA entails exposing reproductively mature fathead minnows (*Pimephales promelas*) to a minimum of three concentrations of a test chemical and appropriate control(s) for 21 days. Successful spawning is established during a preexposure period of at least 14 days. Each of the four replicate tanks in each treatment level contains four females and two males.

Endpoints (= key endpoints):* Survival; behavior; body length; body weight; fecundity* (# of spawns; # of eggs/female reproductive day); fertilization success* (# fertile eggs/female reproductive day; % fertile eggs); gonadal histopathology*; gonadosomatic index (GSI)*; appearance and secondary sex characteristics* (overall body coloration, vertical banding; fatpad [weight, score, and index]; tubercles [count and score]; ovipositor size).

Biochemical measures:* Vitellogenin; estradiol; testosterone.

Interpretation: The FSTRA as presented is intended to serve in a screening capacity to provide an indication of potential endocrine activity, not to confirm any specific mechanism, mode of action, or adverse effect. Therefore, a significant effect in one or more of the key endpoints of this assay (fecundity, fertilization success, histopathology, GSI, biochemical measures, and secondary sex characteristics) should be considered indicative of possible endocrine system disturbance. The suite of endpoints included is necessary to provide a fully comprehensive assessment of the disrupting potential to the HPG axis in a representative fish.

It is important to note, however, that if a given exposure level results in substantial mortality or other overt signs of toxicity, responses in other endpoints may be due to general toxicity, not necessarily mediated primarily via interaction with the endocrine system. The lower treatment level(s) should be examined for effects outside of the range of general toxicity. If all test concentrations exhibit mortality, then the assay would need repeating before an inference on possible endocrine activity can be made.

It is recognized that some endpoints may be responsive to nonendocrine stresses in addition to endocrine-mediated pathways, particularly fecundity. Although reductions in fecundity indicate adverse organismal and, potentially, population level effects (i.e., reproductive toxicity), these cannot be definitively distinguished from direct endocrine-mediated effects by this assay when changes in other core endpoints are not present. Nevertheless, reductions in fecundity are considered a positive effect in this assay because they may be endocrine-mediated and should be considered in concert with results of the other assays in the Tier 1 battery. Results that would be considered equivocal for this single assay should be considered indications of potential endocrine activity and evaluated in light of the WoE from the other assays in the Tier 1 battery of assays for the EDSP.

Strengths:

- Incorporates a standard, easily acquired laboratory model species
- Straightforward, cost-effective, reasonably short-term assay
- Detects (anti-)estrogen and (anti-)androgen perturbations in addition to disruptors of the entire HPG axis
- Employs an intact HPG axis and hence is relevant to other taxa when conserved elements of the HPG axis are considered

Weaknesses:

- Inherent technical difficulties in testing substances that are poorly soluble in water in aquatic systems, and methods for delivering such substances to the test system.
- Some measurements (e.g., plasma steroids) will require specialized technical expertise.

OPPTS 890.1400 Hershberger (Rat)

Purpose: A short-term in vivo assay to detect androgenic or antiandrogenic chemicals or chemicals that inhibit 5α-reductase, based on the changes in the weight of five androgen-dependent tissues in the castrated male rat.

Design: There are two versions of the Hershberger assay: an immature version and a peripubertal version employing castrated rats. In addition, each version can be run to detect AR agonists or antagonists. When screening for potential androgenic activity, the test substance is administered daily by oral gavage or subcutaneous injection for a period of 10 consecutive days. Test substances are administered to a minimum of two treatment groups of experimental animals using one dose level per group. When screening for potential antiandrogenic activity, the test substance is administered daily by oral gavage or subcutaneous injection for a period of 10 consecutive days in concert with daily TP doses (0.2 or 0.4 mg/kg/day) by subcutaneous injection. Graduated test substance doses are administered to a minimum of two treatment groups of experimental animals using one dose level per group. In both the agonist and antagonist procedures, the animals are necropsied approximately 24 h after the last dose.

Endpoints: The assay is based on statistically significant changes in weight in androgen-dependent tissues. The five tissues weighed in the castrated male are ventral prostate, seminal vesicle (plus fluids and coagulating glands), levator ani-bulbocavernosus muscle, paired Cowper's glands, and the glans penis (GP). In the immature version, the GP cannot be detached and measured, but the testes and epidimydes are weighed in the intact weanling.

Interpretation: A positive result is a statistically significant change in the weight of two of the tissues.

Strengths:

- Relatively rapid screen that is quite specific to androgenic effects.
- In vivo procedure incorporates metabolism; thus, it can detect chemicals that need activation.
- Oral administration will model a primary exposure route and incorporates ADME.

Weakness:

- The growth response of the individual androgen-dependent tissues is not entirely of androgenic origin, that is, compounds other than androgen agonists can alter the weight of certain tissues. However, the growth response of several tissues concomitantly substantiates a more androgen-specific mechanism.

OPPTS 890.1450 Female Pubertal (Rat)

Purpose: This assay is capable of detecting chemicals with estrogenic/anti estrogenic activity, or agents which alter pubertal development via changes in steroidogenesis, or hypothalamic–pituitary regulation of the ovary and thyroid homeostasis (Table 36.3).

Design: Test chemical is administered daily by gavage from post natal day (PND) 22 to PND 42 (21 days) to 15 females per dose. Two doses plus vehicle control are employed. The animals are weighed daily, and examined for vaginal opening from PND 22 until opening is complete. After vaginal opening, vaginal smears are taken daily. Additional measures are taken at necropsy.

Endpoints: Growth (daily body weight); Age and weight at vaginal opening; Organ weights [Uterus (blotted), Ovaries (paired), Thyroid, Liver, Kidneys (paired), Pituitary, Adrenals (paired)]; Histology [Uterus, Ovary, Thyroid (colloid area and follicular cell height), Kidney]; Blood Chemistry (standard panel); Hormones [Serum or plasma thyroxine (T_4), total serum or plasma TSH]: Estrus cyclicity; Age at first estrus after vaginal opening; Length of cycle; Percent of animals cycling; Percent of animals cycling regularly.

Interpretation: Results are evaluated for evidence of interaction of the test chemical with the endocrine system, primarily estrogen- and thyroid-related. Body weight, organ weight, and hormone values for the control animals are subject to performance criteria for mean and coefficient of variation. Thyroid endpoints are generally interpreted separately from the sex-hormone-related endpoints.

TABLE 36.3
Potential Changes Indicative of Different Modes of Action in Female Pubertal Assay

Estrogen Agonist	Steroidogenesis Inhibition	Disruption of HPT Axis	Thyrotoxicants
Early VO, pseudoprecocious puberty	Delayed VO	Alterations in VO	↓ T_4
↓ BW at VO	Delayed first estrus	Alterations in cyclicity	Alterations in TSH
Early first estrus	Persistent diestrus	Altered ovarian, uterine, or pituitary wts	Changes in thyroid histology
Altered organ histology	↓ Uterine wt	Altered organ histology	Changes in thyroid wt
Persistent estrus	Altered organ histology		Changes in liver wt/enzyme profile
↓ Ovarian wt			
↑ Uterine wt			

Notes: Changes in hypothalamic–pituitary function may advance or delay puberty, modify ovarian cycling by inducing early cycles, alter the regular cycles, and alter tissue weight depending on whether the agent activates or inhibits pubertal development.

Strengths:

- Intact mammalian in vivo system, and thus addresses ADME concerns
- Apical assay covering several modes of interaction, including ones not covered by other assays in battery
- Redundant endpoints, maximizing chance for detection while minimizing false negatives
- Covers pubertal period of development
- Well-established relationship between endpoints and endocrine system

Weakness:

- Protocol is not as diagnostic for specific MOAs as other assays in the battery such as uterotrophic for ER agonist.

OPPTS 890.1500 Male Pubertal (Rat)

Purpose: Provide information obtained from an in vivo mammalian system that is useful in determining the potential of chemicals or mixtures to interact with the endocrine system. Detect chemicals with antithyroid, androgenic, or antiandrogenic [AR or steroid-enzyme-mediated] activity or agents which alter pubertal development via changes in gonadotropins, prolactin, or hypothalamic function (Table 36.4).

Design: Test chemical is administered daily by gavage from PND 23 to PND 53 (31 days) to 15 males per dose level at two dose levels plus vehicle control. The animals are weighed daily, and examined for preputial separation from PND 30 until separation is complete. The other measurements are taken at necropsy.

Endpoints: Growth (daily body weight); Age and weight at preputial separation; Organ weights (seminal vesicle plus coagulating gland, ventral prostate, dorsolateral prostate, levator ani plus bulbocavernosus muscle complex, epididymis, testis, thyroid, liver, kidney, adrenal, pituitary); Blood Chemistry (standard panel); Hormone levels (serum testosterone, total serum thyroxine, total serum thyroid stimulating hormone); and Histology (epididymis, testis, thyroid, kidney.

Interpretation: Results are evaluated for evidence of interaction of the test chemical with the endocrine system, primarily androgen- and thyroid-related. Body weight, organ weight, and hormone values for the control animals are subject to performance criteria for mean and coefficient of variation. Thyroid endpoints are generally interpreted separately from the androgen-related endpoints.

Strengths:

- Intact mammalian in vivo system and thus addresses ADME concerns
- Apical assay covering several modes of interaction, including ones not covered elsewhere
- Redundant endpoints, maximizing chance for detection while minimizing false negatives
- Covers pubertal period of development
- Well-established relationship between endpoints and endocrine system
- Endpoints easy to measure

Weaknesses:

- Variability of hormone measurements, particularly testosterone
- Relatively long duration

OPPTS 890.1600 Uterotrophic (Rat)

Purpose: To detect estrogenic chemicals through a simple in vivo assay based on uterotrophic response (increase in uterine weight).

Design: There are two versions of the uterotrophic assay an immature version and an ovariectomized adult version. In both versions, two concentrations of test substance are administered orally or sc to ovariectomized or immature female rats for a minimum of 3 consecutive days. Estrogenic substances cause a uterotrophic response that is due to the imbibition of water and the growth of cells.

Endpoints: Uterine weight is measured and compared with controls.

TABLE 36.4
Potential Changes Indicative of Different Modes of Action in Male Pubertal Assay

Androgen Antagonist	Steroidogenesis Inhibitor or HPG Suppression	Hypothyroidism
↑ Age at puberty	↑ Age at puberty	↓ T_4
↓ Wts ventral prostate, seminal vesicles, LABC, epididymis	↓ Wts ventral prostate, seminal vesicles, LABC, epididymis	↑ TSH
↑ Testosterone	↓ Testosterone or no effect	↑ Follicular cell height
		↓ Colloid area
		↑ Liver wt for agents that induce hepatic clearance of thyroxine or no effect

Notes: Results are evaluated by weight-of-evidence. Redundancy of endpoints must be incorporated into evaluation. Thyroid endpoints are generally interpreted separately.

Interpretation: A statistical increase in uterine weight compared with controls is a positive result.

Strengths:

- Relatively rapid screen that is quite specific to estrogenic effects.
- In vivo procedure incorporates metabolism; thus, it can detect chemicals that need activation.

Weakness:

- Uterotrophic response is not due exclusively to estrogenic chemicals, so a uterotrophic response should be confirmed by corroborating information such as ER binding or TA.

Tier 2 Testing

The three goals of Tier 2 assays include the following:

1. Determining whether a substance may cause endocrine-mediated effects through or involving estrogen, androgen, or thyroid hormone systems
2. Determining the consequences to the organism of the activities observed in Tier 1
3. Establishing the relationship between doses of an endocrine-active substance administered in the test and the effects observed

The Tier 2 tests are longer in duration than Tier 1 tests, are designed to encompass critical life stages and processes as well as a broad range of doses, and are intended to be administrated by a relevant route of exposure. Effects associated with endocrine disruption may not be expressed until later in the test subject's life or may not appear until the reproductive period is reached. Therefore, Tier 2 tests usually encompass two generations and include effects on fertility and mating, embryonic development, sensitive neonatal growth and development, and transformation from the juvenile life stage to sexual maturity. The two-generation reproduction study or the extended one-generation reproduction test (refer to Chapter 34 for a discussion of these study designs) is the mammalian multigeneration test that will enable EPA to obtain a more comprehensive profile of the biological consequences of a chemical exposure and identify the dose or exposure that caused the consequences. The proposed Tier 2 nonmammalian ecotoxicology tests include Amphibian 2-Generation, Avian 2-Generation, Fish Life cycle, and the Invertebrate (Mysid) Life cycle. The validation of these tests is underway. Discussions of the endocrine systems of these species, study designs, endpoints, and validation methods are contained in the respective Draft Detailed Review Papers located on the EDSP website[58–60] and will not be discussed further in this chapter.

Hazard Assessment

According to the EPA, Tier 1 screening and Tier 2 testing data collected as part of EDSP will help to identify and characterize the hazard (the potential to cause harm). When EPA integrates and interprets all of the endocrine disruptor–related hazard data in consideration with other available hazard information, the EPA will perform a hazard assessment and then conduct an exposure assessment by looking at the amount of chemical to which wildlife or humans are likely to be exposed. The final step in the process is the risk assessment, through which EPA integrates the information about the potential harm of a chemical with the likelihood that someone or something will be exposed. Based on scientifically sound risk assessment, the agency can make risk management decisions regulating the chemical(s). For more information about how EPA conducts health risk assessments, read EPA's Office of Pesticide Programs' fact sheet entitled "Assessing Health Risks from Pesticides."[61]

WoE: A WoE approach is an interpretive process that considers all scientifically relevant information in an integrative analysis.[62,63] This process takes into account various kinds of available evidence, quality and quantity of that evidence, and strengths and limitations associated with each type of evidence and explains how the various types of evidence fit together to support a conclusion. EPA's Science Policy Council[64] recommended the use of five general assessment factors: (1) soundness, (2) applicability and utility, (3) clarity and completeness, (4) uncertainty and variability, and (5) evaluation and review.

Soundness: Scientific and technical procedures, measures, methods, or models employed to generate the information are reasonable for, and consistent with, the intended purpose. The following should be considered: (1) adequacy of the test methods to detect the effect of interest; (2) conduct of studies according to the scientific method of hypothesis development and testing through observation, experimentation, and verification; (3) ability to distinguish between a specific versus a nonspecific outcome according to the intended purpose of the study; and (4) interpretation of results and conclusions that are statistically significant, biologically plausible, and consistent with the data.

Applicability and utility: The information is relevant for the agency's intended use. The following should be considered: (1) appropriateness of test materials and methods, study design, and endpoints based on rationale, objectives, and hypotheses related to the intended purpose of the study; (2) evidence of competence in collection, analysis, presentation, and interpretation of data and conclusions; and (3) reliability of information from traditional as well as new methodologies.

Clarity and completeness: The degree of clarity and completeness with which the data, assumptions, methods, QA, sponsoring organizations, and analyses employed to generate the information is documented. The following should be considered: (1) transparency of authors, coauthors, contributors, and acknowledgement of respective institutions or organizations as well as sponsors; (2) background information or rationale, study objectives, hypotheses that are being tested, and experimental design, including controls and the number of observations/groups related to the intended purpose of the study; (3) degree of standardization or scientifically valid methodology that supports repeatability with accuracy and precision; (4) availability of raw data; (5) statistical analysis

approach; and (6) interpretation of statistical significance, plausibility of biological outcomes, and scientifically sound conclusions.

Uncertainty and variability: The uncertainty and variability (quantitative and qualitative) in the information or the procedures, measures, methods, or models are evaluated and characterized. The following should be considered: (1) citation of references pertaining to the specificity and sensitivity of test methods or models, experimental designs, or endpoints; (2) evidence of reproducibility or repeatability of the QA; (3) performance criteria and QC or assurance measures that may include historical or reference control information, coefficients of variation, good laboratory practice (GLP) compliance, or independent peer review; and (4) the number of animals or observations/groups and statistical analysis approach to sufficiently and adequately detect differences between or among groups.

Evaluation and review: The information or the procedures, measures, methods, or models are independently verified, validated, and peer-reviewed. The following should be considered: (1) explanation or reference of the process for verification or validation to evaluate relevance and reliability of test methods and endpoints as specific and sensitive units of measure; (2) general acceptance of the method in the peer-reviewed literature; (3) availability of validation results; and (4) availability of performance or evaluation criteria.

Future EDSP Plans

In 2010 and 2011, EPA's Office of the Inspector General (OIG) evaluated the EDSP and concluded that, without a better defined universe of chemicals, the agency will not be able to estimate longer-term resource needs for completion of milestones for the program. Therefore, the OIG recommended that the agency first define and identify the universe of chemicals for EDSP screening and testing. In response, the agency has decided to incorporate a discussion of the universe of chemicals into both the EDSP21 Work Plan[65] and the EDSP Comprehensive Management Plan.[40] Both documents are intended to provide primary guidance regarding the strategic direction and management of the EDSP for a period of at least 5 years. The EDSP21 Work Plan describes an approach for using computational or in silico models and molecular-based in vitro HTP assays to prioritize and screen chemicals to determine their potential to interact with the estrogen, androgen, or thyroid (E, A, or T) hormonal systems. There are two important drivers for this work plan. First, EPA is statutorily required to complete a first round of registration review of previously registered pesticides by October 1, 2022. Each review takes about 5–6 years, and currently, the agency initiates the review of approximately 70–80 active ingredients each year. So far, only a small percentage of the pesticide-active ingredients under review have received test orders for screening under the EDSP. Also, Tier 1 screening has yet to begin for a large number of active ingredients. Second, the president's proposed fiscal year 2012 budget for the agency states as follows: "In FY 2012, EPA will begin a multiyear transition from the EDSP to validate and more efficiently use computational toxicology methods and high throughput screens that will allow the agency to more quickly and cost-effectively assess potential chemical toxicity" (President's Budget FY2012).

OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals

The OECD-proposed approach is more complex in terms of the number of levels employed. However, in the OECD scheme, it is possible to enter and exit at any test level depending on the outcome of evaluations. The OECD "Guidance Document on the Assessment of Chemicals for Endocrine Disruption"[66] describes the testing guidelines used in the endocrine disruptor framework. The OECD conceptual framework can be summarized as follows:

Level 1 (sorting and prioritization) is based upon existing information such as the following:

- Physical and chemical properties, for example, molecular weight (MW), reactivity, volatility, and biodegradability
- Human and environmental exposure, for example, production volume, release, and use patterns
- Hazard, for example, available toxicological data

Level 2 (in vitro assays) provides mechanistic data such as the following:

- ER binding assay (U.S. EPA OPPTS 890.1250)
- AR binding assay (U.S. EPA OPPTS 890.1150)
- Performance-based test guideline (PBTG) for stably transfected transactivation in vitro assays to detect *estrogen receptor agonists* (TG455)
- Aromatase assay (U.S. EPA OPPTS 890.1200)
- H295R steroidogenesis assay (draft OECD TG, U.S. EPA OPPTS 890.1550)
- Stably transfected human AR transcriptional activation assay (AR STTA)
- TR binding affinity (guidance not written)
- Aryl hydrocarbon receptor (AhR) binding affinity (guidance not written)
- Thyroid function in vitro (guidance not written)
- Fish hepatocyte Vitellogenin (VTG) assay (guidance not written)
- Yeast transactivation assays (YES and YAS) (guidance not written)

Level 3 (in vivo assays) provide data about single endocrine mechanisms and effects, utilizing the following:

- Mammalian in vivo assays
  - Hershberger bioassay in rodents (TG441) (androgenic related)
  - Uterotropic bioassay in rodents (TG440) (estrogenic related)
- Nonmammalian in vivo assays
  - FSTRA (TG229)
  - AMA (TG231)

Level 4 (in vivo assays) provides data about multiple endocrine mechanisms and effects, utilizing the following:

- Mammalian in vivo assays
  - Enhanced repeated dose 28-day oral toxicity study in rodents (TG407)[67] (endpoints based on endocrine mechanisms)
  - Pubertal development and thyroid function assay in peripubertal male rats (PP male assay) (U.S. EPA OPPTS 890.1500)
  - Pubertal development and thyroid function assay in peripubertal female rats (PP female assay) (U.S. EPA OPPTS 890.1450)
  - Adult intact male assay
  - Nonmammalian in vivo assay
  - twenty-one-day fish assay (TG 230) (estrogenic related)
  - FSTRA (TG 229)
  - Androgenized female stickleback screen (AFSS) (draft OECD TG)
  - Fish sexual development test (FSDT) (TG234)

Level 5 (in vivo assays) provides data on effects from endocrine and other mechanisms

- Mammalian in vivo assays
  - One-generation reproduction toxicity (TG415 enhanced)[68]
  - Two-generation reproduction toxicity (TG416 enhanced)[69]
  - Reproduction/developmental toxicity screening test (TG421 enhanced)[70]
  - Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (TG 422 enhanced)[71]
- Nonmammalian in vivo assays
  - Fish life cycle toxicity test (FLCTT) (see similar design in U.S. EPA OPPTS 885.4700)
  - Avian reproduction test (TG206)
  - Medaka multigeneration test (MMGT) (draft OECD TG)
  - Amphibian development, growth, and reproduction assay (ADGRA) (draft OECD TG)
  - Avian two-generation test (ATGT) (draft OECD TG)
  - Invertebrate development and reproduction assays

The framework should not be considered as all inclusive at the present time. At levels 3, 4, and 5, the framework includes assays that are either available or for which validation is under way. With respect to the latter, these have been provisionally included. Once developed and validated, they will be formally added to the framework. Level 5 should not be considered as including definitive tests only. Tests included at that level are considered to contribute to general hazard and risk assessment. Level 5 ecotoxicology tests should include endpoints that indicate mechanisms of adverse effects and potential population damage.

The assessment of each chemical should be based on a case-by-case basis, taking into account all available information, bearing in mind the function of the framework levels. Entering at all levels and exiting at all levels is possible in the OECD framework and depends upon the nature of existing information needs for hazard and risk assessment purposes. For example, when a multimodal model has been performed and it covers several of the single endpoint assays of a lessor level, then that model would replace the use of those single endpoint assays.

In Vitro Assays

Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists (TG455)[72] This performance-based test guideline (PBTG) describes in vitro assays, which provides the methodology of stably transfected transactivation to detect ER agonists. It comprises mechanistically and functionally similar test methods for the identification of ER agonists and should facilitate the development of new similar or modified test methods. The two reference test methods that provide the basis for this PBTG are the stably transfected TA (STTA) assay using the ERα-HeLa-9903 cell line, derived from a human cervical tumor, and the BG1Luc ER TA assay using the BG1Luc-4E2 cell line, derived from a human ovarian adenocarcinoma. The cell lines used in these assays express ER and have been stably transfected with an ER responsive luciferase reporter gene. The assays are used to identify chemicals that activate the ER following ligand binding, after which the receptor-ligand complex binds to specific DNA response elements and transactivates the reporter gene, resulting in increased cellular expression of a marker enzyme (e.g., luciferase in luciferase-based systems). The enzyme then transforms the substrate to a bioluminescent product that can be quantitatively measured with a luminometer. These test methods are being proposed for screening and prioritization purposes but also provide mechanistic information that can be used in a WoE approach.

H295R Steroidogenesis Assay (TG456)[73] This Test Guideline describes an in vitro screen for chemical effects on steroidogenesis, specifically the production of 17β-estradiol (E2) and testosterone (T). The human H295R adrenocarcinoma cell line, used for the assay, expresses genes that encode for all the key enzymes for steroidogenesis. After an acclimation period of 24 h in multi well plates, cells are exposed for 48 h to seven concentrations of the test chemical in at least triplicate. Solvent and a known inhibitor and inducer of hormone production (see Guideline) are run at a fixed concentration as negative and positive controls. At the end of the exposure period, cell viability in each well is analyzed. Concentrations of hormones in the medium can be measured using a variety of methods including commercially available hormone measurement kits and/or instrumental techniques such as LC–MS. Data are expressed as fold change relative to the solvent control and the lowest-observed-effect-concentration. If the assay is negative, the highest concentration tested is reported as the no-observed-effect-concentration.

BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists (TG457)[74] This Test Guideline describes an in vitro assay that provides concentration–response data for substances with in vitro ER agonist and antagonist activity. The test system utilizes the BG1Luc4E2 cell line derived from a human ovarian adenocarcinoma and stably transfected with an ER responsive luciferase reporter gene. This cell line can evaluate TA mediated by ERα and ERβ. The cells are plated into 96-well plate and exposed to 7 (range finder tests) and 11 (comprehensive test) noncytotoxic concentrations of the test chemical for 19–24 h to induce the reporter gene product (luciferase). Its activity is measured in a luminometer. Acceptance or rejection of a test is based on the evaluation of reference standard and control results from each experiment conducted on a 96-well plate. A positive response is identified by a concentration–response curve containing at least three points with nonoverlapping error bars, as well as a change in amplitude (normalized relative light unit) of at least 20% of the maximal value for the reference substance (17β-estradiol for the agonist assay and raloxifene HCL/17β-estradiol for the antagonist assay).

In Vivo Tests—Mammalian

Hershberger Bioassay in Rats (TG441)[75]

The Hershberger bioassay is an in vivo short-term screening test. It evaluates the ability of a chemical to elicit biological activities consistent with androgen agonists, antagonists, or 5α-reductase inhibitors. The current bioassay is based on the changes in the weight of five androgen-dependent tissues in the castrate-peripubertal male rat: the ventral prostate, seminal vesicle (plus fluids and coagulating glands), levator ani-bulbocavernosus muscle, paired Cowper's glands, and the GP. In order to establish whether a test substance can have androgenic or antiandrogenic action, two—respectively three—dose groups of the test substance, plus positive and vehicle (negative) controls, are normally sufficient. The test substance is administered by gavage or subcutaneous injection daily for 10 consecutive days. To test for antiandrogens, the test substance is administered together with a reference androgen agonist. Each treated and control group should include a minimum of six animals. The animals are necropsied approximately 24 h after the last administration of the test substance. The tissues are excised and their fresh weights determined. A statistically significant increase (androgenic) or decrease (antiandrogenic) in the weights of two of the five tissues indicates a positive response in this assay.

Uterotrophic Bioassay in Rodents (TG440)[76] The uterotrophic bioassay is an in vivo short-term screening test. It is based on the increase in uterine weight or uterotrophic response. The uterotrophic bioassay relies for its sensitivity on an animal test system in which the hypothalamic–pituitary–ovarian axis is not functional. Two estrogen-sensitive states in the female rodent meet this requirement: (1) immature females after weaning and prior to puberty and (2) young adult females after ovariectomy with adequate time for uterine tissues to regress. The test substance is administered daily by oral gavage or subcutaneous injection. Each treated and control group should include at least six animals. Graduated test substance doses are administered to a minimum of two treatment groups of experimental animals using one dose level per group and an administration period of 3 consecutive days for immature female method and a minimum administration period of 3 consecutive days for ovx-adult method. The animals are necropsied approximately 24 h after the last dose. For estrogen agonists, the mean uterine weight of the treated animal groups relative to the vehicle group is assessed for a statistically significant increase. A statistically significant increase in the mean uterine weight of a test group indicates a positive response in this bioassay. The report should include daily body weights, daily record of the status of animal, wet and blotted uterine weight, and daily food consumption values.

Repeated Dose 28-Day Oral Toxicity Study in Rodents (TG407)[70] This method provides information on health hazard likely to arise from exposure to test substance via oral administration. The method is based on the repeated oral administration of the substance of interest during one limited period (one dose level daily during 28 days). This guideline is intended primarily for use with rodents (rat preferably). At least 10 animals (5 females and 5 males) should be used for each dose level. Three tests groups, at least, should be used. The test compound is administered by gavage or via the diet or drinking water. A limit test may be performed if no effects would be expected at a dose of 1000 mg/kg bw/day. The report of this study will include results from clinical and functional observations, body weight and food/water consumption measurements, hematology, and clinical biochemistry, as well as gross necropsy and histopathology.

One-Generation Reproduction Toxicity Study (TG415)[71] This test guideline for reproduction testing is designed to provide general information concerning the effects of a test substance (solid, liquid, gas, or vapor) on male and female reproductive performance. The test substance is administered orally in graduated doses to several groups of males and females.

Males should be dosed during growth and for at least one complete spermatogenic cycle; females of the parent generation should be dosed for at least two complete estrous cycles. The animals are then mated. The test substance is administered to both sexes during the mating period and thereafter only to females during pregnancy and for the duration of the nursing period. This test guideline is intended primarily for use with the rat or mouse. Each test and control group should contain a sufficient number of animals to yield about 20 pregnant females at or near term. Three test groups, at least, should be used. It is recommended that the test substance be administered in the diet or drinking water. A limit test may be performed if no effects would be expected at a

dose of 1000 mg/kg bw/day. The results of this study include measurements (weighing and food consumption) and daily and detailed observations, each day preferably at the same time, as well as gross necropsy and histopathology. The findings of a reproduction toxicity study should be evaluated in terms of the observed effects, necropsy, and microscopic findings. A properly conducted reproduction test should provide a satisfactory estimation of a no-effect level and an understanding of adverse effects on reproduction, parturition, lactation, and postnatal growth.

Two-Generation Reproduction Toxicity (TG416)[72] This test guideline for two-generation reproduction testing is designed to provide general information concerning the effects of a test substance on the integrity and performance of the male and female reproductive systems, and on the growth and development of the offspring. The test substance is administered daily in graduated doses to several groups of males and females.

Males and females of the parent generation (5–9 weeks old) should be dosed during growth, during their mating, during the resulting pregnancies, and through the weaning of their first-generation offspring. The administration of the substance is continued to first-generation offspring during their growth into adulthood, mating, and production of a second generation (until the weaning). The rat is the preferred species for testing. Each test and control group should contain a sufficient number of animals to yield preferably not less than 20 pregnant females at or near parturition. At least three dose levels and a concurrent control shall be used. It is recommended that the test substance be administered orally (by diet, drinking water, or gavage). A limit test may be performed if no effects would be expected at a dose of 1000 mg/kg bw/day. The results of this study include measurements (weighing, sperm parameters, estrous cycle parameters, and offspring parameters) and clinical daily observations, as well as gross necropsy and histopathology. The findings of this two-generation reproduction toxicity study should be evaluated in terms of the observed effects, including necropsy and microscopic findings. A properly conducted reproductive toxicity test should provide a satisfactory estimation of a no-effect level and an understanding of adverse effects on reproduction, parturition, lactation, and postnatal development including growth and sexual development.

Reproduction/Developmental Toxicity Screening Test (TG421)[73] The test substance is administered in graduated doses to several groups of males and females. Males should be dosed for a minimum of 4 weeks. Females should be dosed throughout the study, so approximately 54 days. This test guideline is designed for use with the rat. It is recommended that each group be started with at least 10 animals of each sex. Generally, at least three test groups and a control group should be used. Dose levels may be based on information from acute toxicity tests or on results from repeated dose studies. The test substance is administered orally and daily. The limit test corresponds to one dose level of at least 1000 mg/kg bw. The results of this study include measurements (weighing and food/water consumption) and daily and detailed observations, preferably each day at the same time, as well as gross necropsy and histopathology. The findings of this toxicity study should be evaluated in terms of the observed effects, necropsy, and microscopic findings. Because of the short period of treatment of the male, the histopathology of the testis and epididymus must be considered along with the fertility data, when assessing male reproductive effects.

Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (TG422)[74] The test substance is administered in graduated doses to several groups of males and females. Males should be dosed for a minimum of 4 weeks; females should be dosed throughout the study (approximately 54 days). Normally, matings of *one male to one female* should be used in this study.

This test guideline is designed for use with the rat. It is recommended that the test substance be administered orally by gavage. This should be done in a single dose daily to the animals using a stomach tube or a suitable intubation cannula. Each group should be started with at least 10 animals of each sex. Generally, at least three test groups and a control group should be used. Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available. The limit test corresponds to one dose level of at least 1000 mg/kg bw. The results of this study include measurements (weighing and food/water consumption) and daily detailed observations (including sensory reactivity to stimuli), preferably each day at the same time, as well as gross necropsy and histopathology. The findings of this toxicity study should be evaluated in terms of the observed effects, necropsy, and microscopic findings. The evaluation will include the relationship between the dose of the test substance and the presence or absence of observations. Because of the short period of treatment of the male, the histopathology of the testis and epididymus must be considered along with the fertility data, when assessing male reproduction effects.

In Vivo Tests—Nonmammalian

Amphibian Metamorphosis Assay (TG231)[77] This test guideline describes an AMA intended to screen substances that may interfere with the normal functioning of the HPT axis. The assay was validated with the species *Xenopus laevis*, which is recommended for use in the guideline. The assay uses three test chemical concentrations and the necessary controls, including a carrier control if necessary. The assay starts with tadpoles at the development stage 51 on the Nieuwkoop and Faber scale and is extended for a duration of 21 days. Four replicate test vessels are used for each treatment level and control(s). After 7 days of exposure, a subset of tadpoles from each treatment level is sampled for the measurement of the length of the hind limb. At termination of 21-day exposure period, developmental stage, snout–vent length, and hind limb length are measured on all remaining tadpoles. A subset of tadpoles from each treatment level is fixed (whole-body or dissected) for histopathology of the thyroid gland.

Short-Term Screening for Estrogenic and Androgenic Activity, and Aromatase Inhibition (TG230)[78] This test guideline describes an in vivo screening assay for certain endocrine active substances where sexually mature male and spawning female fish are held together and exposed to a chemical during a limited part of their life cycle (21 days). This assay covers the screening of estrogenic and androgenic activities, and aromatase inhibition. The assay was validated on the fathead minnow (*Pimephales promelas*), the Japanese medaka (*Oryzias latipes*), and the zebrafish (*Danio rerio*); however, zebrafish does not allow the detection of androgenic activities. At termination of the 21-day exposure period, depending on the species used, one or two biomarker endpoint(s) are measured in males and females as indicators of estrogenic, aromatase inhibition or androgenic activity of the test chemical; these endpoints are vitellogenin and secondary sexual characteristics. Vitellogenin is measured in fathead minnow, Japanese medaka, and zebrafish, whereas secondary sex characteristics are measured in fathead minnow and Japanese medaka only.

Fish Short-Term Reproduction Assay (TG229)[79] This test guideline describes an in vivo screening assay for fish reproduction where sexually mature male and spawning female fish are held together and exposed to a chemical during a limited part of their life cycle (21 days). The short-term reproduction assay was validated in the fathead minnow (*Pimephales promelas*), and this is the recommended species. The assay is run with three test chemical concentrations and the necessary controls, including a carrier control if necessary. For the fathead minnow, four replicate test vessels are used for each treatment level and control(s). During the conduct of the assay, the egg production is measured quantitatively daily in each test vessel. At termination of the 21-day exposure period, two biomarker endpoints are measured in males and females separately as indicators of endocrine activity of the test chemical; these endpoints are vitellogenin and secondary sexual characteristics. Gonads of both sexes are also preserved, and histopathology may be evaluated to assess the reproductive fitness of the test animals and to add to the WoE of other endpoints.

Fish Sexual Development Test (TG234)[80] This test guideline describes an assay that assesses early life-stage effects and potential adverse consequences of putative endocrine disrupting chemicals (EDCs) (e.g., estrogens, androgens, and steroidogenesis inhibitors) on fish sexual development. In the test, fish are exposed, from newly fertilized egg until the completion of sexual differentiation at about 60 days posthatch, to at least three concentrations of the test substance dissolved in water. In each treatment level and control(s) group(s), a minimum of four replicates are recommended. At termination of the test, two core endpoints are measured in each fish: vitellogenin concentration from head and tail or from blood sampling, and proportion of males, females, intersex, and undifferentiated fish through gonadal histology. In fish species possessing a genetic sex marker, the genetic sex is identified to determine sex reversal in individual fish. The combination of the two core endocrine endpoints, vitellogenin concentration and phenotypic (and possibly genotypic) sex ratio, enables the test to indicate the mode of action of the test chemical.

Fish Life Cycle Toxicity Test (OPPTS 885.4700)[81] The basic FLCTT as described by Benoit,[82] the U.S. EPA, and others does not contain endpoints that solely respond to endocrine disrupters. However, many of the endpoints in this apical test are nevertheless affected by estrogen, androgen, thyroid, steroidogenesis endocrine disruptors (EATS EDs). Of particular interest in the context of estrogens, androgens, and steroidogenesis disrupters are time to sexual maturity, sex ratio of adults, fecundity, and fertility, but other endpoints may also be responsive to some EDs (e.g., growth may respond to some thyroid disrupters).

This assay is designed primarily as an apical test for chemicals with suspected reproductive or long-term toxicity. It has not been validated for publication as a test guideline but has been widely used for several decades by regulatory agencies for assessing possible chronic effects in fish. The endpoints are all apical measures of development, growth, or reproduction. Exposure of the test organisms (fathead minnow *Pimephales promelas*,[82] but other species can also be successfully used with minor changes in the protocol, including sheepshead minnow *Cyprinodon variegatus*, zebrafish *Danio rerio*, and medaka *Oryzias latipes*) usually continues from the freshly fertilized eggs of the F_0 generation to the fry or young fish of the F_1 generation (4–8 weeks posthatch in the case of fathead minnow).[82,83]

The purpose of this test guideline is to determine the effects on the reproduction of a substance administered with food to birds. Birds are fed a diet containing the test substance in various concentrations for a period of not less than 20 weeks. A minimum of three dietary concentrations of the test substance is required. The maximum recommended test concentration is 1000 ppm. Birds may be kept in pens as pairs (at least 12 pens per test group) or as groups of one male and two or three females (at least 8–12 pens per group). Birds are induced, by photoperiod manipulation, to lay eggs. Eggs are collected over a 10-week period, artificially incubated and hatched, and the young maintained for 14 days. Suitable facilities for rearing birds, preferably indoors, are necessary. Mortality of adults, egg production, cracked eggs, egg shell thickness (at least two eggs from each pen), viability, hatchability, and effects on young birds are observed during the study.

Technical Questions and Answers Concerning the Tier 1 Tests

The U.S. EPA has issued clarifications about technical aspects of the conduct of Tier 1 assays for the EDSP in response to questions raised by Test Order recipients and others. In some cases, these questions pointed out simple but significant errors in test guidelines (e.g., incorrect references to tables and misplaced decimal points). In other cases, questions pointed out ambiguous language that required clarification; and in yet other cases, questions were raised about whether alternative

techniques had been considered by the EPA and rejected, or whether they might be acceptable to use. These items must be reviewed prior to following any of the published guidelines. The following documents are available on the U.S. EPA website's EDSP[84] and review the EPA's response to questions.

- Clarifications on Technical Aspects of the EDSP Tier 1 Assays[85]
- Responses to EDSP Tier 1 Technical Questions Received from CeeTox Contract Laboratory—Generic Technical Questions Regarding the Conduct of In Vitro EDSP Tier 1 Assays[86]
- Responses to EDSP Tier 1 Technical Questions Received from Integrated Laboratory Systems—Conducting In Vivo Mammalian Studies[87]
- Responses to EDSP Tier 1 Technical Questions Received from Huntingdon Life Sciences—Conducting the EDSP Aromatase Assay Using Guideline 890.1200[88]
- Responses to EDSP Tier 1 Technical Questions Received from Huntingdon Life Sciences—Regarding the Estrogen Receptor Binding Assay[89]
- Responses to EDSP Tier 1 Technical Questions Received from CeeTox Contract Laboratory—Assessing False Positives in the Estrogen Receptor Transcriptional Activation (ERTA) Assay[90]

Standard Evaluation Procedures and Data Entry Spreadsheet Templates for EDSP Tier 1 Assays

EPA's OCSPP has developed a set of standard evaluation procedures (SEPs),[91] which provide guidance for the review and evaluation of environmental and human health effects data submitted in response to Test Orders for the 890 Guideline Series for the EDSP Tier 1 battery. There is an individual SEP for each of the eleven 890 Guideline Series EDSP Tier 1 assays that comprise the battery. The objective of the SEPs is to assist EPA reviewers and support comprehensive and consistent evaluations of major scientific topics; the SEPs also provide interpretive scientific and policy guidance where appropriate. The SEPs provide a venue for standardizing data organization and evaluation procedures with the goal of increasing the efficiency, thoroughness, and consistency with which individual study evaluations are prepared and assist the scientists to reach clear, reasonable, and concise conclusions that are scientifically robust.

Using the guidance within the relevant SEP, EPA reviewers will develop data evaluation records (DERs) for each of the submitted assays. The DERs reflect how well the assay conforms to the Series 890 EDSP Test Guidelines and document basic study information such as materials, methods, results, and significant deviations from the study protocol or guideline recommendations as well as their potential impacts. The DER also provides the registrant's conclusions, the EPA reviewer's conclusions, and any other information about the performance of the study that affects the interpretation of the data within the context of the EDSP.

The SEPs do not provide guidance for making the determination that a chemical that has been screened using the EDSP Tier 1 assays should move on to Tier 2 testing. The EPA has submitted a separate draft guidance document[92] of the WoE.

OCSPP has also developed a set of data entry spreadsheet templates (DESTs) that Test Order recipients may choose to use as a method for reporting all raw data in electronic format for the 890 Guideline Series for the EDSP Tier 1 battery. There is a DEST for each of the eleven 890 Guideline Series EDSP Tier 1 assays that comprise the battery. EPA encourages Test Order recipients to use the DEST as a method for standardizing raw data reporting and to assist EPA reviewers in the comprehensive and consistent evaluations of the raw data.

BIOCHEMICAL ASSESSMENT

The diagnosis of endocrine disorders as well as an understanding of the mechanisms of hormonal action was significantly advanced when methods became available to measure hormones in the blood.[93] Until about the 1960s, the measurement of hormones usually involved in vivo bioassays. The discovery of mAb techniques revolutionized the measurement of hormones. Subsequently, antibody-based competitive protein-binding assays (i.e., RIAs) were perfected with the availability of radioactive, or tracer, hormones.

Evaluation of endocrine function is an important aspect in determining the mechanism of endocrine toxicity.[22] Measurement of hormone and hormonal metabolite levels in the blood or urine can reveal important information regarding the site of endocrine toxicity. These sites can include the endocrine tissue itself (e.g., the site of hormone synthesis and release), the endocrine target tissue (e.g., the hormone receptor and intracellular signaling pathways), and the mechanism of transport from the endocrine tissue to the target cell receptor to exert an endocrine effect (e.g., the hormone carrier proteins). Furthermore, toxic actions on feedback control mechanisms can augment or inhibit endocrine responses.

There are limitations to the biochemical assessment of the endocrine system. For example, factors that may have modulatory effects on endocrine target cells cannot always be identified by simple evaluation of blood hormone levels. For this reason, biochemical assessment of the endocrine system frequently offers a macroscopic survey of toxicology mechanisms. Determination of pathophysiology and molecular endocrine toxicity is limited due to the complex interplay of the numerous and frequently redundant regulatory and counterregulatory feedback loops.[94] Biochemical assessment of physiologic conditions can be confounded by several external factors, such as stress, extreme temperatures, or changes in the diurnal variation of sleep patterns. Similarly, the biochemical assessment can be confounded by internal factors such as the female menstrual cycle in both the reproductive and nonreproductive organs. Finally, the sensitivity and specificity of hormonal testing vary greatly among different hormonal systems.

In Vivo Bioassays

An in vivo bioassay determines the biological activity of a hormone by noting its effect on a live animal or isolated organ preparation compared to a known standard preparation. Bioassays provide a means of assessing endocrine status, and although more accurate measurement of hormonal levels can now be achieved with RIA, bioassays are sometimes useful when RIA is unable to distinguish active from inactive hormonal metabolites or precursors.[95] Historically, bioassays have used hypoglycemia to measure insulin, bone growth to measure GH, and ovarian weight change to measure gonadotropins. The main drawbacks to in vivo bioassays are that they are frequently insensitive and nonspecific, and comparisons to the dose–response curve for a standard preparation are imprecise. Some of these in vivo bioassays but definitely not all of these assays were earlier discussed in the Endocrine Disruptor Screening Program section.

In Vitro Bioassays

In vitro bioassays employ endocrine-responsive tissue cell culture lines that can assess the amount of biologically active hormone in sera or other fluids such as urine. The hormonal activity can be determined by measuring a cellular response to a hormone. Classically, hormonally induced changes in adenylate cyclase activity (with changes in cAMP levels) have been used, but more recently, changes in intracellular calcium levels, phosphoinositol metabolites, and protein phosphorylation have been used to assess hormonal activity. Other in vitro bioassays can examine changes more distal to the receptor and signal transduction mechanisms and note changes in enzymatic activity or steroidogenesis. Finally, some in vitro bioassays can assess the mitogenic responses to a given hormone. A shortcoming to in vitro bioassays is that there may be coexisting stimulatory or inhibitory substances in the matrix that can confound the observed response.

Cell Culture Systems

Examination of dispersed or cultured cells from any level of the hypothalamus–pituitary–adrenocortical (HPA) axis can provide better information than simple biochemical assays because an intact cell is much more complicated than an isolated reaction and, hence, one step closer to examining the intact organism.

Corticotropes

Mammalian corticotropes[96–98] have been used to study the actions of corticotropin-releasing hormone (CRH) and/or arginine vasopressin (AVP), also known as vasopressin, argipressin, or antidiuretic hormone (ADH), as well as the effects of other agents on the synthesis/release of ACTH (or corticotropin), into the culture medium, or on changes in messenger ribonucleic acid (mRNA) levels. These in vitro systems must be carefully examined since many additional factors can alter the responsiveness of the corticotrope in vivo (e.g., cortisol levels, CRH and/or AVP receptor levels, and other circulating or local factors such as pituitary adenylate cyclase–activating polypeptide [PACAP]).

Adrenal Cortical Cells

Mouse models of cultured adrenal cells also may be used to develop EDC screening assays. However, mouse adrenal models often differ markedly from results seen in humans.[99] A human adenocarcinoma cell line (H295R) has been developed as a screening assay for chemical factors that interfere with steroidogenesis using production of progesterone, testosterone, and E2 as end products (OPPTS 890.1550 steroidogenesis—human cell line [H295R]), and this system possibly could be validated for measuring GC growth-hormone-releasing hormone (GHRH) as well. An in vitro method for assessing pesticide effects on adrenal cells of rainbow trout has been reported recently[100] and could provide the basis for a simple screening assay for specifically directed at fish.

Glucocorticoid Growth-Hormone-Releasing Hormone Target Cells

Some in vitro systems have been described for looking at metabolic actions of GCs on mammalian uterine cells,[101] liver cells,[102–105] or adipose cells.[106,107] Additional in vitro systems have been explored in fish liver.[108,109]

Gene Activation Assays

Following the exposure of a cell to a GC, GC antagonist, or GC agonist, expression of genes can be monitored by isolating mRNA, forming the corresponding cDNA, and identifying the specific genes involved using DNA microarrays. DNA technology has resulted in the development of DNA microarrays for various species for whom the genome has been cataloged (e.g., human, *Xenopus*, zebrafish). These microarrays are available from commercial sources. However, many of the genes activated in these arrays have yet to be linked to a known GC function.

Radioimmunoassay

Measurement of endocrine values was revolutionized in 1959 when Yalow and Berson published their work on the RIA to detect nanomolar concentrations of hormones in human subjects.[20] Immunoassays are based on the fact that specific proteins can distinguish three-dimensional structures at the molecular level. When specific proteins are produced by a biological organism, their specificity can be discriminated and tightly bound to those molecules in a highly complex mixture providing a means to measure these proteins in a controlled situation. RIAs use radioactive isotopes to identify these specific proteins. Classic bioassays were vastly surpassed in sensitivity, specificity, and facilitation by RIA. In addition, RIA allows measurement of biological materials not previously detectable by chromatographic or spectrophotometric techniques.[110] RIAs can be used to measure hormones that cannot be radiolabeled to detectable levels in vivo. They are also used for hormones that cannot fix complement when bound to antibodies, or they can be used to identify cross-reacting antigens that compete and bind with the antibody.

Generally, immunoassays can be divided into two categories: competitive inhibition and noncompetitive inhibition. Competitive assays use a single specific antibody type that is fixed to a surface and a corresponding analog of the protein to carry the label. The protein in the sample competes with the labeled analog for binding positions on the antibody. Once the unbound analog is separated, the amount of label remaining is inversely related to the amount of bound protein. Competitive inhibition of radiolabeled hormone antibody binding by unlabeled hormone (either as a standard or an unknown mixture) is the principle of most RIAs. A standard curve for measuring antigen (hormone) binding to antibody is constructed by placing known amounts of radiolabeled antigen and the antibody into a set of test tubes. Varying amounts of unlabeled antigen are added to the test tubes. Antigen–antibody complexes are separated from the antigen, and the amount of radioactivity from each sample is measured to detect how much unlabeled antigen is bound to the antibody. Smaller amounts of radiolabeled antigen–antibody complexes are present in the fractions containing higher amounts of unlabeled antigen. Usually, a standard curve is constructed that measures the percent of radiolabeled antigen bound with the concentration of unlabeled antigen present.

Noncompetitive assays utilize two specific antibodies to sandwich the protein being examined. One antibody (termed the *capture protein*) is immobilized to a surface and a second (termed the *label protein*) carries the label. In the assay, the protein being examined is bound simultaneously by both the capture and label proteins. At completion of the assay, the unbound label protein is separated and measured; the remaining label is measured and is directly proportional to the protein concentration in the sample. Sandwich assays are generally limited to those proteins of sufficient size to be able to bind two materials simultaneously, typically proteins and microorganisms. Competitive assays are compatible with a wide variety of proteins and are used for the majority of low-molecular-weight proteins. Although several methods exist for the separation of antigen–antibody complexes, two methods are most commonly employed in RIAs. The first, the double-antibody technique, precipitates antigen–antibody complexes out of solution by utilizing a second antibody, which binds to the first antibody. Although other means of antigen–antibody precipitation exist, they can sometimes chemically alter antigen–antibody binding properties. The drawback to the double-antibody technique is its expense, which makes this technique uneconomical for RIA screening procedures.

Another commonly used method is the dextran-coated activated charcoal technique. Addition of dextran-coated activated charcoal to the sample followed by immediate centrifugation absorbs free antigen and leaves antigen–antibody complexes in the supernatant fraction. Although it is economical, a drawback to this technique is that it works best only when the molecular weight of the antigen is 30 kDa or less. Also, sufficient carrier protein must be present to prevent adsorption of unbound antibody.

Once a standard curve has been constructed, the RIA can determine the concentration of hormone in a sample (usually plasma or urine). The values of hormone levels are usually accurate when the RIA is used, but certain factors (e.g., pH or ionic strength) can affect antigen binding to the antibody; thus, controlled and similar conditions must be used when assessing the standard and the sample.

Difficulties with RIAs include a lack of specificity. This problem is usually due to nonspecific cross-reactivity of the antibody. Another complication is that, because the assays require the use of radioactive materials by definition, the laboratory must be regulated by the Nuclear Regulatory Commission (NRC). Despite the more complex and involved RIA and mAb methodologies, they are of immense value for measuring various tropic hormones. RIA represents an analytical approach of great sensitivity, and such techniques have been applied to numerous biological substances, many of which cannot be assessed by other techniques. Unlike bioassays that often require large amounts of tissue (or blood), the greater sensitivity of the RIAs or mAb techniques allows the use of smaller samples of biological fluids. Some of these RIA methodologies are more useful than others, and their usefulness to some extent depends on the degree of hormonal cross-reactions or, in the case of mAb methods, their degree of sensitivity.

Although the RIA technique requires specialized equipment, it is extremely sensitive and extremely specific. It remains the least expensive method to perform such tests. Today, it has been supplanted by the ELISA method, where the antigen–antibody reaction is measured using colorimetric signals instead of a radioactive signal. However, because of its robustness, consistent results, and low price per test, RIA methods are again becoming popular.

Enzyme-Linked Immunosorbent Assay

The ELISA is comparable to the immunoradiometric assay (IRMA) except that an enzyme tag is attached to the antibody instead of a radioactive label. ELISAs produce an end product that can be assessed with a spectrophotometer. The hormone is bound to the enzyme-labeled antibody, and the excess antibody is removed for IRMAs. After excess antibody has been removed or the second antibody containing the enzyme has been added (two-site assay), the substrate and cofactors necessary are added to visualize and record enzyme activity. The level of hormone present is directly related to the level of enzymatic activity. The sensitivity of the ELISAs can be enhanced by increasing the incubation time for producing substrate. Sometimes, the substrate formed may yield a color change so that detection of the hormone being measured can be determined visually.

Indirect ELISA

Indirect ELISA adopts the following mechanism:

1. A buffered solution of the antigen to be tested for is added to each well of a microtiterplate, where it is given time to adhere to the plastic through charge interactions.
2. A solution of nonreacting protein, such as bovine serum albumin or casein, is added to block any uncoated plastic surface in the well.

3. The primary antibody is added, which binds specifically to the test antigen coating the well.
4. A secondary antibody is added, which will bind the primary antibody. This secondary antibody often has an enzyme attached to it.
5. A substrate for this enzyme is then added. Often, this substrate changes color upon reaction with the enzyme. The color change shows the secondary antibody has bound to primary antibody, which strongly implies the donor has had an immune reaction to the test antigen. This can be helpful in a clinical setting, and in research.
6. The higher the concentration of the primary antibody present in the serum, the stronger the color change. Often, a spectrometer is used to give quantitative values for color strength.

The enzyme acts as an amplifier; even if only few enzyme-linked antibodies remain bound, the enzyme molecules will produce many signal molecules. Within common-sense limitations, the enzyme can go on producing color indefinitely, but the more primary antibody is present in the donor serum, the more secondary antibody + enzyme will bind, and the faster the color will develop. A major disadvantage of the indirect ELISA is the method of antigen immobilization is not specific; when serum is used as the source of test antigen, all proteins in the sample may stick to the microtiter plate well, so small concentrations of analyte in serum must compete with other serum proteins when binding to the well surface. The sandwich or direct ELISA provides a solution to this problem, by using a *capture* antibody specific for the test antigen to pull it out of the serum's molecular mixture.

ELISA may be run in a qualitative or quantitative format. Qualitative results provide a simple positive or negative result (yes or no) for a sample. The cutoff between positive and negative is determined by the analyst and may be statistical. Two or three times the standard deviation is often used to distinguish positive from negative samples. In quantitative ELISA, the OD of the sample is compared to a standard curve, which is typically a serial dilution of a known-concentration solution of the target molecule. For example, if a test sample returns an OD of 1.0, the point on the standard curve that gave OD = 1.0 must be of the same analyte concentration as the sample.

Sandwich or Direct ELISA

A sandwich ELISA adopts the following mechanism:

1. Plate is coated with a capture antibody.
2. Sample is added, and any antigen present binds to capture antibody.
3. Detecting antibody is added and binds to antigen.
4. Enzyme-linked secondary antibody is added and binds to detecting antibody.
5. Substrate is added and is converted by enzyme to detectable form.

A less-common variant of this technique, a *sandwich* ELISA, is used to detect sample antigen. The steps are as follows:

1. A surface is prepared to which a known quantity of capture antibody is bound.
2. Any nonspecific binding sites on the surface are blocked.
3. The antigen-containing sample is applied to the plate.
4. The plate is washed to remove unbound antigen.
5. A specific antibody is added and binds to antigen (hence the *sandwich*: the Ag is stuck between two antibodies).
6. Enzyme-linked secondary antibodies are applied as detection antibodies that also bind specifically to the antibody's Fc (fragment, crystallizable) region (nonspecific).
7. The plate is washed to remove the unbound antibody–enzyme conjugates.
8. A chemical is added to be converted by the enzyme into a color or fluorescent or electrochemical signal.
9. The absorbency or fluorescence or electrochemical signal (e.g., current) of the plate wells is measured to determine the presence and quantity of antigen.

Competitive ELISA

A third use of ELISA is through competitive binding. The steps for this ELISA are somewhat different from the first two examples:

1. Unlabeled antibody is incubated in the presence of its antigen (sample).
2. These bound antibody/antigen complexes are then added to an antigen-coated well.
3. The plate is washed, so unbound antibody is removed. The more antigen in the sample, the less antibody will be able to bind to the antigen in the well, hence *competition*.
4. The secondary antibody, specific to the primary antibody, is added. This second antibody is coupled to the enzyme.
5. A substrate is added, and remaining enzymes elicit a chromogenic or fluorescent signal.
6. The reaction is stopped to prevent the eventual saturation of the signal.

Some competitive ELISA kits include enzyme-linked antigen rather than enzyme-linked antibody. The labeled antigen competes for primary antibody binding sites with the sample antigen (unlabeled). The more antigen in the sample, the less labeled antigen is retained in the well and the weaker the signal. For example, for the detection of HIV antibodies, the wells of microtiter plate are coated with the HIV antigen. Two specific antibodies are used, one conjugated with enzyme and the other present in serum (if serum is positive for the antibody). Competition occurs between the two antibodies for the same antigen. Sera to be tested are added to these wells

and incubated at 37°C and then washed. If antibodies are present, the antigen–antibody reaction occurs. No antigen is left for the enzyme-labeled specific HIV antibodies. These antibodies remain free upon addition and are washed off during washing. Substrate is added, but there is no enzyme to act on it, so positive result shows no color change.

Multiple and Portable ELISA

A new technique[111] uses a solid phase made up of an immunosorbent polystyrene rod with 8–12 protruding ogives, the roundly tapered end of a two- or three-dimensional object. The entire device is immersed in a test tube containing the collected sample, and the following steps (washing, incubation in conjugate, and incubation in chromogens) are carried out by dipping the ogives in the microwells of standard microplates filled with reagents.

The advantages of this technique are as follows:

1. The ogives can each be sensitized to a different reagent, allowing the simultaneous detection of different antibodies and/or different antigens for multiple-target assays.
2. The sample volume can be increased to improve the test sensitivity in clinical (blood, saliva, and urine), food (bulk milk, and pooled eggs), and environmental (water) samples.
3. One ogive is left unsensitized to measure the nonspecific reactions of the sample.
4. The use of laboratory supplies for dispensing sample aliquots, washing solution, and reagents in microwells is not required, facilitating the development of ready-to-use lab kits and on-site testing.

Issues with ELISAs

ELISAs are very good at detecting single analytes within a fairly narrow concentration range.

- If the concentration of analyte is very high or low, then many dilutions of the sample may need to be made in order to read off a standard curve. This can mean an assay must be repeated to get accurate results.
- Each substrate used in ELISA reaches a certain color intensity after which it is not possible to determine differences; this means that usually ELISAs only have a limited *range*, for example, 1–1000 μg/mL. These ranges may be too narrow for all applications.
- ELISAs are quite lengthy assays, taking anything up to 1 day to run. This means that repeat samples, whether you have 1 repeat or 50 repeat samples, require the same time to run repeat assays.
- ELISAs only measure one analyte; it is rarely possible to *multiplex*, that is, to measure many analytes in the same sample. This means separate assays must be established in order to measure many analytes.
- There are many washing steps in ELISA that wash away unbound analyte. Antibodies bind with varying affinity (strength of binding) and avidity (numbers of binding sites). There is a risk that antibodies with low binding affinity will be washed away and false negatives or lower than true results will be obtained.
- ELISAs typically require reasonably large sample volumes for testing. This may mean that for smaller animal species, satellite animals are required in order to collect enough samples for testing.

Immunoradiometric Assays

IRMAs are like RIAs in that a radiolabeled substance is used in an antibody–antigen reaction. The radioactive label, however, is attached to the antibody instead of the hormone. This assay is based upon the reversible and noncovalent binding of an antigen by a specific antibody labeled with a radioisotope. Further, excess of antibody, rather than limited quantity, is present in the assay. All of the unknown antigen becomes bound in IRMA, rather than just a portion as in RIA. IRMAs are more sensitive. In the one-site assay, the excess antibody that is not bound to the sample is removed by the addition of a precipitating binder. In the two-site assay (i.e., sandwich technique), a hormone with at least two antibody-binding sites is adsorbed onto a solid phase, to which one of the antibodies is firmly attached (either the walls of the assay tube itself or beads that are added to the patient sample in assay buffer). After binding to the antibody is completed, a second antibody labeled with ^{125}I is added to the assay. This antibody reacts with the second antibody-binding site to form the sandwich, which is composed of antibody-hormone-labeled antibody. In contrast to RIA and similar competitive protein-binding assays, the amount of hormone present is directly proportional to the amount of radioactivity measured in the assay.[112]

Enzyme-Multiplied Immunoassay Technique

Using enzyme-multiplied immunoassay technique (EMIT) assays, enzyme tags replace the radiolabels; however, the antibody binding alters the enzyme characteristics, allowing for measurement of hormone without separating the bound and free components (i.e., homogeneous assay). EMIT assays are used to monitor urine for drugs, but because of a lack of sensitivity, they have not been used to assess hormones. No extraction is required, and the assay can be completed within a few minutes.[113] The enzyme is attached to the hormone or drug being tested. This enzyme-labeled antigen is incubated with the sample and with antibody to the hormone or drug. Binding of the antibody to the enzyme-linked hormone either physically blocks the active site of the enzyme or changes the protein conformation so the enzyme is no longer active. When antibody binding has occurred, the enzyme substrate and cofactor are added, and the enzyme activity can be measured. If the sample contains hormone or drug, it will compete with enzyme-linked hormones for antibody binding. The enzyme will not be blocked by the antibody, and more enzyme activity will be measurable.

Enzyme-Linked Immunosorbent Spot Assay

The enzyme-linked immunosorbent spot (ELISPOT) assay is a common method for monitoring immune responses in humans and animals. It was developed by Cecil Czerkinsky in 1983. The ELISPOT assay is based on, and was developed from, a modified version of the ELISA. ELISPOT assays were originally developed to enumerate B cells secreting antigen-specific antibodies and have subsequently been adapted for various tasks, especially the identification and enumeration of cytokine-producing cells at the single-cell level. At appropriate conditions, the ELISPOT assay allows visualization of the secretory product of individual activated or responding cells. Each spot that develops in the assay represents a single reactive cell. Thus, the ELISPOT assay provides both qualitative (type of immune protein) and quantitative (number of responding cells) information.

By virtue of exquisite sensitivity of the ELISPOT assay, frequency analysis of rare cell populations (e.g., antigen-specific responses), which were not possible before, are now relatively easy. This exceptional sensitivity is, in part, because the product is rapidly captured around the secreting cell: before it is either diluted in the supernatant, captured by receptors of adjacent cells, or degraded. This makes ELISPOT assays much more sensitive than conventional ELISA measurements. Limits of detection are below 1/100,000 rendering enumerate the actively producing cells. This allows much of the analysis process to be automated and permits a greater level of accuracy than what can be achieved using manual inspection.

Procedure: As noted earlier, the ELISPOT assays employ a technique very similar to the sandwich ELISA technique. Either a monoclonal (preferred for greater specificity) or polyclonal capture antibody that is coated aseptically onto a polyvinylidenefluoride (PVDF)-backed microplate. These antibodies are chosen for their specificity for the analyte in question. The plate is blocked, usually with a serum protein that is nonreactive with any of the antibodies in the assay. After this, cells of interest are plated out at varying densities, along with antigen or mitogen, and then placed in a humidified 37°C CO_2 incubator for a specified period of time (Figure 36.2).

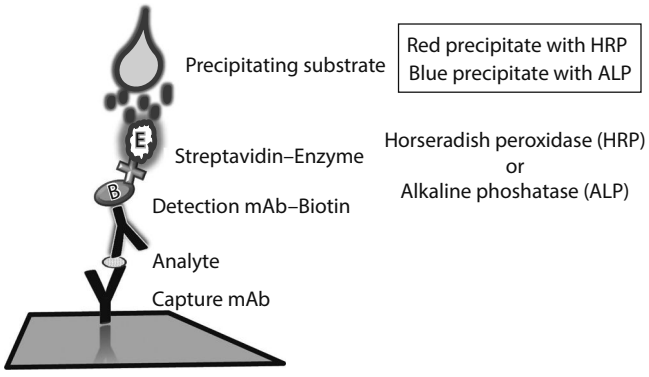

FIGURE 36.2 ELISPOT.

Cytokine (or other cell product of interest) secreted by activated cells is captured locally by the coated antibody on the high surface area PVDF membrane. After washing the wells to remove cells, debris, and media components, a biotinylated polyclonal antibody specific for the chosen analyte is added to the wells. This antibody is reactive with a distinct epitope of the target cytokine and thus is employed to detect the captured cytokine. Following a wash to remove any unbound biotinylated antibody, the detected cytokine is then visualized using an avidin-horseradish peroxidase (HRP), and a precipitating substrate (e.g., 3-amino-9-ethylcarbazole (AEC), 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT). The colored end product (a spot, usually a blackish blue) typically represents an individual cytokine-producing cell. The spots can be counted manually (e.g., with a dissecting microscope) or using an automated reader to capture the microwell images and to analyze spot number and size.

FluoroSpot Assay

The FluoroSpot assay is a modification of the ELISPOT assay and is based on using multiple fluorescent anticytokines, which makes it possible to spot two cytokines in the same assay.

Monoclonal Antibodies

All of the major hormones can be assessed using mAb techniques. In 1975, Köhler and Milstein[21] first discovered mAbs and devised an immunological method for producing large quantities of mAbs that could be targeted to specific proteins. Once mAbs for a given substance have been produced, they can be used to detect the presence of this substance. The western blot test and immuno dot blot tests detect the protein on a membrane. They are also very useful in immunohistochemistry, which detect antigen in fixed tissue sections and immunofluorescence test, which detect the substance in a frozen tissue section or in live cells. mAb techniques do provide a means of producing a specific antibody for binding antigen; this technique is useful for studying protein structure relations (or alterations) and has been used for devising specific RIAs. The advantages and disadvantages of mAbs compared to polyclonal antisera are listed in Table 36.5.

TABLE 36.5
Advantages and Disadvantages of Monoclonal Antibodies Compared to Polyclonal Antisera

Advantages	Disadvantages
Sensitivity	Overly specific
Quantities available	Decreased affinity
Immunologically defined	Diminished complement fixation
Detection of neoantigens on cell membrane	Labor intense; high cost

Source: Adapted from Srikanta, S. et al., *Diabetes*, 34, 300, 1985.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a sensitive technique to amplify a piece of DNA, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of repeated cycles of heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Two (one for each strand of DNA in a DNA double helix) sequence-specific primers (short DNA fragments) are designed to selectively target the DNA sequence of interest; these along with a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for subsequent replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR is used for a wide range of applications.

PCR uses a heat-stable DNA polymerase (Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*). This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, that is, alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary first to physically separate the two strands of a DNA double helix at a high temperature, to two single strands, in a process called DNA melting. At a lower temperature, the DNA primers will selectively bind each strand of their target sequence, and this event then *primes* the synthesis of the complement strand by DNA polymerase, using the original strand as a template (Figure 36.3).

The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions. PCR can be combined with reverse transcription, to detect RNA in a reaction termed reverse transcriptase polymerase chain reaction (RT-PCR).

Because the target DNA sequence is of a specific size, the DNA fragment can be run on an agarose gel, separated, and sized against a predefined DNA ladder by a process called gel electrophoresis (Figure 36.4). Because DNA is negatively charged, using agarose as a molecular sieve, and applying a current across the gel, the DNA will migrate towards the positive electrode. The agarose will separate DNA fragments of different sizes as smaller molecules find it easier to migrate through this matrix. Using an intercalating dye that specifically binds DNA, the fragments can be visualized, and a band of specific size, under ultraviolet (UV) light.

Applications for PCR include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases. PCR can be employed to detect mRNA for hormones, growth factors, polypeptides, receptors, and other proteins involved with the endocrine system.

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) is the use of PCR to determine the quantities of starting amounts of the target DNA. The simplest way to achieve this is by using RT PCR, or kinetic PCR, which is a technique based on PCR, and is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification of one or more specific sequences in a DNA sample, in real time, within the reaction tube. Although standard PCR can be semi quantitative, the term "qPCR" is now synonymously used to refer to RT PCR.

The procedure follows the general principle of PCR; its key feature is that the amplified DNA is detected as the reaction progresses, in real time, a fairly new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for detection of products in RT PCR are: non specific fluorescent dyes that intercolate with any double-stranded DNA, and also sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter, which permits detection only after hybridization of the probe with its complementary DNA (cDNA) target sequence (Figure 36.5).

Frequently, RT PCR is combined with reverse transcription to quantify RNA in a reaction termed RT-qPCR. Because the term "RT" is originally designated to mean "reverse transcription" in RT-PCR, the use of "RT" to mean "real time" PCR is an incorrect abbreviation. Sometimes, the term "quantitative real-time PCR (qRT-PCR)" is used to specify the use of real-time for qPCR, as opposed to qPCR done by standard endpoint PCR. It is important to clarify what the client means by the term "RT."

TaqMan Probes

TaqMan[114] probes are hydrolysis probes that are designed to increase the specificity of RT-PCR assays. The method was first described by researchers in 1991,[115] and the technology was subsequently developed for diagnostic assays and for research applications. TaqMan probe–based assays are widely used in RT-PCR in research and medical laboratories for gene expression assays, pharmacogenomics, human leukocyte antigen (HLA) genotyping, determination of the viral load in clinical specimens (HIV, tuberculosis, and hepatitis), bacterial identification[116] assays, DNA quantification, single-nucleotide polymorphism (SNP) genotyping, and verification of microarray results.

The TaqMan probe principle relies on the 5′–3′ exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophore-based detection.[117] As in other RT-PCR methods, the resulting fluorescence signal permits quantitative measurements of the accumulation of the product during the exponential stages of the PCR; however, the TaqMan probe significantly increases the specificity of the detection.

TaqMan probes consist of a fluorophore covalently attached to the 5′-end of the oligonucleotide probe and a quencher at the 3′-end.[118] Several different fluorophores

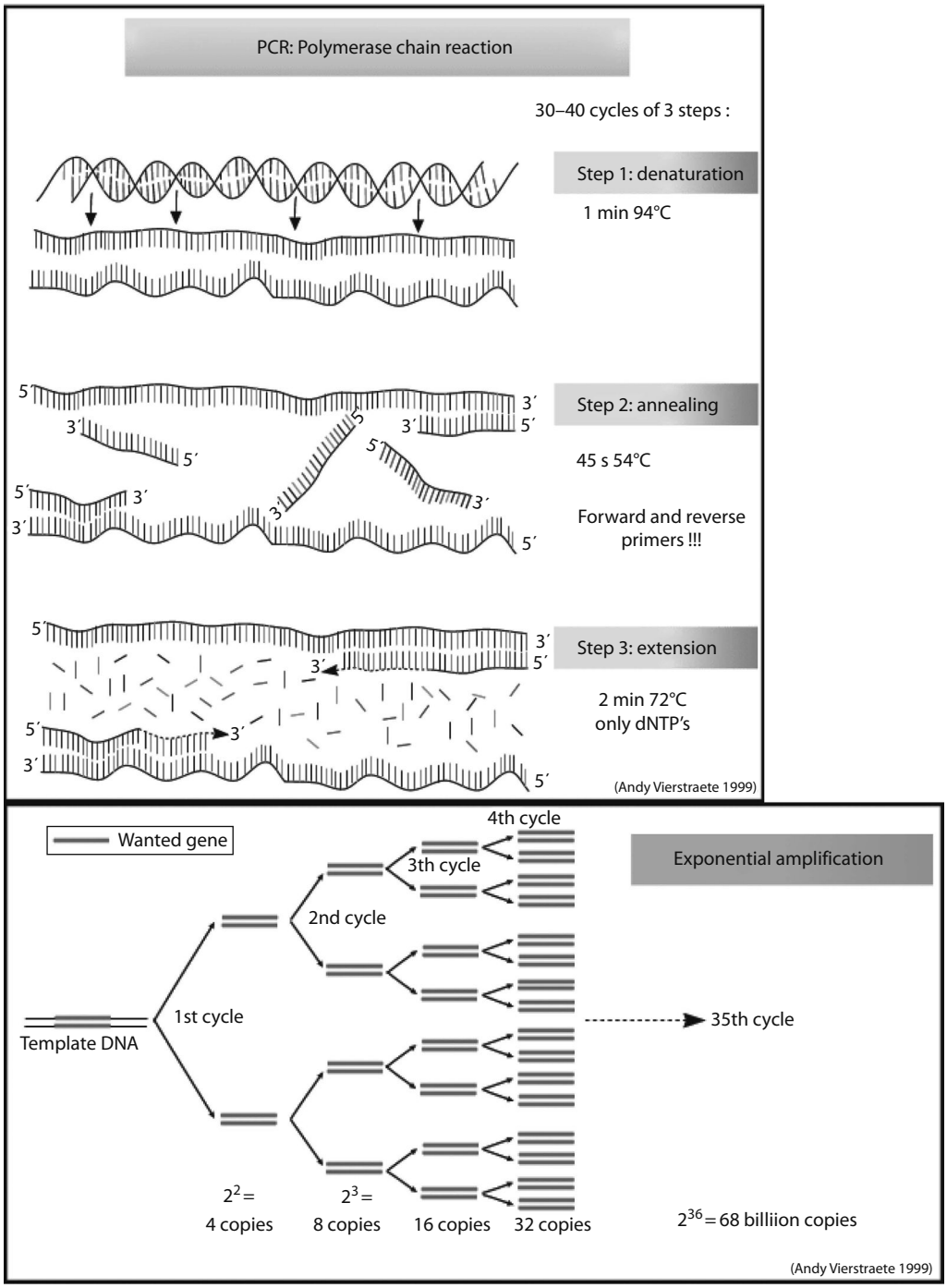

FIGURE 36.3 Polymerase chain reaction. Because both strands are copied during PCR, there is an *exponential* increase in the number of copies of the gene. Suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be two copies; after two cycles, there will be four copies; three cycles will result in eight copies; and so on. (From Vierstraete, A., *Principle of the PCR*, University of Ghent, revised May 20, 2012. http://users.ugent.be/~avierstr/principles/pcr.html.)

(e.g., 6-carboxyfluorescein or tetrachlorofluorescein) and quenchers (e.g., tetramethylrhodamine or dihydrocyclopyrroloindole tripeptide minor groove binder) are available.[119] The quencher molecule quenches the fluorescence emitted by the fluorophore when excited by the cycler's light source via fluorescence resonance energy transfer.[120] As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals.

TaqMan probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes the nascent strand, the 5′–3′ exonuclease activity of the polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore.

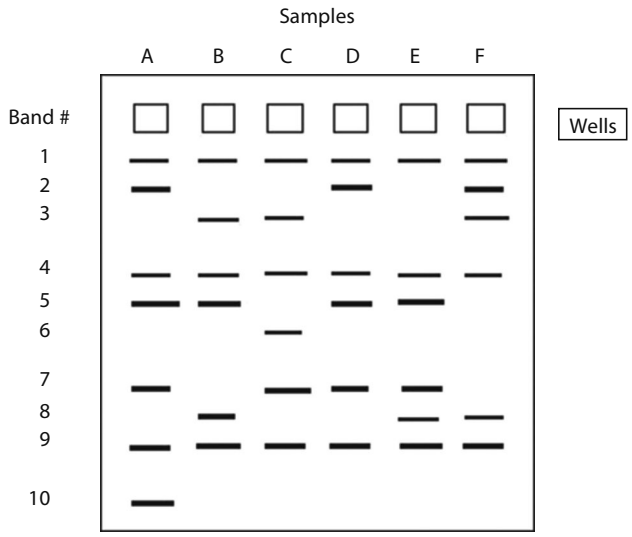

FIGURE 36.4 Gel electrophoresis. The agarose gel provides a matrix with pores to allow molecules to travel through and be sorted by size. The DNA molecules are loaded into the wells. The negative pole is located closest to the wells. The positive pole is located at the other end of the gel. An electric current causes the negatively charged DNA molecules to move toward the positive pole. Longer DNA fragments take longer to work their way through the pores of the gel matrix; they do not travel as far through the gel as the shorter fragments in the same amount of time. Each band in the drawing of the agarose gel represents many DNA fragments that are the same size. The bands nearest the wells (containing the longest DNA fragments) traveled the slowest (Band #1). The bands farthest from the wells (containing the shortest DNA fragments) traveled the fastest (Bands #10).

Hence, fluorescence detected in the RT-PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR.

Gene Expression

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins, but the product is a functional RNA in nonprotein coding genes such as ribosomal RNA (rRNA), transfer RNA (tRNA), or small nuclear RNA (snRNA) genes. Several steps in the gene expression process, including the transcription, RNA splicing, translation, and posttranslational modification of a protein,, may be modulated. Gene regulation gives the cell control over structure and function and is the basis for cellular differentiation, morphogenesis, and the versatility and adaptability of any organism.

The effect of an endocrine modulator or disruptor can influence gene expression via transcriptional control. By increasing or decreasing the rate of gene transcription, one can effectively modify gene expression. By quantitating the amount of mRNA for a given gene, it is possible to measure the effect on transcriptional gene expression.[121] Because mRNA represents a relatively small fraction of RNAs in the cell, methods have been developed that are sensitive and specific for detecting changes in mRNA levels and can distinguish between tRNA and rRNA. The following experimental techniques are used to measure gene expression and are listed in roughly chronological order, starting with the older, more established technologies. They are divided into two groups based on their degree of multiplexity. Low-to-mid-plex techniques include reporter gene, northern blot, western blot, fluorescent in situ hybridization, and RT-PCR while higher-plex techniques include SOLiD-SAGE assay (serial analysis of gene expression), DNA microarray, tiling array, and RNA-sequencing (RNA-Seq).

Reporter genes can also be used to assay for the expression of the gene of interest, which may produce a protein that has little obvious or immediate effect on the cell culture or organism. In these cases, the reporter is directly attached to the gene of interest to create a gene fusion. The two genes are under the same promoter elements and are transcribed into a single mRNA molecule. The mRNA is then translated into protein. In these cases, it is important that both proteins be able to properly fold into their active conformations and interact with their substrates despite being fused. In building the DNA construct, a segment of DNA coding for a flexible polypeptide linker region is usually included so that the reporter and the gene product will only minimally interfere with one another.

Reporter genes can be used to assay for the activity of a particular promoter in a cell or organism. In this case, there is no separate *gene of interest*; the reporter gene is simply placed under the control of the target promoter, and the reporter gene product's activity is quantitatively measured. The results are normally reported relative to the activity under a *consensus* promoter known to induce strong gene expression.

A northern blot is a general blotting procedure[122] that starts with extraction of total RNA from a homogenized tissue sample or from cells. Eukaryotic mRNA can then be isolated through the use of oligo (dT) cellulose chromatography to isolate only those RNAs with a poly(A)tail.[123,124] RNA samples are then separated by gel electrophoresis. The RNA samples, now separated by size, are transferred to a nylon membrane through a capillary or vacuum blotting system. Once the RNA has been transferred to the membrane, it is immobilized through covalent linkage to the membrane by UV light or heat. After a probe has been labeled, it is hybridized to the RNA on the membrane. The membrane is washed to ensure that the probe has bound specifically and to avoid background signals from arising. The hybrid signals are then detected by x-ray film and can be quantified by densitometry. To create controls for comparison in a northern blot, samples not displaying the gene product of interest can be used after determination by microarrays or RT-PCR.[125]

The western blot (sometimes called the protein immunoblot) is a widely accepted analytical technique used to detect specific proteins in the given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein.[126,127]

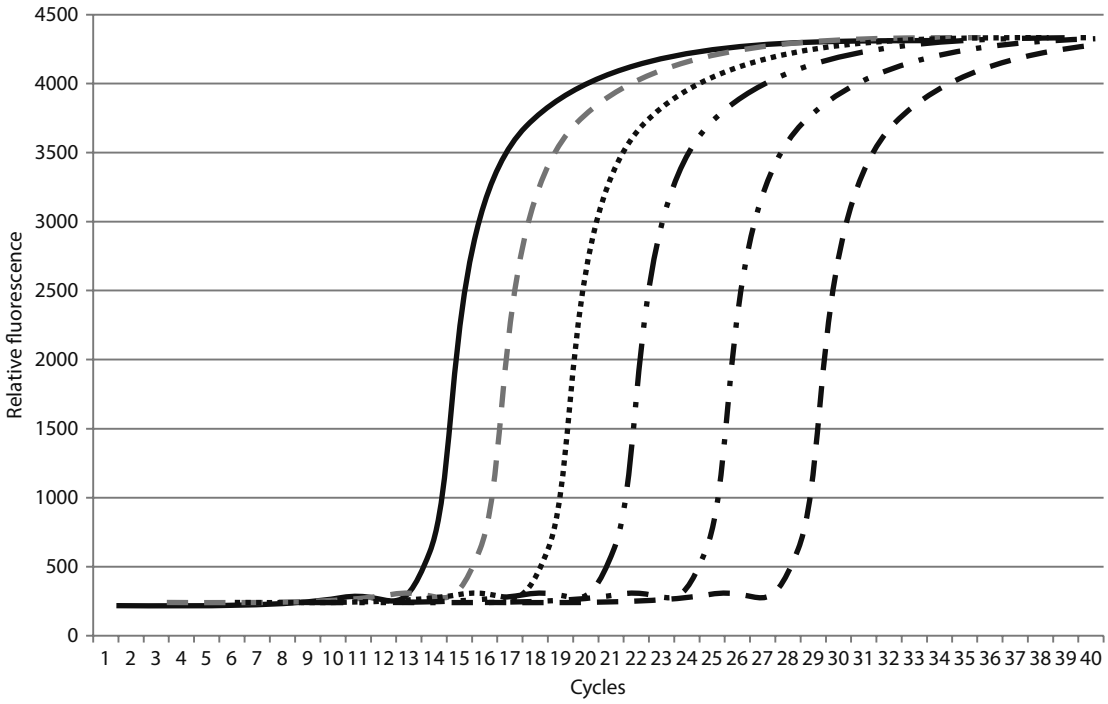

FIGURE 36.5 Concentration curves.

There are now numerous companies that specialize in providing antibodies (both monoclonal and polyclonal antibodies) against thousands of different proteins.[128] Commercial antibodies can be expensive, although the unbound antibody can be reused between experiments. This method can be used for hormone gene expression.

Fluorescence in situ hybridization (FISH) is a cytogenetic technique developed by biomedical researchers in the early 1980s[129] that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence complementarity. Fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosomes. FISH is often used for finding specific features in DNA for use in genetic counseling, medicine, and species identification. FISH can also be used to detect and localize specific RNA targets (mRNA, Inc [Incompatibility] RNA [a natural antisense RNA with 72 nucleotides forming a stem-loop structure], and micro-RNA) in cells, circulating tumor cells, and tissue samples. In this context, it can help define the spatial-temporal patterns of gene expression within cells and tissues.

RT-PCR is one of many variants of PCR that is commonly used in molecular biology to detect RNA expression levels.[130] RT-PCR is often confused with real-time polymerase chain reaction (qPCR).[131] However, they are separate and distinct techniques. While RT-PCR is used to qualitatively detect gene expression through creation of cDNA transcripts from RNA, qPCR is used to quantitatively measure the amplification of DNA using fluorescent probes. qPCR is also referred to as quantitative PCR, quantitative real-time PCR,[132] and real-time quantitative PCR.[133]

Although RT-PCR and the traditional PCR both produce multiple copies of particular DNA isolates through amplification, the applications of the two techniques are fundamentally different. The traditional PCR is simply used to exponentially amplify given DNA sequences. RT-PCR is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement through the use of reverse transcriptase. Subsequently, the newly synthesized cDNA is amplified using traditional PCR.

In addition to qualitative study of gene expression, RT-PCR can be utilized for quantification of RNA[134] by incorporating qPCR into the technique. The combined technique, described as quantitative RT-PCR[135] or real-time RT-PCR[136] (sometimes even quantitative real-time RT-PCR[137]), is often abbreviated as qRT-PCR,[138] RT-qPCR,[139] or RRT-PCR.[140] Compared to other RNA quantification methods, such as northern blot, qRT-PCR is considered to be the most powerful, sensitive, and quantitative assay for the detection of RNA levels. It is frequently used in the expression analysis of single or multiple genes and expression patterns for identifying infections and diseases.

SAGE is a technique used by molecular biologists to produce a snapshot of the mRNA population in a sample of interest in the form of small tags that correspond to fragments of those transcripts. The original technique was developed by Dr. Victor Velculescu in 1995.[141] Several variants have been developed since, most notably a more robust version,

LongSAGE,[142] Robust LongSAGE,[143] and the most recent SuperSAGE.[144] Many of these have improved the technique with the capture of longer tags, enabling more confident identification of a source gene.

A DNA microarray[145] (also known as a biochip or DNA chip) is a collection of microscopic DNA spots attached to a solid surface. DNA microarrays are used to measure the expression levels of large numbers of genes simultaneously. Each DNA spot contains picomoles of a specific DNA sequence, known as *probes* (or *reporters or oligos*). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called antisense RNA) sample (called *target*) under high-stringency conditions. Probe–target hybridization is usually detected and quantified by the detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Tiling arrays[146] are a subtype of microarray chips. Like traditional microarrays, they function by hybridizing labeled DNA or RNA target molecules to probes fixed onto a solid surface. Tiling arrays differ from traditional microarrays in the nature of the probes. Instead of probing for sequences of known or predicted genes that may be dispersed throughout the genome, tiling arrays probe intensively for sequences that are known to exist in a contiguous region. This is useful for characterizing regions that are sequenced, but whose local functions are largely unknown. Tiling arrays aid in transcriptome mapping as well as in discovering sites of DNA/protein interaction, of DNA methylation, and of sensitivity to DNase.[147] In addition to detecting previously unidentified genes and regulatory sequences, improved quantification of transcription products is possible. Specific probes are present in millions of copies (as opposed to only several in traditional arrays) within an array unit called a feature, with anywhere from 10,000 to more than 6,000,000 different features per array. Variable mapping resolutions are obtainable by adjusting the amount of sequence overlap between probes, or the amount of known base pairs between probe sequences, as well as probe length. Tiling arrays are a useful tool in genome-wide association studies.

RNA-seq,[148] also called whole transcriptome shotgun sequencing[149] (WTSS), refers to the use of HTP sequencing technologies to sequence cDNA in order to get information about a sample's RNA content. The technique has been rapidly adopted in studies of diseases like cancer. With deep coverage and base-level resolution, next-generation sequencing provides information on differential expression of genes, including gene alleles and differently spliced transcripts; non coding RNAs; posttranscriptional mutations or editing; and gene fusions.[150] The technique has been dubbed *a revolutionary tool for transcriptomics*.[151]

In Situ Hybridization and Immunohistochemistry

In situ hybridization is a type of hybridization that utilizes labeled nucleic acid probes (either DNA or RNA), which permits the detection and localization of mRNA in tissue samples with labeled nucleic acid probes (DNA or RNA). Tissue samples (or cultured cells) are treated to increase permeability, thus allowing the desired probe to enter the cell. The samples are fixed, embedded, and thinly sectioned prior to hybridization. Hybridization is permitted at elevated temperatures, and the excess probe is then removed. A complementary probe is labeled with an antigenic, fluorescent, or radioactive tag to precisely locate and quantify the probe remaining in the tissue. Radiolabeled probes work well for abundant mRNAs and can be detected using autoradiography. Other methods of signal detection include fluorescence, such as FISH, which is a cytogenetic technique used to detect and localize specific DNA sequences on chromosomes. Fluorescence microscopy is used to localize specific DNA sequences on chromosomes. For example, a probe is constructed and tagged with fluorophores for specific targets. A chromosome preparation is done with the chromosomes fixed to a glass substrate whereupon the probe is applied to the DNA of the chromosome and allowed to hybridize. Unbound probe is removed. If a very limited amount of probe remains, fluorescent-tagged antibodies or streptavidin is bound to the tagged molecules, resulting in amplified fluorescence. This preparation is embedded and subsequently examined under the fluoroscence microscope. Low-abundance mRNAs can be detected by in situ PCR, which amplifies the amount of nucleic acid target in the tissue. This technique is particularly useful for detecting hormone or receptor subtypes that are biologically unique by combining immunogenic features with related biological molecules. Generally, probe specificity parameters are first tested by northern blot analysis.

Immunochemistry refers to a process of detecting and localizing gene products (e.g., proteins) in cells of a tissue section utilizing antibodies.[152] The antibody is tagged with a color-producing tag, which can include such materials as alkaline phosphatase or HRP. Primary monoclonal or polyclonal antibodies can be used to bind specific epitopes of a hormone or receptor, which can then be visualized with a secondary marker, such as fluorochrome-conjugated secondary antibody or streptavidin–biotin labels. Selection of a fluorochrome depends on microscopy wavelength and filters, the stability of the signal, the type of the tissue being examined, and whether or not double-labeling is necessary. Nonfluorescent markers, such as immunoperoxidase or immunogold conjugates, are suitable for bright-field microscopy and offer increased stability over fluorochrome conjugates. The technique is widely used in basic research to help characterize the distribution and localization of biomarkers in different parts of a tissue. Some specific markers are known for specific cancers, such as carcinoembryonic antigen, a marker for colon cancer; CD15 and CD30, markers for Hodgkin's disease (cluster of differentiation [CD] molecules are recognized by specific sets of antibodies, used to identify the cell type, stage of differentiation, and activity of a cell); and prostate-specific antigen, a marker for prostate cancer; and CD117, a marker for gastrointestinal stromal tumors.[13]

Immunohistochemistry is a powerful complement to in situ hybridization, but limitations include the lack of antibody

specificity, denaturation of the antigen during fixation, and cell membrane permeabilization, which refers to the changes that must occur in the plasma membrane to allow for the entry of impermeable fluorescent probes and the binding of antibodies to their respective antigens.

PITUITARY GLAND

Overview

The pituitary gland, which includes the neurohypophysis and the adenohypophysis, is located at the base of the brain in a small cavity called the *sella turcica* (Turkish saddle), which is a saddle-shaped depression in the sphenoid bone at the base of the human skull (see Figure 36.6).[153] The pituitary secretes several tropic hormones that regulate the activities of cells within distant endocrine glands. The hypothalamic–pituitary axis is the union formed by the interaction between the hypothalamus and pituitary gland. It exerts control over many parts of the endocrine system. This axis functions by interacting between the nervous and endocrine systems; the nervous system regulates the endocrine system, and endocrine activity modulates the activity of the central nervous system (CNS). The pituitary is physically attached to the brain by the hypophyseal stalk connected through the median eminence. The median eminence is integral to the hypophyseal portal system, which connects the hypothalamus with the anterior lobe of the pituitary gland. The anterior lobe of the pituitary (i.e., adenohypophysis) receives releasing and inhibitory hormones on their way to the medium eminence, where they collect before entering the portal system. The hypothalamus and the anterior pituitary secrete a number of important hormones (Table 36.6). The posterior pituitary (i.e., neurohypophysis) is primarily a conduit for the hypothalamus. It does not produce its own hormones; instead, it stores and releases the hormones oxytocin and vasopressin into the blood stream.

Pituitary and Target Organ Relationships

To understand the toxicology of the endocrine system, it is necessary to grasp the concept of hormonal feedback systems (see Figure 36.1). Although measurement of specific hormone levels might not be feasible or economical for the general toxicologic screening of a substance, some bioassays and microscopic techniques do yield useful information. The reduction in animal growth rates, although in most instances caused by diminished nutritional intake, might be due to the suppression of pituitary GH secretion. Similarly, a decrease in testicular weight following the administration of certain chemicals can be due to interference with pituitary gonadotropins and not a simple direct toxic effect upon the primary target.[154,155]

Chemically induced changes that affect pituitary–target organ relationships seldom are manifested after a single administration of a toxic substance; rather, compounds that have the potential to exert deleterious effects on the endocrine system ordinarily require multiple administrations and longer durations of time before hormonally complex events can occur. Whereas chemically induced stress could provoke a rapid response in catecholamine secretion and production of GCs, other hormonal responses would not be as immediate. For those chemicals that initiate the induction of hepatic microsomal enzyme systems that affect hormone metabolism (i.e., catabolism), it may be a week or longer before any changes are detected in the endocrine system. Even those chemicals or drugs purposely designed to suppress a particular hormone target organ secretion (e.g., antithyroidal agents) may exhibit an onset of action of several days.

Many steps are involved in the regulation of target organs by the endocrine system (see Figure 36.1). No less than four possible sites can hormonal messages be disrupted by various toxic agents. Chemicals, including certain classes of therapeutic drugs, can interfere with the release of tropic hormones or can affect their synthesis. Still other toxic agents can exert disruptive actions on the CNS or at

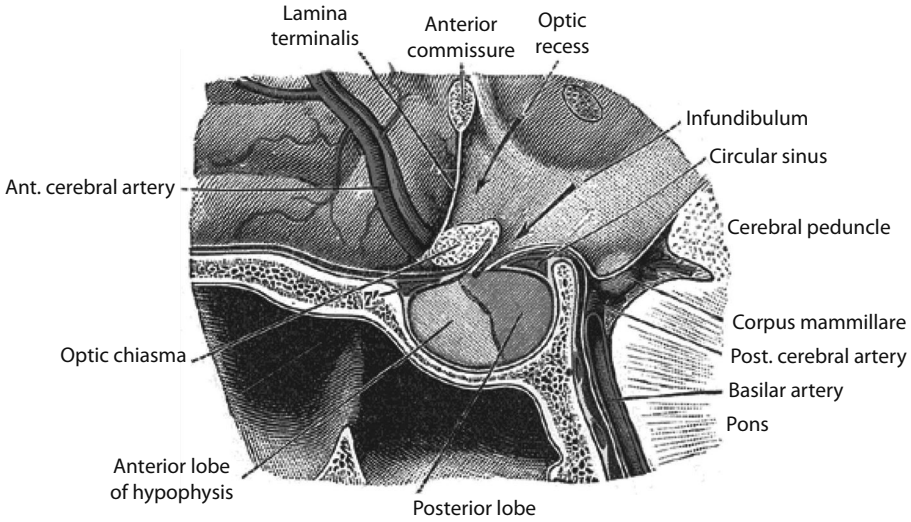

FIGURE 36.6 Pituitary gland. (From Gray's Anatomy.)

TABLE 36.6
Major Hormones Secreted by the Adenohypophysis and Their Respective Releasing Hormones

AdenohypophySeal Tropic Hormone	Hypothalamic-Releasing Factors	Hypothalamic Release–Inhibiting Factors	Target	Effect
ACTH	CRH	—	Adrenal gland	Secretion of GLs (Gls and androgens) in adrenocortical cells
Follicle-stimulating hormone (FSH), luteinizing hormone (LH)	Gonadotropin-releasing hormones (GnRH)	—	Ovary, testis	Females: maturation of ovarian follicles Males: spermatogenesis; enhances production of androgen-binding protein by the Sertoli cells of the testis
LH	GnRH	—	Gonads	Females: ovulation Males: stimulates Leytid cell production of testosterone
GH or HGH, Somatotropin	Growth-hormone-releasing hormone (GHRH); somatotropin	Somatotropin release–inhibiting factor (SRH)	Liver, adipose tissue	Promotes growth; lipid metabolism Releases insulin-like growth factor-1 from liver
Prolactin (PRL)	Prolactin-releasing hormone (PRH)	Prolactin-inhibitory hormone (PIH); prolactin-releasing hormone (PRH); dopamine	Ovaries and mammary glands	Secretion of estrogens and progesterone; milk production in mammary glands; sexual gratification after sexual acts
TSH	TRH	Somatostatin	Thyroid gland	Secretion of thyroid hormones (thyroxine (T) and triiodothyronine (T_3)
Melanocyte-stimulating hormone (MSH or α-MSH)	Melanocyte-stimulating release hormone (MRH)	Melanocyte-inhibiting hormone (MIH)	Melanocytes	Synthesis of melanin by melanocytes in skin and hair

Sources: Adapted from Gardner, D.G. and Nissenson, R.A., in *Basic and Clinical Endocrinology*, 7th edn., Greenspan, F.S. and Gardner, D.G., eds., McGraw-Hill, New York, pp. 61–84, 2004; Porterfield, S.P., ed., *Endocrine Physiology*, 2nd edn., Mosby, St. Louis, MO, 2001; Wikipedia. Anterior Pituitary, 2014, http://en.wikipedia.org/wiki/Anterior_pituitary.

the hypothalamus or target organ itself in the biosynthesis of target organ hormone regulatory secretions. Thus, various chemicals have several sites of action on adenohypophyseal–target organ feedback systems.

The sites of a chemical's action may differ in their sensitivity. Target organs such as the gonads are frequently sensitive to toxic substances, particularly because rapidly dividing cells (e.g., spermatogonia) are often vulnerable to chemicals and drugs. Furthermore, stress can affect the secretory activity of certain of the hypothalamic-releasing hormones and hence alter pituitary–target organ relationships (e.g., ACTH and prolactin). Often, toxic agents bind to circulating blood proteins and alter the ratio of free to bound target organ hormones. Such changes in binding also can modify the pituitary–target organ relationship. Numerous target organs, then, can be affected by chemical perturbation.

Adenohypophysis

The adenohypophysis, also called the *anterior pituitary*, comprises the anterior lobe of the pituitary gland (see Figure 36.6). Hormone secretion from the anterior pituitary gland is regulated by hormones secreted by the hypothalamus. Neuroendocrine neurons in the hypothalamus project axons to the median eminence, at the base of the brain. At this site, these neurons can release substances into small blood vessels that travel directly to the anterior pituitary gland (the hypothalamo-hypophysial portal vessels). The anterior pituitary gland secretes hormones that regulate processes such as growth, stress, and reproduction. The major hormones of the adenohypophysis and their hypothalamic-releasing hormones and targets, as well as effects, are shown in Table 36.6. The glycoproteins include TSH, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG). Each hormone is a heterodimer of two noncovalently associated subunits α and β, which are encoded by separate genes situated on different chromosomes. It is the β subunit that generally confers the unique biological specificity of each hormone. This biological activity is dependent on the intact dimers; free subunits are biologically inactive.

Accurate measurement of these proteins to understand their biological functions and molecular mechanisms of the functions is crucial. Because tropic hormones are either

protein or glycoproteins with half-lives of 20 min to 6 h, traditional laboratory methods, such as RIA and ELISA, are not able to measure multiple proteins with a small sample volume. MILLIPLEX® MAP Human Pituitary Magnetic Bead Panel 1[156] as well as the rat, dog, and nonhuman primate panels can be used for the simultaneous measurement of the following analytes in any combination: ACTH, FSH, GH, LH, and TSH. This system allows simultaneous identification of pituitary hormones with antibodies chemically attached to fluorescently labeled microbeads. The beads are resuspended in assay buffer, and the reaction mixture can be quantified using a protein array reader. This multiplex assay can analyze these seven proteins simultaneously and uses a small sample volume 25 μL.

Although bioassays may be useful for certain of the adenohypophyseal hormones, such tests are often inaccurate or have been replaced by more sensitive methods. Bioassays, however, might be employed when there is only a secondary interest in determining if a particular toxicologic agent is affecting tropic hormone levels. Sometimes, a target organ that is known to be directly influenced by a particular tropic hormone can be measured and hence provide some general insight into the nature of the chemically induced alterations in the endocrine system. Target organs whose secretions (e.g., estrogen, testosterone, and T_4) act back upon the adenohypophysis are usually low-molecular-weight hormones.

MEASUREMENT OF ANTERIOR PITUITARY HORMONES

Adrenocorticotropin

ACTH stimulates the growth and the maintenance of the adrenal gland, in addition to stimulating the adrenal cortex to secrete cortical steroids. Many pathologic states can alter ACTH secretion. Stress, caused by a variety of environmental or chemical stimuli, including surgery, trauma, infection, hypoxia, and anxiety, can cause a rapid elevation in ACTH blood levels. ACTH and cortisol exhibit diurnal variations, with the highest levels occurring in the morning (about 8:00 a.m.) and the lowest levels in late afternoon. The hypothalamic–pituitary–adrenal axis has many modulators, many of which are mediated through the CNS and stimulate or inhibit the secretion of ACTH. Measurements of plasma ACTH are extremely useful in the diagnosis of both Cushing's syndrome and adrenal insufficiency.

Prior to the development of RIA, the bioassays employed for the determination of ACTH were highly complex, time-consuming, and costly in terms of the number of animals used. Gravimetric assay of adrenal glands represented one of the simplest methods for indirectly evaluating ACTH activity. This bioassay uses hypophysectomized animals; injections of ACTH-like material can enhance the weight of the adrenal glands. Their sensitivity was such that the normal early morning peak of ACTH could not be determined.

The introduction of immunoassay methodology enabled the measurement of low-normal ACTH concentrations. Immunological recognition of ACTH by the antibodies employed offered improvements with regard to specificity. The development of two-site immunometric assays further improved specificity and the ability to measure low-normal ACTH concentrations without the need to extract large volumes of plasma. The quantification of ACTH is now routinely performed in clinical laboratories, with nonradioisotopic methods becoming increasingly popular. The highly sensitive IRMA ACTH assay has been very useful in diagnosing adrenal deficiencies.[157] In addition to the MILLIPLEX® MAP Pituitary Magnetic Bead Panel, other methods such as ELISA (manual), two-site IRMA (manual), double-antibody RIA (manual), one-step immunochemiluminometric assay, and two-site sequential immunochemiluminometric assay. The current commercially available two-site immunometric assays have an analytical sensitivity between 0.6 and 9 pg/mL (0.12–19.8 pmol/L).[158] Plasma ACTH concentrations are usually between 10 and 60 pg/mL (2.2 and 13.3 pmol/L) at 8 a.m. with the values decreasing during the waking hours. The plasma ACTH values are usually less than 20 pg/mL (4.5 pmol/L) at 4 p.m. and usually less than 5 pg/mL (1.1 pmol/L) within 1 h after the usual time of falling sleep. This circadian rhythm in plasma ACTH concentrations is the cause of the parallel changes in cortisol secretion by the adrenal glands and the resulting rhythm in serum cortisol concentrations.

Because ACTH stimulates increases in plasma cortisol and corticosterone and elevates urinary 17-hydroxycorticosteroids and 17-ketosteroids, these steroid levels can also be used to assess ACTH. ACTH causes involution of the thymus gland and deposition of hepatic glycogen and leads to a decrease in circulating eosinophils in hypophysectomized rodents. ACTH can also cause depletion of adrenal ascorbic acid. In addition to those assays for ACTH that rely on adrenal gland responses (see section on adrenal glands), radioligand–receptor assays have been developed for ACTH. Often, cortisol levels are used to assess ACTH. Cortisol can be determined using commercially available antibody-coated tube RIA kits.

Thyroid-Stimulating Hormone

TSH is a glycoprotein that is synthesized and secreted by the thyrotrope cells of the anterior pituitary gland. This hormone regulates the growth and proliferation of cells of the thyroid gland. In this case, the hypothalamus produces TRH, which subsequently stimulates the anterior pituitary to produce and release TSH. TSH, in turn, stimulates the thyroid gland to secrete the hormone T_4, which has only a slight effect on metabolism. T_4 is converted to triiodothyronine (T_3), which is the active hormone that stimulates metabolism. About 80% of this conversion is in the liver and other organs, and 20% in the thyroid itself.[159] Somatostatin is also produced by the hypothalamus and has an opposite effect on the pituitary production of TSH, decreasing or inhibiting its release.

TSH is a glycoprotein and consists of two subunits, the α and β subunits. The α subunit (i.e., chorionic

gonadotropin alpha) is nearly identical to that of hCG, LH, and FSH. The α subunit is thought to be the effector region responsible for stimulation of adenylate cyclase (involved in the generation of cAMP). The α chain has a 92-amino acid sequence. The β subunit (TSHB) is unique to TSH and therefore determines its receptor specificity. The β chain has a 118-amino acid sequence. TSH is secreted throughout life but particularly reaches high levels during the periods of rapid growth and development.

These tyrosine-based hormones function to increase the basal metabolic rate and make changes to the rate of protein, carbohydrate, and fat synthesis. T_3 and T_4 (thyroid) as well as somatostatin (hypothalamus) negatively feedback to inhibit TSH production. The TSH receptor site is complex and involves an extracellular domain as well as a transmembrane component.[160] The TSH receptor is found mainly on thyroid follicular cells.[161] Stimulation of the receptor increases T_3 and T_4 production and secretion. Stimulating antibodies to this receptor mimic TSH and can cause Graves' disease. In addition, hCG shows some cross-reactivity to the TSH receptor and therefore can stimulate production of thyroid hormones. In pregnancy, prolonged high concentrations of hCG can produce a transient condition termed gestational hyperthyroidism.[162]

In addition to the MILLIPLEX MAP Pituitary Magnetic Bead Panel, other methods such as immunochemistry assays are also available for measuring TSH.[163,164] A liquid-phase, two-site IRMA has been described for human TSH (hTSH). The TSH IRMA is based on the simultaneous addition of affinity-purified sheep anti-hTSH IgG-^{125}I and rabbit anti-hTSH antiserum. This assay is specific for hTSH and exhibits no cross-reactivity with other pituitary glycoprotein[165]; thus, the IRMAs for TSH may be more specific than current RIAs for TSH. The one-step IRMA method involves the use of monospecific antibody against two immunogenic sites on the TSH molecule.[166] Other assays for thyrotropin involve a combination of bioluminescence and immunoassay techniques.[167] The therapeutic target range TSH level for patients on treatment ranges between 0.3 and 3.0 μIU/mL.[168]

Growth Hormone (Somatotropin)

GH is also referred to as *somatropin*, *somatotropin*, or *somatotrophin*. Somatotropic cells constitute 30%–40% of anterior pituitary cells. They release GH in response to GHRH, or somatocrinin, or are inhibited by growth-hormone-inhibiting hormone (GHIH) (somatostatin), both received from the hypothalamus via the hypophyseal portal system vein and the secondary plexus. Somatotrope cells are classified as acidophilic cells. These cells take years to grow and mature very slowly. If these cells grow large enough, they can impair vision, cause headaches, or damage other pituitary functions.

Human GH is a peptide composed of 191 amino acids with a molecular weight of 22,000 Da. The structure contains four helices that precisely interact with the GH receptor. GH is secreted by the somatotrope cells found in the anterior pituitary. It has been found that transcription factor Pit-1 stimulates the development of somatotrope cell and subsequently the production of GH. GHRH released from the hypothalamus promotes the secretion of GH, while somatostatin from the periventricular nucleus inhibits GH production, as does the concentration of GH and insulin-like growth factor 1 (IGF-1), a protein similar in structure to insulin that is produced by target cells and the liver and is involved in the regulation of metabolism. GH exerts its actions on a variety of cells to stimulate lipolysis, protein anabolism, and hyperglycemia. Its actions on bone and cartilage are mediated primarily through IGF-1. A deficiency in GH leads to a reduction in the incorporation of amino acids into protein. GH causes a marked stimulation of cartilaginous growth at the epiphyses of long bones.

A constant flow of stimulating and inhibiting peptides enhances or suppresses the rate of secretion of GH. In addition, many physiological events effect changes in GH secretion. Many of the agents and physiological factors that can affect the rate of GH secretion are listed in Table 36.7. Hypoglycemia or insulin can cause a sudden and dramatic increase in serum GH. Starvation can affect GH levels, while cold, stress, or surgical trauma can lead to an increase in serum GH. Drugs and chemicals that affect catecholamine neurotransmission and the autonomic nervous system can influence GH secretion.

It has been shown that most of the physiologically critical GH secretions generally occur as several large surges over the course of the day that may extend from 10 to 30 min, with the largest GH peak occurring during the first hour of sleep.

TABLE 36.7
Effects of Various Factors on GH Levels

Increased GH Levels	Decreased GH Levels
Apomorphine	Dietary carbohydrate and GCs
Argininea[a]	Genetic conditions
Clonidine[a]	Sleep
a-Deoxyglucose	Somatostatin
Dietary protein	Pituitary adenoma
L-DOPA[a]	Severe head injury
Endorphins	Autoimmune disease
Enkephalins	Interference with the blood supply to the pituitary gland
Epinephrine	Pituitary damage radiotherapy
Estradiol	
Exercise	
Insulin (hypoglycemia)[a]	
Norepinephrine	
Prostaglandins	
Serotonin	
Stress	
Substance P	
TRH	
Vasopressin	

[a] Used as a provocative test for the diagnosis of GH disorders.

The frequencies are most pronounced (8 peaks per 24 h), and the levels of GH are highest during early childhood and at puberty during adolescence, whereas the mature adult averages approximately 5 peaks per 24 h.

GH has been assessed using bioassays. Some GH assays simply use a 10-day body weight gain test in hypophysectomized female rats. The hormone has also been assayed by measuring the width of the tibial epiphyseal growth plate. Sensitive RIAs have been developed for experimental animals (e.g., rat) and for humans. Human GH concentrations can be measured with double-antibody RIAs. Immunocytologic assays for GH can also be performed on epon-embedded, semi-thin sections of tissue using the avidin–biotin–peroxidase complex technique.[169] Microdissection techniques[170] and other in vitro assays for the release of GH have been employed.[163]

Over the last few decades, measurement technology has evolved from less sensitive, mainly radioactive assays based on polyclonal antisera, to the latest generations of highly sensitive chemiluminescence methods employing mAbs. Sensitive RIAs have been developed for experimental animals (e.g., rat) and for humans. Human GH concentrations can be measured with double-antibody RIAs. In addition, an ultrasensitive immunofluometric assay[171] and a chemiluminescence-based GH assay[172] are now available.

Whether GH is assessed using bioassays or by the more accurate and sensitive RIA procedures, the experimental design of either acute or chronic toxicity tests must closely monitor the nutritional and behavioral status of the animals. Several toxic agents can affect dietary intake and hence reduce body weight. Experimental designs using paired-feeding protocols are often necessary for interpreting GH activity in any well-designed toxicology protocol.

Gonadotropins (FSH and LH)

Gonadotropins are hormones secreted by the gonadotrope cells located in the anterior pituitary gland. The two primary gonadotropins are the FSH and LH, both of which are glycoproteins that contain α and β peptide subunits. The α units in both hormones are essentially identical, but there is some functional and immunologic specificity with the β subunits of the gonadotropins, including hCG, which is produced by the placenta during pregnancy.

Gonadotropin receptors are found on the surface of the gonads, and these are connected to the G-protein system. G-proteins are guanine nucleotide–binding proteins, a group of specific proteins involved in second-messenger feedback systems. Second messengers are used in signal transduction to relay signals within a particular cell in response to an external signal received by a transmembrane receptor. These signals are relayed by cyclic AMP when the receptor is bound by the G-protein.

The critical gonadotropins, FSH and LH, are secreted from the adenohypophysis as directed by the gonadotropin-releasing hormone (GnRH) from the hypothalamus. Their principal stimulatory actions and primary targets are the testes or ovaries. FSH stimulates follicular development in the ovary and spermatogenesis in the testes. LH, also referred to as *interstitial-cell-stimulating hormone* (ICSH) in the male, causes luteinization of the ovary and stimulates androgen production in testicular Leydig cells. The gonads contain receptors for LH and FSH, which are involved in intracellular signaling.[26]

In the human female, blood levels of FSH and LH vary according to the phase of the menstrual cycle. In human males, although some diurnal fluctuation in FSH and LH may occur, blood levels are noncyclic.

In response to FSH, Sertoli cells in the seminiferous tubules secrete a number of proteinaceous substances, including androgen-binding protein (ABP), which binds testosterone within the testes presumably to maintain high levels of testosterone at the site of the developing germ cells. ABP has been used as an indicator of toxicologic insult following the administration of potentially damaging chemicals or drugs. FSH or LH levels can be assessed by direct measurement of the hormones or by indirect assays that reflect an influence of these hormones on their target tissues. RIAs are available to measure gonadotropins.

Earlier methods in classical endocrinology studies often employed bioassays. FSH has been bioassayed by assessing its ability to enhance ovarian weight in immature rats treated with a placental gonadotropin. A sensitive assay for LH once employed ovarian ascorbic acid depletion. The bioassay of gonadotropins has largely been abandoned because of low sensitivity and expense. The sensitivity of RIAs offers substantial advantages over bioassays, even though the biological and immunological activities do not always correlate.

Indirect measurement of gonadotropins in women often includes the measurement of ovarian steroids or the study of vaginal cytology (see later section on gonads). In men, indirect assays include the measurement of androgens or the histological assessment of spermatogenesis. Chromosome studies to rule out Klinefelter's syndrome or Turner's syndrome can also be of some value when assessing hypogonadism for pituitary gonadal activity. Localization of FSH and LH and their receptors can be detected using immunoperoxidase techniques.

In addition to the MILLIPLEX® MAP Pituitary Magnetic Bead Panel, other methods such as ELISA, two-site IRMA, double-antibody RIA, one-step immunochemiluminometric assay, and two-site sequential immunochemiluminometric assay. Normal FSH and LH levels will differ depending on a person's age and gender. The FSH levels for males are 0–5.0 mIU/mL (before puberty); 0.3–10.0 mIU/mL (during puberty); and 1.5–12.4 mIU/mL (adult) while for females the levels are 0–4.0 mIU/mL (before puberty); 0.3–10.0 mIU/mL (during puberty); 4.7–21.5 mIU/mL (during menstruation); and 25.8–134.8 mIU/mL (postmenopausal).[173] The LH levels for males are <0.5 mIU/mL (before puberty) and 1.7–8.6 mIU/mL (adult) while for females the levels are 0.5 mIU/mL (before puberty); during child-bearing potential (2.4–12.6 mIU/mL [follicular phase], 14.0–95.6 mIU/mL [ovulation phase], and 1.0–11.4 mIU/mL [luteal phase]; and 7.7–58.5 mIU/mL [postmenopause]).[174]

Inhibin (A and B)

Inhibin, a proteinaceous substance found in the mammalian gonad, is involved in the negative feedback regulation of FSH secretion.[175] Dimeric inhibin is produced by the testes in the male (B only) and ovary in the female (A and B), together with the fetoplacental unit in pregnancy (A only). These various inhibin forms act in direct negative feedback on the pituitary production of FSH in the control of folliculogenesis and spermatogenesis. Inhibin is a heterodimer, where the α and β subunits are linked by disulfide bonds. In men, inhibin can be detected in the testes, seminal plasma, rete testes fluid, and spermatozoa. The Sertoli cells are the only testicular cells that secrete an inhibin-like substance referred to as *Sertoli cell factor* (SCF). Inhibin, as well as SCF, can suppress the pituitary secretion of FSH. Inhibin (A or B) is synthesized in granulosa cells, and production is stimulated by FSH and estradiol. The biological activities of various inhibin preparations, including SCF, can be assessed in vitro using pituitary cell cultures.[176,177] This in vitro assay consists of evaluating the degree of suppression of basal FSH release following an incubation with the test material relative to that of a control culture. In addition to RIAs,[178] several ELISAs for inhibin A and B have been developed.[179,180]

Prolactin

Prolactin is a protein hormone whose amino acid composition is quite similar to GH. It is synthesized and secreted by lactotrope cells in the adenohypophysis. Prolactin is a single-chain polypeptide structure of 199 amino acids resulting in a molecular weight of about 24,000 Da. Interestingly, it is also produced in decidua and the breast tissue. Prolactin secretion is initiated in the hypothalamus by Pit-1 transcription factor, which binds to the prolactin genes at several sites. Estrogen also enhances the growth of lactotrope cells. Dopamine suppresses the production of prolactin and itself is controlled by estrogen, which inhibits the release of dopamine. Prolactin causes initiation and maintenance of lactation in women. It has no known physiological function in men. In rodents, prolactin maintains the corpus luteum. In many species, milk ejection cannot be produced by suckling unless prolactin first stimulates the myoepithelial cells of the mammary glands.

Prior to the development of sensitive RIAs for prolactin, it was bioassayed. Prolactin has the ability to stimulate the crop sac of the pigeon. The prolactin blood test is based on an immunometric solid phase direct sandwich ELISA method. The samples and diluted antiprolactin HRP conjugate are added to the wells coated with mAb to prolactin. Prolactin in the patient's serum binds to antiprolactin MAb on the well and the antiprolactin HRP then binds to prolactin. Unbound protein and HRP conjugate are washed off by wash buffer. Upon the addition of the substrate, the intensity of color is proportional to the concentration of prolactin in the samples. A standard curve is prepared relating color intensity to the concentration of the prolactin garnered with the prolactin blood test. Reference ranges vary from laboratory to laboratory but are generally within the following values: adult male and female (0–20 ng/mL) and pregnant female (20–400 ng/mL).

Prolactin can also be identified in tissues using in vitro immunoassays, but a number of test system conditions in vitro can affect the detectable levels of prolactin (Table 36.8).[181] It is important to determine the optimal conditions for assessing tissue prolactin. Many drugs, chemicals, or even some physiological conditions can affect blood levels of prolactin (Table 36.9). Several of the actions of these agents are mediated by dopaminergic mechanisms. Prolactin secretion can be increased by physical exercise, coitus, suckling, and surgical stress. Such factors must be taken into consideration when assessing prolactin levels in toxicologic protocols.

TABLE 36.8
Conditions Affecting Immunoassay of Prolactin and GH

Duration of incubation
Incubation temperature
pH
Homogenate or tissue fraction
Concentration of test system constituents:
Cysteamine
Reduce glutathione
EDTA
Urea
Sodium dodecyl sulfate
Iodoacetate

TABLE 36.9
Effect of Various Agents on Blood Prolactin Levels[a]

Increase Prolactin Levels	Decrease Prolactin Levels
Atropine	Acetylcholine
Chlorpromazine	Apomorphine
Diethyl ether	Bromocriptine
β-Endorphin	Bulimia
Estrogens	Dopamine
Haloperidol	L-DOPA
Histamine	β-Hydroxy-GABA
Met-enkephalin	Iproniazid
Methyl-DOPA	Somatostatin
α-Methyl-*p*-tyrosine	Thyroxine (T_3)
Nicotine	
Opiates	
Perphenazine (and other phenothiazines)	
Prolactinoma	
Prostaglandin E	
Reserpine	
Supiride	
Tricyclic antidepressants	
TRH	
Vasopressin	

[a] Response may vary quantitatively depending on the dose and the particular species.

POSTERIOR PITUITARY

The posterior pituitary gland, or neurohypophysis, comprises the posterior lobe of the pituitary gland (see Figure 36.6). It consists primarily of neuronal axon nerve fiber projections from the hypothalamus. The hormones of the posterior pituitary are produced in the hypothalamus and are transported through the hypophyseal vein to the posterior pituitary, where they are stored until needed for use. The posterior pituitary is not a gland in the conventional sense, but rather an extension of the hypothalamus from the supraoptic nuclei (SON) and paraventricular nuclei (PVN) of the hypothalamus.

Neurohypophyseal Peptides

The posterior pituitary contains a group of peptides known as neurophysins, as well as oxytocin and vasopressin (ADH). The neurophysins appear to be synthesized in the same hypothalamic neurons as the nonapeptides oxytocin and vasopressin. Whereas the physiological function of oxytocin and vasopressin are well established, less is known about the biological function of the neurophysins. It appears that oxytocin and vasopressin are packaged with the protein neurophysin into discrete granules, which then move down the SON and PVN of the hypothalamus axon and are stored in the posterior pituitary. When the hypothalamus is stimulated, these hormones are then released into the bloodstream.

The principal physiological function of vasopressin is conservation of fluids, and it exerts its action on the renal tubule, leading to the reabsorption of water. The release of vasopressin is mediated by neural impulses from osmoreceptors located in the hypothalamus. The principal physiological function of oxytocin is to stimulate uterine smooth musculature and to aid in the process of lactation. Oxytocin is released in response to suckling, and it may also play a role in parturition.

Drugs, other hormones, and physiological state can affect the secretion of vasopressin or oxytocin (Table 36.10). Nonspecific stress also can stimulate the release of ADH and hence is an important variable to consider in any toxicologic protocol involved with monitoring water balance. Some chemicals and drugs affect the central release of posterior pituitary hormones at the source, whereas others may block their peripheral actions. Physiological factors also affect the secretory rate of oxytocin, and considerable differences exist among species. Suckling and mammary duct dilation and cervical distension lead to enhanced secretion of oxytocin. Estrogens and pregnancy enhance the sensitivity of the uterine smooth muscle to oxytocin.

Oxytocin

Oxytocin is a oligopeptide that contains 9 amino acids (termed a nonapeptide) and has a molecular weight of 1007 Da. Oxytocin is produced by the oxytocin-producing magnocellular neurosecretory cells located within the SON and PVN. The oxytocin neurons affect CRH. The oxytocin receptor is a G-protein-coupled receptor, a protein family of transmembrane receptors that transduce extracellular signals into intracellular signals and requires magnesium and cholesterol. Oxytocin is released after the stimulation of the nipples and distention of the vagina and cervix to prepare the female for birthing and breastfeeding. It is also released in males and females during orgasm and has been shown to be involved in bonding between individuals.

In females, the three critical actions involving oxytocin are as follows[182]:

- Uterine contraction, which is critical for cervical dilation in late labor and for blood-clotting mechanisms with parturition
- Letdown reflex, which occurs during lactation and permits the milk to accumulate in the collecting

TABLE 36.10
Effects of Various Factors on the Release or Action of Neurohypophyseal Hormones

Enhanced Release	Inhibited Release	Blocked Peripheral Action
Vasopressin		
Acetylcholine	Ethanol	Lithium
Nicotine	Caffeine	β-Adrenergic agonists
α-Adrenergic agonists		Tetracyclines
Vincristine		
Clofibrate		
Stress		
Increased plasma osmolality		
Oxytocin		
Prostaglandin E_2	Ethanol	Propranolol
Prostaglandin $F_{1\alpha}$	Methalibure	Vasopressin analogs
Suckling	Oxytocin analogs	
Mammary duct dilation		
Distention of the cervix		

chambers for the infant to suckle (suckling activity at the nipple stimulates increased secretion of oxytocin)
- Maternal behavior, which appears to have an oxytocin chemical component in some female animals such as voles and sheep

In males, the actions of oxytocin include the following[183]:

- Facilitation of sperm transport with orgasm (which releases oxytocin into the bloodstream)
- Enhanced sexual arousal

General actions of oxytocin that affect both sexes include the following:

- Secretion into the bloodstream at orgasm in both males and females[184]
- Reduced anxiety, blood pressure, and cortisol levels, as well as mediation of fight-or-flight behavior
- Increased tolerance to pain
- Stimulation of sodium excretion from kidneys (natriuresis)
- Possible cardiac development involvement (specifically, by enhancing cardiomyocyte development)[185]

Recent studies have begun to investigate oxytocin's role in various behaviors, including orgasm, social recognition, pair bonding, anxiety, and maternal behaviors.[186] For this reason, it is sometimes referred to as the *love hormone*. The inability to secrete oxytocin and feel empathy is linked to sociopathy, psychopathy, narcissism, and general manipulativeness.[187] However, there is some evidence that oxytocin promotes *tribal* behavior, incorporating the trust and empathy of ingroups with their suspicion and rejection of outsiders.[188]

The biologically active form of oxytocin, commonly measured by RIA and/or high-performance liquid chromatography (HPLC) techniques, is also known as the octapeptide *oxytocin disulfide* (oxidized form), but oxytocin also exists as a reduced dithiol nonapeptide called oxytoceine.[189] A solid-phase RIA for the direct measure of plasma oxytocin has been developed that is rapid, relatively sensitive, and reproducible and does not require the prior extraction of plasma samples.[190]

Oxytocin may also be measured by bioassay. Because oxytocin can stimulate the contraction of smooth muscles, several bioassays have been developed using isolated muscle strips. Oxytocin can be assayed employing the mammotonic activity of the hormone on strips of lactating rat mammary gland. The increment of tension developed by the muscle strip is used as an index of oxytocic activity. A four-point assay can be carried out using United States Pharmacopeia (USP) posterior pituitary standard as a reference.

Arginine Vasopressin

AVP, also known as vasopressin, argipressin, or ADH, is a neurohypophysial hormone found in most mammals. Vasopressin is an oligopeptide that contains 9 amino acids (termed a nonapeptide) and has a molecular weight of about 1084 Da. Vasopressin is produced by the vasopressin-producing magnocellular neurosecretory cells located within the hypothalamic SON and PVN. Interestingly, vasopressin is also secreted from the parvocellular neuroendocrine neurons found in the paraventricular nucleus, which transports the hormone to the anterior pituitary, where it becomes a releasing factor for ACTH.

Vasopressin is secreted from the posterior pituitary gland in response to reductions in plasma volume, increases in the plasma osmolality, and cholecystokinin (CCK) secreted by the small intestine:

- Secretion *in response to reduced plasma volume* is activated by pressure receptors in the veins, atria, and carotids.
- Secretion *in response to increases in plasma osmotic pressure* is mediated by osmoreceptors in the hypothalamus.
- Secretion *in response to increases in plasma CCK* is mediated by an currently unknown pathway.

The neurons that make AVP, in the SON and PVN, are themselves osmoreceptors, but they also receive synaptic input from other osmoreceptors located in regions adjacent to the anterior wall of the third ventricle. These regions include the organum vasculosum of the lamina terminalis and the subfornical organ.

Vasopressin is also released into the brain by neurons of the suprachiasmatic nucleus of the hypothalamus, which generates a circadian rhythm of neuronal activity, ultimately regulating many different body functions over a 24 h period. In addition, vasopressin released into the brain has been shown to be involved with delayed reflexes, memory, aggression, blood pressure modulation, and temperature regulation.

Peripheral actions of vasopressin primarily include activities at three receptor sites:

- V1a, which is located in various tissues and is involved with vasoconstriction, gluconeogenesis in the liver, platelet aggregation, and release of factor VIII
- V1b, which is located in the anterior pituitary and is involved with corticotropin secretion
- V2, which is located primarily in the distal convoluted tubules and collecting ducts of the kidneys, where it is involved with the control of free water reabsorption in the cortical collecting ducts of the renal medulla

Vasopressin has two effects in the kidney by which it contributes to increased urine osmolarity (increased concentration) and decreased water excretion:

1. Increasing the water permeability of distal tubule and collecting duct cells in the kidney, thus allowing water reabsorption and excretion of more concentrated urine, that is, antidiuresis. This occurs through V2 receptor–mediated insertion of water channels (aquaporin-2) into the apical membrane of distal tubule

and collecting duct epithelial cells.[191] Aquaporins allow water to move down their osmotic gradient and out of the nephron, increasing the amount of water reabsorbed from the filtrate (forming urine) back into the bloodstream. Vasopressin also increases the concentration of calcium in the collecting duct cells, by episodic release from intracellular stores.

2. Increasing permeability of the inner medullary portion of the collecting duct to urea by regulating the cell surface expression of urea transporters,[192] which facilitates its reabsorption into the medullary interstitium as it travels down the concentration gradient created by removing water from the connecting tubule, cortical collecting duct, and outer medullary collecting duct.

Vasopressin also acts on the cardiovascular system by increasing peripheral vascular resistance (vasoconstriction) and thus increasing arterial blood pressure. While this effect appears small in healthy individuals, however, it becomes an important compensatory mechanism for restoring blood pressure in hypovolemic shock.

Vasopressin released within the brain has many actions:

- Vasopressin released from centrally projecting hypothalamic neurons is involved in aggression, blood pressure regulation, and temperature regulation.
- It is likely that vasopressin acts in conjunction with CRH to modulate the release of corticosteroids from the adrenal gland in response to stress, particularly during pregnancy and lactation in mammals.
- Recent evidence suggests that vasopressin may have analgesic effects. The analgesia effects of vasopressin were found to be dependent on both stress and gender.[193]
- It has been implicated in memory formation, including delayed reflexes, image, and short- and long-term memory, though the mechanism remains unknown; these findings are controversial.
- Selective AVPr1a blockade in the ventral pallidum has been shown to prevent partner preference, suggesting that these receptors in this ventral forebrain region are crucial for pair bonding.[194]

Evidence for this partner preference comes from studies in several species, which indicate that the precise distribution of vasopressin and vasopressin receptors in the brain is associated with species-typical patterns of social behavior. In particular, there are consistent differences in the distribution of AVP receptors between monogamous species and promiscuous species, and sometimes in the distribution of vasopressin-containing axons, even when closely related species are compared.[195] Studies involving either injecting AVP agonists into the brain or blocking the actions of AVP support the hypothesis that vasopressin is involved in aggression toward other males. There is also evidence that differences in the AVP receptor gene between individual members of a species might be predictive of differences in social behavior. One study has suggested that genetic variation in male humans affects pair-bonding behavior. The brain of males uses vasopressin as a reward for forming lasting bonds with a mate, and men with one or two of the genetic alleles are more likely to experience marital discord. The partners of the men with two of the alleles affecting vasopressin reception state disappointing levels of satisfaction, affection, and cohesion.[196] Vasopressin receptors in monogamous prairie voles are distributed along the reward circuit pathway, specifically in the ventral pallidum. These pathways are activated when AVP is released during social interactions such as mating. The activation of the reward circuitry leads to conditioned partner preference and thereby initiates the formation of a pair bond.[197]

ADH can be measured by RIA or bioassay. With bioassay, ADH or extracts of posterior pituitary tissue can be measured for their antidiuretic activity in the rat using ether anesthesia and a constant water load; the diminution in urine flow is an index of antidiuretic activity. Unanesthetized trained dogs also have been employed in the bioassay, but stress must be minimized because it can reduce urinary output and hence the ADH assay. Simple and rapid RIAs for vasopressin are available.[198] Because vasopressin is present in biological fluids in low concentrations (picograms), tests must be sensitive. It ordinarily has been difficult to measure basal levels or small fluctuations resulting from particular experimental designs.

In vitro assays include a quantitative RIA. Arg^8-Vasopressin immunoassay is a competitive enzyme immunoassay (EIA) designed to measure Arg^8-Vasopressin in cell culture supernates, saliva, urine, serum, and plasma. This assay is based on the competitive binding technique in which Arg^8-Vasopressin present in a sample competes with a fixed amount of alkaline phosphatase–labeled Arg^8-Vasopressin for sites on a rabbit polyclonal antibody. During the incubation, the polyclonal antibody becomes bound to the goat antirabbit antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. Immediately following the color development, the absorbance is read at 405 nm. The intensity of the color is inversely proportional to the concentration of Arg8-Vasopressin in the sample.

THYROID GLAND

The thyroid gland controls how quickly the body uses energy, makes proteins, and controls how sensitive the body is to other hormones. It is unique among endocrine organs in three ways; it is able to store large amounts of hormones with slow turnover rates, providing prolonged protection against depletion should synthesis cease[199]; it has a web of feedback systems providing redundant and compensatory responses to maintain thyroid hormone signaling, and it is one of the largest endocrine glands, weighing about 3 g in neonates and 18–60 g in adults. Located in the neck anterior to the spinal cord between vertebrae C-5 and T-1 (see Figure 36.7) and below the thyroid cartilage (which forms the laryngeal prominence or "Adam's apple"), it is shaped like a butterfly,

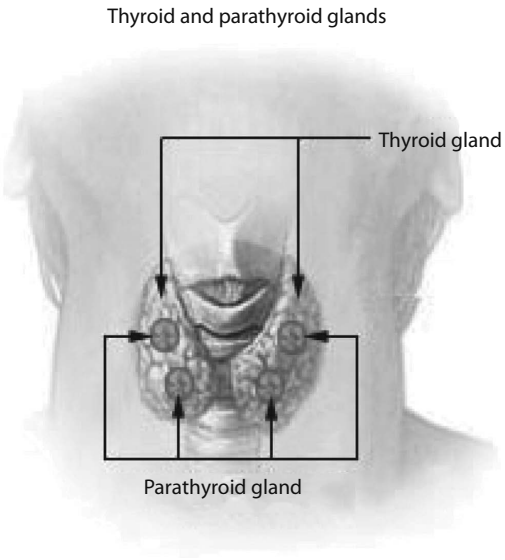

FIGURE 36.7 Parathyroid and thyroid glands. (Courtesy of U.S. National Cancer Institute Surveillance, Epidemiology, and End Results [SEER] Program.)

with the wings extending over each side of the trachea. The thyroid gets its name from the Greek word for "shield," due to the shape of the related thyroid cartilage.

The follicle is the unit of thyroid structure, consisting of closed sacs, containing homogeneous, slightly acidophilic colloid, that are lined by epithelium cells. The sacs are relatively uniform in size but appear in variable sizes in histological sections. Follicles are grouped into lobules, each supplied by a terminal branch of an extremely profuse blood supply. Estimates of blood flow to the parenchyma of the thyroid exceed the blood flow to the kidneys.[200] Iodine is selectively absorbed using the sodium/iodine transporter by these follicles whose purpose is to secrete the hormones T_3 and T_4. The follicles also contain a protein, thyroglobulin. Iodination of thyroglobulin to T_3 and T_4 is catalyzed by thyroid peroxidise. Parafollicular cells (C cells) are found throughout the gland; these cells secrete calcitonin (also known as thyrocalcitonin), which participates in the regulation of Vitamin D, bone mineralization, and reduction of calcium in the blood.

The secretory process of the thyroid gland is modulated by the HPT axis, which is highly conserved among vertebrate species and is mediated by a negative feedback relationship. TSH[201,202] is a glycoprotein hormone synthesized and secreted by thyrotrope cells in the anterior pituitary gland and regulates endocrine function of the thyroid gland. The nuclear thyroid receptor TRβ2, expressed in the pituitary gland and hypothalamic paraventricular nucleus, appears to be the predominant mediator of the negative feedback action of thyroid hormones on TSH release.[203] TSH stimulates the thyroid gland to increase the secretion and release of T_3 and T_4. The initial step in the synthesis of these thyroid hormones is the uptake of iodide (normally derived from dietary iodine) into the follicular cells of the thyroid in response to TSH (Figure 36.8). Internalized iodide is then oxidized and chemically combined

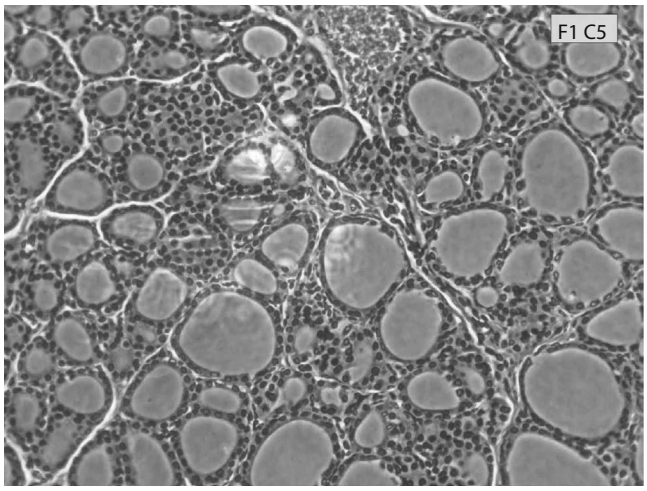

FIGURE 36.8 Histology of rat thyroid follicular cells. (From U.S. EPA Guideline Female Pubertal Assay.)

with the tyrosine components of the protein, thyroglobulin, to form either monoiodotyrosyl or diiodotyrosyl residues. Monoiodotyrosyl and diiodotyrosyl combine to form triiodothyronine, aided by the enzyme thyroid peroxidase (TPO). As much as 40% of the T_4 is converted to T_3 by the liver, spleen, and kidney; hence, T_3 is many more times active than T_4, although the ratio can be altered by certain physiological states.[204] T_3 and T_4 remain incorporated in the thyroglobulin, stored in the follicular colloid material of the gland (see Figure 36.8) until released in response to TSH. The release results from the proteolytic cleavage of thyroglobulin by lysosomes into the thyroid hormones and component amino acids. Monoiodotyrosine and diiodotyrosine are then enzymatically degraded, liberating iodide, which is eventually reincorporated into protein by the gland. Normally, thyroglobulin does not reach the circulation but remains inside the thyroid cell.

Once released into circulation, T_3 and T_4 may be free in the blood or bound in plasma to specific transporter proteins. Although there are species differences in the protein-binding patterns of the thyroid hormones, the primary binding protein in humans is thyroxine-binding globulin (TBG) as well with transthyretin and to a lesser extent albumin. TBG is an acidic glycoprotein (molecular weight 40,000) synthesized primarily in the liver that binds T_4 with a relatively high binding affinity and T_3 with a lower binding affinity. TBG has no inhibitory function unlike many other members of this class of proteins. Researchers have identified two forms of inherited TBG deficiency: the complete form (TBG-CD), that results in a total loss of TBG, and the partial form (TBG-PD), that reduces the amount of this protein or alters its structure.[205] These deficiencies are usually identified during routine blood tests that measure thyroid hormones. Although inherited TBG deficiency does not cause any health problems, it can be mistaken for more serious thyroid disorders (such as hypothyroidism). Therefore, it is important to diagnose inherited TBG deficiency to avoid unnecessary clinical intervention.

A second transport protein, thyroxine-binding prealbumin, although present in higher amounts than TBG, has a

TABLE 36.11
Diseases of the Thyroid Gland

Type of Effect	Disease	Specific Condition
Hypothyroidism (myxedema)	Hashimoto's thyroiditis or thyroiditis	Autoimmune disorder where antibodies destroy thyroid cells
	Ord's thyroiditis	Atrophic autoimmune disorder common in Europe
	Postpartum thyroiditis	Inflammation and dysfunction of thyroid after delivery, due to antibodies
	Silent thyroiditis	A variant of chronic autoimmune thyroiditis similar to postpartum thyroiditis
	Postoperative thyroidism	Development of hypothyroidism upon surgical removal of the thyroid
	Acute and subacute thyroiditis	Inflammation and dysfunction of the thyroid
	Iatrogenic hypothyroidism	Dysfunction of the thyroid after exposure to irradiation
Hyperthyroidism (thyrotoxicosis)	Graves' disease	Autoimmune disorder where antibodies stimulate the thyroid cells to overproduce
	Thyroid storm	An acute, life-threatening, thyroid-hormone-induced hypermetabolic state
	Toxic thyroid nodule	Autonomously functioning thyroid nodules that secrete excess thyroid hormone
	Toxic nodular struma (Plummer's disease)	Enlarged thyroid gland containing small, rounded masses called *nodules* that secrete excess thyroid hormone
	Hashitoxicosis	Autoimmune disorder where thyroid cells are sporadically stimulated to overproduce
	De Quervain thyroiditis (giant cell thyroiditis)	Viral infection that initially causes oversecretion and then undersecretion of thyroid hormone
	Iatrogenic hyperthyroidism	Overtreatment of hypothyroidism with thyroid hormone replacement
Additional thyroid pathology	Goiter	Swelling of the thyroid gland because of lack of iodine (endemic); toxic goiter (from inflammation, neoplasm); nontoxic goiter (caused by autoimmune or chemical reaction); diffuse/multinodular goiter (spread throughout the thyroid)
	Thyroid adenoma	Usually benign tumors of the follicular epithelium of the thyroid gland
	Thyroid cancer	Cancer in one of the following forms: papillary, follicular, medullary, or anaplastic
	Cretinism (congenital hypothyroidism)	A congenital defect that results in an underdevelopment of the thyroid; thought to be a result of iodine deficiency

Sources: Data from Greenspan, F.S., in *Basic and Clinical Endocrinology*, 7th edn., Greenspan, F.S. and Gardner, D.G., eds., McGraw-Hill, New York, 2004, pp. 215–294; Wikipedia, http://en.wikipedia.org/, 2006.

lower binding affinity for the thyroid hormones and is considered of secondary physiologic importance. In humans and most other mammals, the thyroid hormones can also bind to albumin following the occupation of the higher-affinity binding sites. As a consequence of this plasma protein binding, less than 0.1% of the total plasma thyroid hormones exist in a free or unbound form. Care must be exercised when monitoring thyroid function in a species such as the rat, which does not possess TBG (a high-affinity binding protein) and therefore has lower plasma levels of protein-bound thyroid hormone. Many factors can affect the measurement of rodent thyroid hormones (see Table 36.12). It is the free hormone that is available for degradation, and the plasma half-life for T_4 would be longer in a species with a TBG than in a species without the protein. The T_4 plasma half-life in the human, which has a TBG, is 5–9 days. In the rat, which does not have a TBG, the T_4 plasma half-life is 12–24 h.

The thyroid gland can be affected by various disease states (Table 36.11), drugs, and environmental agents. Antithyroidal drugs can alter the biosynthesis of thyroid hormones. Some agents interfere with the uptake of iodine or by inhibiting TPO, resulting in a reduction in T_3 and T_4 levels, while other chemicals affect thyroid hormone secretion (Table 36.12). Industrial or environmental agents that affect thyroid function in humans typically do so by interfering with the intrathyroidal synthesis or secretion of thyroid hormone (i.e., primary hypothyroidism).[16] The liver regulates T_3 and T_4 levels through up- and downregulation of catabolic reactions that removes these hormones from the blood. The route of elimination is through conjugation and excretion through the bile.

TABLE 36.12
Chemicals Producing Abnormal Thyroid Function

Blocks Iodide Trapping	Blocks Iodide Oxidation	Mechanism Not Established
Chlorate	Amphenone	Acetazolamide
Hypochlorite	Carbimazole	Auranofin
Iodate	Cobalt	Amiodarone
Nitrate	Methimazole	Chlorpromazine
Perchlorate	*p*-Aminosalicylate	Chlortrimeton
Periodate	Phenylbutazone	Frentizole
Pertechnetate	Phenylindanedione	Thiopental
Propylthiouracil	Propylthiouracil	Tolbutamide
Thiocyanate	Resorcinol	

Note: Iodide is an iodine ion (I^-).

TABLE 36.13
Factors Influencing TSH, T_3, and T_4 Levels in Rat Plasma

Sex of animal
Age of animal
Time of day
Stage of estrous cycle
Strain of animal
Environmental temperature
Blood collection technique
Animal handling
Locomotor activity of animal

TABLE 36.14
Laboratory Assessment of the Thyroid

Tests assessing metabolic effect of thyroid:
- Serum cholesterol
- Basal metabolic rate
- Cardiac rate (i.e., systolic time intervals)

Tests assessing thyroid function:
- TRH stimulation
- Thyroid suppression test
- Serum TSH
- ^{131}I uptake; T_3 uptake

Tests assessing blood levels (free and bound):
- Serum total T_3 and total T_4
- Serum free (unbound) T_4
- Reverse T_3 (rT_3)
- TBG

Source: Adapted from Dayan, C.M., *Lancet*, 357, 619, 2001.

The thyroid hormones, especially T_3, are important in thermoregulation and regulation of metabolism. Polychlorinated biphenyls (PCBs) affect thyroid hormone metabolism. Amiodarone, an antiarrhythmic drug, is an iodinated benzofuran derivative with a chemical structure similar to thyroxine that may cause hyperthyroidism. PCBs are goitrogenic in some animals. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) can reduce T_4 levels in experimental animals, and dichlorodiphenyltrichloroethane (DDT) can produce avian hypothyroidism. Hydroxyphenols (e.g., resorcinol) and hydroxypyridines have been shown to inhibit TPO; likewise, phthalates (also known as plasticizers) can be degraded by certain bacteria producing dihydroxybenzoic acid (DHBA), which also can inhibit TPO.[16] The development of the brain is susceptible to hypothyroidism, and endocrine fetal hypothyroidism, regardless of the cause, is considered teratogenic.[206] In addition to the various drugs, chemicals, and environmental pollutants that can affect the thyroid gland, other factors can influence the measurement of TSH, T_3, and T_4 levels (see Table 36.13).

Measurement of Thyroid Hormones

Assays for thyroid hormones are used to monitor and confirm the extent of hyper- or hypothyroidism. A large number of laboratory tests are available that can be used to assess the function of the thyroid gland (Table 36.14). Laboratory tests can be divided into various categories, including those that directly measure thyroid function (e.g., ^{131}I uptake), those that measure blood levels (free vs. bound), those that measure metabolic actions (e.g., cholesterol lowering), and those that provide insight into the modulations of thyroid function by the adenohypophysis (e.g., thyroid suppression test).

TSH (thyrotropin) is generally elevated in hypothyroidism and decreased in hyperthyroidism.. Free thyroxine (Free T_4) and free triiodothyronine (Free T_3) are generally elevated in hyperthyroidism and decreased in hypothyroidism (Table 36.15).

TABLE 36.15
Reference Values for TSH, Free T_3, and Free T_4

Thyroid-Stimulating Hormone

Patient Type	Lower Limit	Upper Limit	Unit
Adults—standard range	0.3	3.0	mIU/L
Infants	1.3	19	mIU/L
Free thyroxine (free T_4)			
Normal adult	0.7	1.4	ng/dL
Infant 0–3 days	2	5.0	ng/dL
Infant 3–30 days	0.9	2.2	ng/dL
Child/adolescent 31 days–18 years	0.8	2.0	ng/dL
Pregnant	0.5	1.0	ng/dL
Free triiodothyronine (free T_3)			
Normal adult	0.2	0.5	ng/dL
Children 2–16 years	0.1	0.6	ng/dL

Source: Adapted from Dayan, C.M., *Lancet*, 357, 619, 2001.

RIAs can be used to determine serum T_3 and T_4 levels. The RIA is based on the binding of endogenous hormone to a specific antibody, thereby displacing a proportional amount of radiolabeled hormone from that antibody. Obviously, the hormone-binding proteins in the sample (e.g., TBG, thyroxine-binding prealbumin, and albumin) tend to compete with the antibody for the hormone. This interference can be prevented by extracting the serum prior to the assay or adding chemical agents to block the binding of hormone to binding proteins. Another difficulty in measuring T_3 or T_4 by RIA is that the hormones are small and similar in structure; hence, it has been difficult to produce a specific antibody for T_3 and T_4 without the need for prior chromatographic separation.

Both T_4 and T_3 can be measured using a competitive protein-binding assay that is based on the displacement of radiolabeled hormone from TBG. The amount of label displaced is proportional to the amount of hormone added to the assay in the sample serum. T_4 and T_3 can be effectively measured using the competitive protein-binding assay, but the hormone must first be extracted from the sample serum to eliminate any interference from endogenous binding proteins, which would tend to compete with the binding protein used in the assay. Nonisotopic immunotechniques can also be used to measure thyroid hormones (e.g., EMIT). The principles of these tests are comparable to RIAs except that the labeled analyte may be an enzyme, as in ELISA.[209] EMIT is an enzymatic method that does not require separation of the free and bound portions of the hormone.

PARATHYROID GLANDS

Parathyroid glands are small glands (size of a grain of rice), usually four or more, located in the neck and situated on the posterior surface of the thyroid gland. The parathyroid glands usually weigh between 25 and 40 mg in adult humans. They produce parathyroid hormone (PTH) (see Figure 36.7) and are regulated independently of the thyroid and the HPT axis. The parathyroid glands are quite easily recognizable histologically from the thyroid as they have densely packed cells, in contrast with the follicle structure of the thyroid. However, fat makes them harder to differentiate from the thyroid tissue at surgery.

The densely packed parathyroid gland contains two primary types of cells: oxyphil cells and chief cells. Oxyphil cells stain lighter, are larger in size, less numerous, usually appear in clusters, and contain large numbers of mitochondria. The specific function of oxyphil cells is unknown. The chief cells synthesize PTH in the rough endoplasmic reticulum; it is packaged by the Golgi apparatus and then stored in secretory granules. PTH is synthesized as a prohormone consisting of 115 amino acids, whereas PTH itself contains 84 amino acids. PTH is a small protein that takes part in the control of calcium and phosphate homeostasis, as well as bone physiology, and has effects antagonistic to those of calcitonin.

The balance of endogenous calcium is maintained by several intrinsic factors that modulate the remodeling of bone and the absorption and excretion of calcium and phosphorus homeostasis. PTH, vitamin D, and calcitonin are the principal factors involved in calcium homeokinesis.[210,211] About 98% of endogenous calcium resides in skeletal tissue, with the remainder sequestered in soft tissues and extracellular fluids. About 50% of serum calcium exists in an ionized form, which is the biologically active form of the cation. Hypocalcemia activates calcium-sensing receptors, which induces the release of PTH, which, in turn, stimulates osteoclasts to break down bone and release calcium into the blood. Although the direct involvement of PTH, calcitonin, and vitamin D is important in modulating calcium, other hormones such as thyroxine, GH, estrogens, and corticosteroids also contribute to the maintenance of calcium homeostasis.[211]

Many conditions are associated with the disorders of parathyroid function and can be divided into those causing hyperparathyroidism and those causing hypoparathyroidism. Hyperparathyroidism is overactivity of the parathyroid glands resulting in excess production of PTH. Excessive PTH secretion may be due to problems in the glands themselves (hyperplasia, adenoma, or rarely carcinoma), referred to as *primary* hyperparathyroidism, which leads to hypercalcemia (raised calcium levels). It may also occur in response to low calcium levels, as encountered in various situations such as vitamin D deficiency or chronic kidney disease; this is referred to as *secondary* hyperparathyroidism. In all cases, elevated PTH levels are harmful to bone, and treatment is often needed.[212]

Hypoparathyroidism is a decreased function of the parathyroid glands with underproduction of PTH. This leads to low levels of calcium in the blood, often causing cramping and twitching of muscles or tetany. The condition can be inherited, but it is also encountered after thyroid or parathyroid gland surgery, and it can be caused by immune system–related damage as well as a number of rarer causes. The diagnosis is made with blood tests as well as other investigations such as genetic testing. The treatment of hypoparathyroidism is limited because at this time there is no artificial form of the hormone that can be administered as replacement; calcium replacement or vitamin D can ameliorate the symptoms but can increase the risk of kidney stones and chronic kidney disease.[213]

The evolution of PTH assays can be divided into three types of methods: the competitive immunoassays (first generation), the immunometric assays (second generation), and the new assays recently described (third generation). In the first generation, a single polyclonal antibody competes for labeled PTH. In the second generation assays, two distinct antibodies (usually monoclonal), directed against different epitopes, bind the PTH forms present in the sample. One of the antibodies is bound to a solid phase, and the other is labeled. In the third generation of assays, the recognition of PTH employs a detection antibody that has specificity for the first four amino acids in the PTH molecule, giving a higher precision and accuracy.

A commonly used in vitro assay involves activation of renal adenylate cyclase in response to PTH. PTH can be measured by immunoassay. The majority of assays are directed

against the more stable *C*-terminal fraction.[22] Assays for this *N*-terminal fragment are available and are better suited to detect rapid fluctuations in PTH levels; however, the *C*-terminal assay is the method of choice for assessing abnormal PTH function, particularly with concomitant hypercalcemia.[214] An mAb assay for human vitamin-D-binding protein is also being used.[215] The antihuman vitamin-D-binding protein antibodies cross-react with monkey and pig vitamin-D-binding protein, but not with vitamin-D-binding protein from the rat, mouse, or chicken.

ADRENAL GLANDS

Also known as *suprarenal glands*, the adrenal glands are triangular-shaped glands located on the anteriosuperior aspect of both kidneys (avian and fish species have interrenal glands). In humans, they are found at the level of the T-12 vertebra; they have their own blood supply through the adrenal arteries and measure about 1/2 by 3 in. and weigh 7–10 g combined in an adult human (see Figure 36.9). They are surrounded by an adipose capsule and renal fascia.

The adrenal gland consists of an outer layer (i.e., adrenal cortex) and an inner layer (i.e., adrenal medulla). The inner and outer layers can be readily distinguished by histologic preparations (Figure 36.10). These glands are regulated by both the endocrine and neural systems. The adrenal medulla is a primary source of the catecholamine hormones (epinephrine and norepinephrine) and responds to

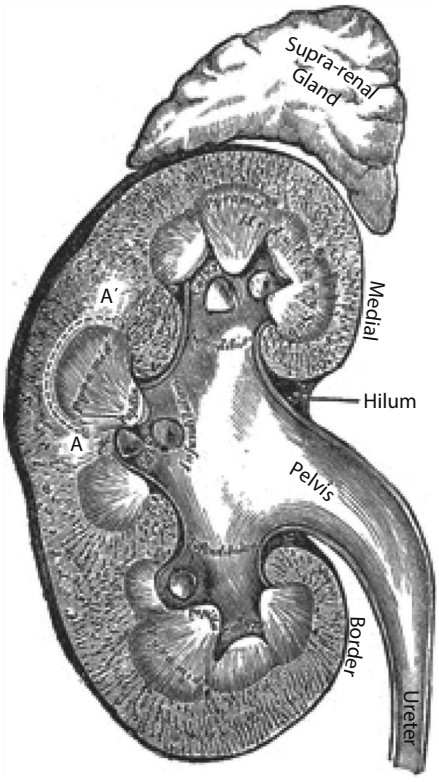

FIGURE 36.9 Relationship of adrenal glands to the kidneys. (Courtesy of U.S. National Cancer Institute Surveillance, Epidemiology, and End Results [SEER] Program.)

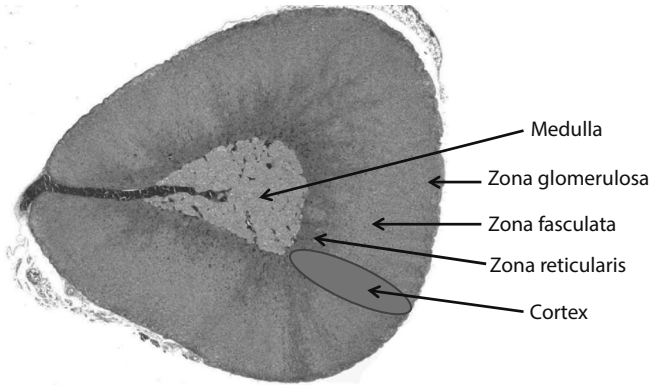

FIGURE 36.10 Histology of rat adrenal cortex (outer area) and medulla (inner area) (original magnification, 126×).

sympathetic nerve stimulation. The release of these hormones produces systemic effects resembling generalized sympathetic nerve stimulation. The adrenal cortex produces two major groups of hormones—namely, mineralocorticoids and GCs. The hypothalamic–pituitary–adrenal axis controls reactions to stress and functions in regulating various body processes such as digestion and the immune system.

Adrenal Medulla

The adrenal medulla can synthesize tyrosine into epinephrine and norepinephrine. The adrenal medulla is considered to be a ganglion of the sympathetic nervous system, as it is derived from neuronic neural crest and from modified neurons. In response to imminent danger, medullary cells release epinephrine and norepinephrine into the bloodstream at a ratio of about 70:30. The adrenal gland also produces androgens and affects kidney function through the secretion of aldosterone, a hormone involved in regulating the osmolarity of blood plasma.

The adrenal medulla is composed primarily of chromaffin cells, which secrete epinephrine (adrenaline), norepinephrine, and enkephalin, which are critical to the fight-or-flight response. Because the autonomic nervous system, specifically the sympathetic division, exerts direct control over the chromaffin cells, hormone release can occur rather quickly. Release of catecholamines is stimulated by nerve impulses, and receptors for catecholamines are widely distributed throughout the body. In response to stressors such as exercise or imminent danger, medullary cells release catecholamines into the blood. Notable effects of adrenaline and noradrenaline include increased heart rate and blood pressure, blood vessel constriction in the skin and gastrointestinal tract, smooth muscle (bronchiole and capillary) dilation, and increased metabolism, all of which are characteristic of the fight-or-flight response.

Adrenal Cortex

Situated along the perimeter of the adrenal gland, the adrenal cortex mediates the stress response through the production of mineralocorticoids and GCs, including

aldosterone and cortisol, respectively. It is also a secondary site of androgen synthesis.

The adrenal cortex is composed of three tissue layers. The external layer, the *zona glomerulosa*, secretes mineralocorticoids such as aldosterone as part of the renin–angiotensin system regulating blood pressure. The intermediate layer, the *zona fasciculata*, produces cortisol, which is involved in the response to stress, increased blood pressure, hyperglycemia, and suppression of the immune system. ACTH causes corticol-producing cells to release GCs as part of the hypothalamic–pituitary–adrenal axis. The inner layer of the adrenal cortex is the *zona reticularis*. The cells of *the zona reticularis* are arranged in a network that has the same functions as cells of the *zona fasciculata*. Evidence suggests that the zona reticularis is the primary source of GCs and adrenal androgens, such as AND (the precursor to testosterone).

The adrenal gland is essential for life, especially the salt-retaining and water-balancing properties of the mineralocorticoids. In the absence of mineralocorticoids, the extracellular fluid potassium concentration rises, and sodium and chloride concentrations fall. In humans, the main mineralocorticoid is aldosterone, although deoxycorticosterone (DOCA), a precursor molecule for the production of aldosterone, also exhibits mineralocorticoid activity. DOCA potency is reported to be only 1/30th that of aldosterone. A rise in serum potassium causes a rise in DOCA secretion.[216] Aldosterone is produced in the cortex of the adrenal gland, and its secretion is mediated principally by angiotensin II but also by ACTH and local potassium levels.[217] The total lack of aldosterone secretion can cause the urinary elimination of up to 20% of the total body sodium in 1 day. The salt elimination can cause a life-threatening reduction in the extracellular and blood volume, which, if untreated, leads to diminished cardiac output and death.

Cortisol, more formally known as hydrocortisone, is the major GC produced in humans and also exhibits a small amount of mineralocorticoid activity. The basic physiological effect of aldosterone is on extracellular fluid volume. It promotes the renal reabsorption of sodium in the ascending portion of the loop of Henle, the distal tubule, and the collecting tubule by acting on the mineralocorticoid receptor. Sodium reabsorption is accompanied by the reabsorption of the chloride anion. In addition to stimulating sodium reabsorption, aldosterone enhances the urinary excretion of potassium and hydrogen ions. The increased elimination of hydrogen ions can lead to alkalosis and an increased extracellular content of bicarbonate ions, which, when combined with an increased extracellular sodium and chloride content, promotes tubular reabsorption of water.[218]

The major GC secreted in humans is cortisol, or hydrocortisone, produced by the *zona fasciculata* of the adrenal gland, although both corticosterone and cortisone possess some GC activity. Their primary functions are to increase blood sugar through gluconeogenesis; suppress the immune system; and aid in fat, protein, and carbohydrate metabolism. With regard to carbohydrate metabolism, the GCs stimulate gluconeogenesis and decrease glucose utilization by the cells leading to hyperglycemia. Cortisol also breaks down protein and lipids, which increases blood glucose, with a resulting increase in glycogen in the liver.[219] The GCs also produce a marked reduction in cellular protein content. An exception to this protein catabolic action is the liver, where protein content increases as does the production of plasma protein by the liver in response to decreased blood glucose levels.

Cortisol suppresses the immune system by downregulating the interleukin-2 receptor (IL-2R) on *helper* T-cells.[220] This results in the inability of interleukin-2 to upregulate the Th2 (humoral) immune response and results in a Th1 (cellular) immune dominance. This leads to a decrease in B-cell antibody production. Cortisol prevents the release of substances in the body that cause inflammation. This is why cortisol is used to treat conditions resulting from overactivity of the B-cell-mediated antibody response, such as inflammatory and rheumatoid diseases, and allergies. Low-potency hydrocortisone, available over the counter in some countries, is used to treat skin problems such as rashes and eczema.

Cortisol is an essential chemical in the restoration of homeostasis after exposure to stress. Among other things, it promotes gluconeogenesis by inhibiting insulin. GCs interfere with the transport of amino acids into extrahepatic cells, and this action combined with continuing protein catabolism in these cells produces an increase in plasma amino acid content. Increased plasma amino acids and their subsequent transport into the liver promote gluconeogenesis (i.e., the conversion of amino acids to glucose). The GCs also promote mobilization of fatty acids from adipose tissue, which raises plasma fatty acid levels. This effect, along with increased oxidation of fatty acids in the cells, is involved in the metabolic conversion from glucose utilization to fatty acid utilization as a source of energy during the periods of stress.

Corticosteroids can bind to plasma protein fractions. A corticosteroid-binding globulin (CBG), also known as transcortin or serpin A6, has a high affinity but a low binding capacity. CBG is produced by the liver and is regulated by estrogens. Therefore, plasma transcortin levels increase during pregnancy (unbound plasma cortisol in term pregnancy is approximately 2.5 times that of nonpregnant women)[221] and are decreased in cirrhosis. Under physiological conditions, most of the hormones (cortisol, corticosterone, aldosterone, and progesterone) are bound to CBG.

It is now recognized that many drugs and chemicals can produce lesions of either the adrenal medulla or the adrenal cortex (Table 36.16). Still other agents can specifically inhibit particular enzymatic steps involved in adrenal steroidogenesis (Figure 36.11). Not only have metyrapone, aminoglutethimide, and *o,p′*-DDD been used as agents to aid in the diagnosis of adrenal cortical dysfunction, but some have also been used as adrenolytic drugs in treating inoperable adrenal adenocarcinomas.

MEASUREMENT OF CORTICOSTEROIDS

Several methods have been used to measure plasma and urinary aldosterone levels. Among them are double-isotope dilution techniques, gas–liquid chromatographic techniques,

TABLE 36.16
Agents Causing Lesions to the Adrenal Gland

Adrenal Cortex	Adrenal Medulla
Adriamycin	Acrylonitrile
Aminoglutethimide	ACTH
4-Aminopyrazolo-(3,4-*d*)pyrimidine	Alloxan
Cadmium	Blocadren
Carbon tetrachloride	Chlordecone
Chloramphenicol	*o*-Chlorobenzylidine
Chlordane	Malononitrile
Chloroform	Cysteamine
Chlorphentermine	Dichloromethane
Chlorpromazine	7,12-Dimethylbenzanthracene
Copper	Estrogens
Cyclosporin	GH
Cyproterone	Interleukin-2
o,p′-DDD	Lactitol
Danazol	Lactose
Dichlorvos	Malathion
7,12-Dimethylbenzanthracene	Mannitol
Etomidate	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyrine (MPTP)
5-Fluorouracil	Neuroleptics
Kepone	Nicotine
Ketoconazole	Pyrazole
Nicotine	Reserpine
Phenobarbital	Retinol acetate
Polychlorinated biphenyls	Sorbitol
Spironolactone	Thiouracil
Tamoxifen	Thyroid hormones
TCDD	TSH
Tetrahydrocannabinol	1,1,2-Trichloroethane
Toxaphene	Xylitol

Source: Colby, H.D. and Longhurst, P.A., in *Endocrine Toxicology*, Atterwill, C.K. and Flack, J.D., eds., Cambridge University Press, Cambridge, U.K., 1992. With permission.

and RIAs.[112] A competitive immunoassay for cortisol reportedly is based on a capillary electrophoresis and laser-induced fluorescence technique.[223] Several analytical procedures have been used for the quantitation of GCs. One of the earliest procedures involved a colorimetric reaction between the GC and a phenylhydrazine reagent. The Porter–Silber method and Zimmerman reactions are colorimetric assays used for measuring corticoids. Both cortisol, which is the primary GC in humans, and corticosterone, which is the primary GC in the rat, have also been measured using a competitive protein-binding assay that takes advantage of the binding affinity of the GC-binding globulin found in the plasma. Many drugs can interfere with the measurement of corticosteroids and ketosteroids; see Table 36.17. With the development of immunochemical techniques, it was found that plasma GCs could be effectively measured using the RIA. A complicating factor was the existence of competition between the endogenous GC-binding globulin and the added antibody for the radioligand.

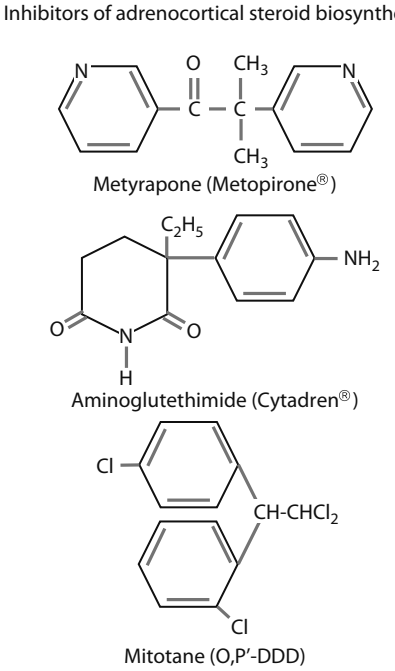

FIGURE 36.11 Agents that inhibit adrenocortical steroid biosynthesis.

TABLE 36.17
Drugs That Interfere with the Measurement of Corticosteroids and Ketosteroids

Antibiotics/antibacterial agents:
- Nalidixic acid
- Sulfamerazine
- Triacetyloleandomycin

Sedatives/tranquilizers:
- Chloral hydrate
- Chlordiazepoxide
- Chlorpromazine
- Ethinamate
- Meprobamate
- Phenaglycodol
- Reserpine

Monoamine oxidase inhibitors:
- Etryptamine

Oral hypoglycemic agents:
- Acetohexamide

Miscellaneous drugs:
- Colchicine
- Phenytoin (DPH)
- Quinidine
- Quinine
- Spironolactone

Note: Ketosteriods have a ketone functional group at the 17 position and include AND, androsterone, estrone, and dehydroepiandrosterone.

Stress can produce a rapid increase in ACTH production, which in turn promotes the secretion of adrenocortical hormones; thus, the conditions under which the animal is prepared for blood sampling can modify the corticoid levels. It has been demonstrated that under ether anesthesia, blood corticoid levels are higher than those levels found after pentobarbital administration, which are higher than those measured following decapitation. Likewise, the time elapsed following the administration of pentobarbital influences blood hormone levels. Thus, stress can be a major problem affecting the interpretation of results. An additional related problem is that fairly large volumes of blood must be drawn for adrenocortical hormone measurement, which also leads to stress.

Noninvasive measures of stress may be obtained by quantifying corticosteroid metabolites excreted in feces. This method has been shown to be useful to assess corticosteroid levels preceding stress in numerous species, including rats and mice.[224] Adrenocortical function can be effectively monitored by measuring corticosteroid levels in 24 h urine samples. Major advantages to this approach are that (1) the animals can be housed stress-free in metabolic cages during toxicity testing and (2) serial measurements can be conducted in the same animal by noninvasive techniques without sacrificing the animal. This approach cannot be used to measure urinary aldosterone in the rat because the primary site of degradation for aldosterone is the liver, and only about 1% of the secreted aldosterone is excreted unchanged in the urine. Nevertheless, relative changes in the urinary corticosteroid patterns could be of value as a screen for adrenotoxicity during chronic toxicity studies. RIA is the method of choice for the determination of urinary corticosteroids.

GONADS

Male Sex Hormones (Androgens)

During mammalian development, gonads are capable of becoming either ovaries or testes. In humans, starting at about week 4, the gonadal rudiments are present within the intermediate mesoderm adjacent to the developing kidneys. At about week 6, epithelial sex cords develop within the forming testes and incorporate the germ cells as they migrate into the gonads. In males, certain Y chromosome genes, particularly SRY, control the development of the male phenotype, including conversion of the early bipotential gonad into testes. In males, the sex cords fully invade the developing gonads. The mesoderm-derived epithelial cells of the sex cords in developing testes become the Sertoli cells, which will function to support sperm cell formation. A minor population of nonepithelial cells appear between the tubules by week 8 of human fetal development. These are Leydig cells, and soon after they differentiate, they begin to produce androgens.

Androgens control and maintain masculine characteristics. These actions are generally mediated by binding to intracellular steroid receptors that specifically bind testosterone and dihydrotestosterone (DHT). Testosterone is produced from cholesterol in the Leydig cells of the male testes and, to a much lesser degree, in the thecal cells of the ovaries. Small amounts of testosterone are produced in both males and females by the *zona reticulosa* of the adrenal glands.

Testosterone is the primary male sex steroid hormone and is found in mammals, reptiles, birds, and other vertebrates.[225,226] It is essential for health and well-being as well as for the prevention of osteoporosis. It acts as a virilizing agent and promotes secondary male sex characteristics such as increased muscle, bone mass, the growth of body hair, and anabolic effects, such as increased bone and muscle strength and size and inhibition of estrogenic effects. In addition, testosterone enhances libido, immune function, normal bone growth, and general well-being. Average plasma concentration in adult human males of testosterone is about 7–8 times as great as the concentration in adult human females' plasma,[227] but because the metabolic consumption of testosterone in males is greater, the daily production of testosterone is about 20 times greater in men.[228] Testosterone binds to sex-hormone-binding globulin (SHBG), which is a glycoprotein that specifically binds testosterone and estradiol. The exact role of SHBG is not clear; however, it is thought that SHBG in concert with other carrier proteins provides a dynamic equilibrium between free and bound androgens in response to fluctuations in the secretion of androgens.[229]

Testosterone not bound to SHBG can bind to ARs or can be reduced to 5α-DHT by 5α-reductase. DHT is a more potent agonist than testosterone. Once activated, the ARs are transported to the nucleus, where they bind to specific areas of chromosomes to form hormone response elements (HREs), which ultimately enhance the appropriate gene transcription activity and result in anabolic or virilizing protein production. Anabolic steroids (e.g., 19-norsteroids) bind the AR, resulting in increased protein synthesis, and simultaneously block the effects of cortisol, thus reducing the rate of catabolism, especially of the muscle mass.

Besides testosterone, other androgens include the following: (1) Dehydroepiandrosterone (DHEA), also called dehydroisoandrosterone or dehydroandrosterone, is a steroid hormone produced in the adrenalcortex from cholesterol. It is the primary precursor of natural estrogens. (2) AND is an androgenic steroid produced by the testes, adrenal cortex, and ovaries that can be converted metabolically to testosterone and other androgens and the parent structure of estrone. AND has been used as an athletic or bodybuilding supplement and is banned by most sporting organizations. (3) Androstenediol is the steroid metabolite thought to act as the main regulator of gonadotropin secretion and is found in approximately equal amounts in the plasma and urine of both males and females. (4) Androsterone is a chemical by-product created during the breakdown of androgens, or derived from progesterone, that also exerts minor masculinizing effects, but with less intensity than testosterone. (5) DHT is a metabolite of testosterone and a more potent androgen than testosterone in that it binds more strongly to ARs. It is produced in the adrenal cortex.

TABLE 36.18
Endpoints Used in Assessment of the Male Reproductive System

Sperm count
Sperm motility (reflectospermiograph)
Sperm head morphology
Testicular morphology
Sperm production rates
Epididymal sperm numbers and transit time
Spermatogenesis (dual-parameter flow cytometry)
Sperm membrane integrity:
  Viability (eosin Y exclusion)
  Hypo-osmotic swelling
Nuclear maturity:
  Acid aniline blue stain
  Nuclear chromatin decondensation (SDS)
Acrosome assessment:
  Normal intact acrosomes
  Acrosin activity
Objective motility assessment:
  Linearity (VSL/VCL)
  ALH
Sperm–oocyte interaction:
  Sperm–zona pellucida binding ratio
  Sperm–oolemma binding ratio
Serum FSH and luteinizing hormone
Serum testosterone and DHT
Gravimetric response (e.g., prostate, seminal vesicles)
Sex accessory organ biochemical constitutions (e.g., fructose)
Hemizona assay (HZA)

Source: Thomas, J.A., in *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 5th edn., Klaassen, C.D., ed., Pergamon Press, New York, pp. 547–581, 1996. With permission.

TABLE 36.19
Characteristics of Normal Human Male Semen

Factor	Measurement
Appearance	Homogeneous, gray-opalescent ejaculate
Volume	>2 mL
Consistency	Not viscous
Liquefaction (conversion to liquid)	Complete within 60 min
Concentration	>20 million sperm per mL
Total count	>40 million sperm per ejaculate
Motility	>50% at 1 h
pH	>7.2
Morphology	>30% normal shape (WHO criteria)
	>14% normal shape (Krueger criteria)
White blood cells	<1 million per mL

Source: ASRM, *Patient Fact Sheet*, American Society for Reproductive Medicine, Birmingham, AL, www.asrm.org, 2001. With permission.

TABLE 36.20
Possible Sites of Action of Agents Affecting the Reproduction

Anatomical Site	Endocrine Effects
CNS	
Cerebral cortex	Altered secretion of FSH and LH
Median eminence	Altered releasing hormone secretion
Adenohypophysis	Changes in gonadotropin secretion
Peripheral target organs	
Ovary	Altered secretion of estrogens
Testes	Altered secretion of androgens
Liver	Increased catabolism of steroids

Source: Thomas, J.A., in *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 5th edn., Klaassen, C.D., ed., Pergamon Press, New York, pp. 547–581, 1996. With permission.

Male reproductive toxicology endpoints can be assessed in several ways (Table 36.18). The components of normal human male semen are listed in Table 36.19. Androgens can be chemically measured, or androgen-dependent organs can be used to determine the endocrine status of male sex hormones. Androgens and other steroids can be biotransformed in several anatomical sites (Table 36.20). In considering these processes, there are numerous potential targets for the action of chemicals ranging from the action of dopamine antagonists on the hypothalamous interrupting the normal secretion of GnRH to the action of estrogens on the pituitary and hypothalamus to interfere with gonadotropins (LH and FSH) production through direct effects on spermatogenesis—where the vast majority of toxicants have their site of action. There are a number of examples of nutritional deficits or over exposure to some vitamins and minerals (vitamin A and zinc) and testicular vasculature effects of cadmium that lead to direct effects on spermatogenesis. CCl_4 can disturb the normal metabolism of sex steroids, leading to changes in clearance, indirectly perturbing the HPG axis and its effect on spermatogenesis.[231] An overview of the hormonal regulation of spermatogenesis in the rat, monkey, and man has identified differences in male fertility between these species. The review reports that FSH and androgens act both separately and synergistically to support a range of events in spermatogenesis, from spermatogonial stem cell division to final sperm release.[232]

Proper functioning of the mammalian testis is dependent upon an array of hormonal messengers acting through endocrine, paracrine, and autocrine pathways. These hormonal messengers are critical not only for regulation of male germ cell development, but also for the proliferation and function of the somatic cell types required for proper development of the testis.[233] These cell types include the interstitial steroidogenic Leydig cells, whose primary function appears to be the production of testosterone; the myoid cells that surround the seminiferous tubules and provide physical support and contractile motion to these structures; and the Sertoli

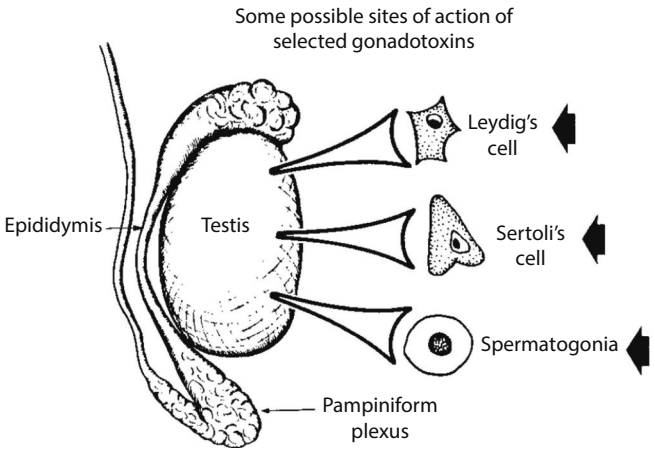

FIGURE 36.12 Testis site of action.

cells, whose direct contact with proliferating and differentiating germ cells within the seminiferous tubules makes them essential for providing both physical and nutritional support for spermatogenesis. Each of these cell types is a direct target for one or more of the hormones whose actions are essential for unimpaired male fertility. Some cell types of the testes are more sensitive to chemical insult than are others. The various subpopulations of cells within the testes include the germ cells, the Sertoli cells, and the Leydig cells (interstitial cells) (Figure 36.12). Cross-sections of the mammalian testes reveal the seminiferous tubules containing the germinal epithelium and the Sertoli cells. Leydig cells lie outside these tubules and are located in the interstitium (Figure 36.13).

Gonadotoxicants may act directly on the testes, indirectly via the CNS, or by a combination of the two. Relative to the germinal epithelium, the Leydig cells are not as sensitive to the toxic effects of chemicals. A pig Leydig cell culture system has been devised to evaluate testicular toxicity.[234] This in vitro system can test the ability of a compound to inhibit steroidogenesis. Some physiochemical characteristics of gonadotoxicants are shown in Table 36.21. The toxicity of a particular gonadotoxicant depends to some extent on its ability to penetrate the blood–testes barrier, which is the barrier formed by tight connections between the Sertoli cells. In younger animals, the testicular gap junctions are still relatively permeable, and the germinal epithelium may be more vulnerable than in the adult testes.

Androgens are steroids (Figure 36.14) that can be measured fluorometrically, by chromatography, and by RIA. They also can be bioassayed using various biological responses such as the stimulation of accessory sex organ weights in castrated animals (e.g., prostate gland or seminal vesicle weights). Still other androgen assessments can be made by measuring male sex accessory gland constituents (e.g., seminal vesicle fructose levels and zinc concentrations).

Androgens possess three primary biological actions: (1) virilizing or masculinizing actions, (2) protein anabolic or myotropic actions, and (3) antiestrogenic properties. All of these biological effects can be bioassayed in experimental animals, with rodents the most commonly used animal

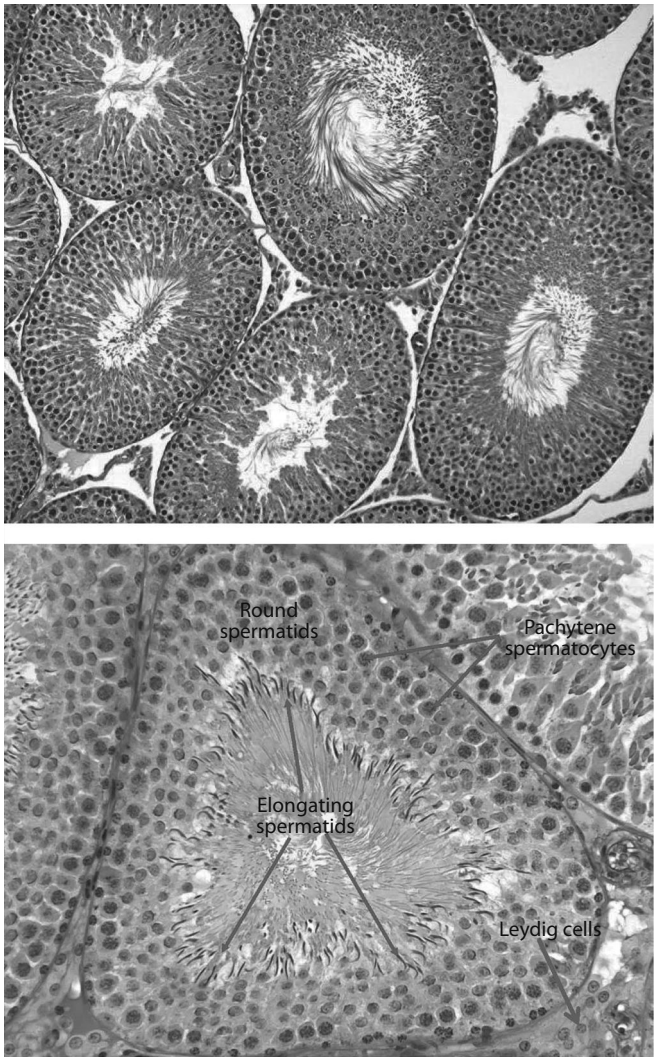

FIGURE 36.13 Normal rat testes.

TABLE 36.21
Some Physicochemical Characteristics of Gonadotoxicants

Usually lipophilic
Avidity/affinity for AR
Permeation of the testes–blood barrier
Diverse chemical structures
Molecular weight frequently less than 400
Proclivity for rapidly dividing cells

model. The actions of male sex hormones (i.e., their masculinizing actions) are usually bioassayed on the basis of gravimetric responses in sex accessory organs (Figure 36.15). Generally, either the rat seminal vesicle or ventral lobes of the prostate gland are used to bioassay androgens. For the bioassay, rats are castrated, and the sex accessory glands are allowed to regress for about 7 days. Using testosterone as a standard, castrated rats are injected for several days, the

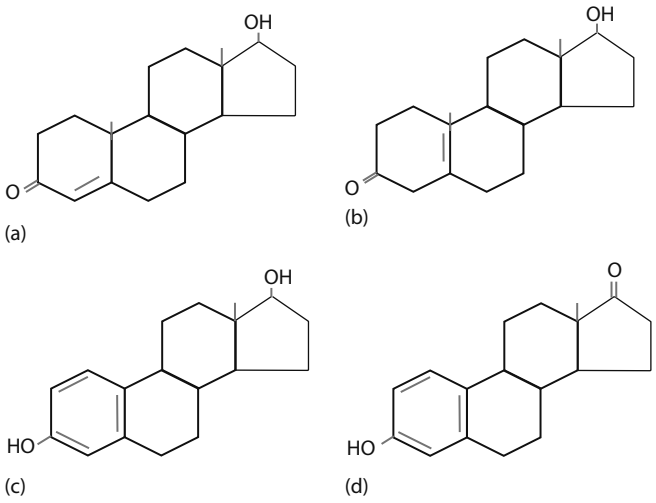

FIGURE 36.14 (a) Testosterone, (b) dihydrotesterone, (c) estradiol-17β, and (d) estrone.

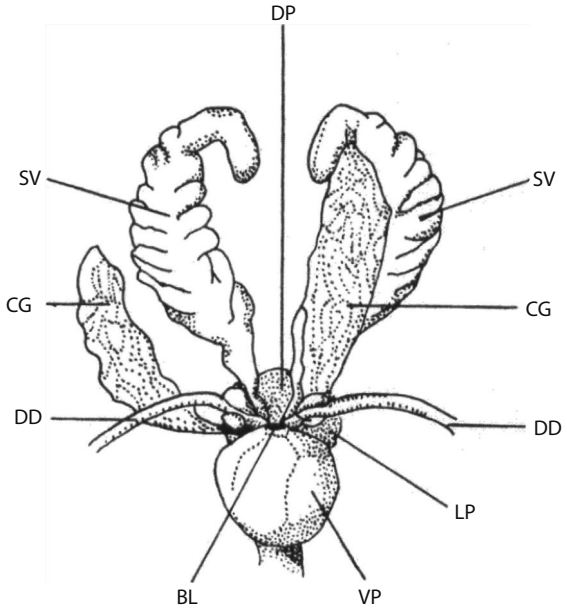

FIGURE 36.15 Anatomical components of rodent sex accessory glands. DD, ductus deferens; BL, bladder; VP, ventral prostate; LP, lateral prostate; CG, coagulating gland (also called anterior prostate); SV, seminal vesicle; DP, dorsal prostate.

animals are killed, and either the seminal vesicles (empty) or the ventral prostate glands are removed and weighed (Figure 36.16). The data reveal that organ weights of both the seminal vesicles and the ventral prostates increase directly proportional to the increase in concentration of injected testosterone. Sometimes, immature rats are used for the bioassay instead of castrated animals.

The rat levator ani muscle weight has been used to bioassay the protein anabolic actions of androgens. The levator ani bioassay was developed following the observations of the myotropic and nitrogen-retaining activities of certain androgenic steroids. This test lacks specificity as a measure of

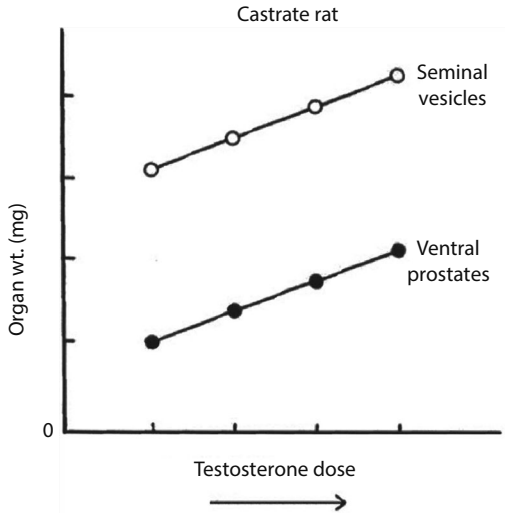

FIGURE 36.16 Bioassay of androgens using rat seminal vesicles or ventral prostate glands from castrated rats.

protein anabolic activity. The levator ani test ordinarily uses immature castrated rats that have been treated for 1 week with the steroid. In addition to androgen bioassay methods using rodent sex accessory organs and muscles, male sex hormones, which exert a renotropic action in mice, can be utilized. Androgens can stimulate the growth of the kidneys in castrated or immature mice.

Chemical indicator tests also have been used to assess androgen activity. Sex accessory organs contain several biochemical constituents that are androgen-dependent and quantifiable[236]; for example, sex accessory gland fructose decreases following castration and can be restored by androgen administration. Sex accessory gland fructose has been used as a sensitive chemical indicator for testosterone and other androgens. Fructose can be measured spectrophotometrically by several colorimetric reactions. Similarly, sex accessory organ citric acid can be used to assess androgenic activity. These chemical indicator tests for androgens are more sensitive than the gravimetric responses used in bioassay procedures. A number of different endpoints to assess male reproductive function have been proposed for food and color additives.[164]

Urinary creatine profiles have been used as a chemical indicator for testicular toxicity.[237] Several chemicals (e.g., methoxyacetic acid)[238] have been assessed for their testicular toxicity, as evidenced by increases in urinary creatine (Table 36.22). Creatine is associated primarily with cells of the seminiferous epithelium. Several chemicals can destroy the germ cells, as reflected by creatinuria. Chemicals that destroy primarily Leydig cells do not lead to creatinuria; for example, ethane dimethane sulfonate (EDS) exerts a direct cytotoxic action on the Leydig cells. Creatinuria is related to testicular degeneration, and a substantial portion of the testicular creatine is associated with the cells of the seminiferous epithelium.

Testosterone levels have been determined using double-isotope derivative methods, gas–liquid chromatography,

TABLE 36.22
Urinary Creatine: A Test for Cell-Specific Testicular Toxicants

Agent	Urinary Creatine	Site of Toxicity
Cadmium (Cd)	↑	Vasculature
2-Methoxyethanol (2-ME)	↑	Early- and late-stage pachytene primary spermatocytes
Methoxyacetic acid (MAA)	↑	Germ cell
di-(*n*-Pentyl) phthalate (DPP)	↑	Initially, Sertoli cells; secondarily, germ cells
Dinitrobenzene (DNB)	↑	Initially, Sertoli cells; secondarily, germ cells
EDS	↔	Leydig cell

Source: Moore, N.P. et al., *Arch. Toxicol.*, 66, 435, 1992. With permission.

and fluorometric procedures. Although these testosterone methods are specific and sensitive, elaborate purification is essential for accuracy, and routine application is often time-consuming. Competitive protein-binding assays and RIAs are available. In the RIA, antiserum against testosterone can be produced in rabbits immunized with testosterone-3-oxime-beef serum albumin. Biological samples must undergo solvent extraction and be eluted on microcolumns. If the extraction and purification of the samples are appropriately carried out, the RIA of testosterone is highly sensitive and accurate; it is capable of detecting testosterone in nanogram to picogram amounts. A method has been described for assessing androgens under field or remote site conditions.[239] mAb techniques can be used for assessing ARs.

Male Infertility/Testicular Toxicity

The inability of a male to achieve a pregnancy in a fertile female is referred to as male infertility. It is estimated that 40%–50% of infertility in humans is due to the male partner.[240] The etiology of male infertility may be genetic (Y chromosome microdeletions to Klinefelter syndrome) or nongenetic (trauma, disease, and cancer). Infertility may or may not be due to direct toxic insults to the testicles. The primary functions of the testes are to produce sperm and to produce androgens, primarily testosterone. Both functions of the testicle are influenced by gonadotropic hormones produced by the anterior pituitary. LH results in testosterone release. The presence of both testosterone and FSH is needed to support spermatogenesis. It has also been shown in animal studies that if testes are exposed to either too high or too low levels of estrogens (such as estradiol; E2), spermatogenesis can be disrupted to such an extent that the animals become infertile.[241]

In mammals, the testes are found outside of the body cavity within the scrotum. Because spermatogenesis cannot occur at body temperature, scrotal temperature is a few degrees lower than internal body temperature. The physiological temperature of the adult human testis is 31°C–35°C.[242] Several mechanisms aid in maintaining testicular temperature within defined limits as environmental conditions change. For efficient dissipation of heat, the scrotum is rich in sweat glands and has only minimal amounts of subcutaneous fat. Importantly, the pampiniform plexus is an effective heat-exchange system that aids in decreasing scrotal temperatures. A two-muscle system lowers and lifts the testis. The tunica dartos is a muscular layer of the scrotum, and the cremaster muscle extends from the body wall through the inguinal canal, surrounding the spermatic cord (see Figure 36.17). When environmental temperature is elevated, the muscles relax in unison, and the testes are lowered from the body, permitting the escape of excess heat. Under cold conditions, the muscles contract, pulling the testes toward the warmth generated by the body.

Numerous factors, both neural and hormonal, may contribute to male infertility. The hormonal factor can be affected by gonadotoxicants, which cause spermatogenic arrest and degeneration of seminiferous tubules, leading to infertility (i.e., low sperm count). Some industrial chemicals can cause sterile tubules and result in infertility[243]; examples include DBCP[243] and alkylating agents (e.g., nitrogen mustards).[244] X-rays[245] and cryptorchidism (undescended testes)[246] can also lead to infertility.

Most testicular toxicity can occur with no change in hormone levels. More broadly, a recent survey of industry practices indicated that most investigative studies of blood hormone concentrations (most often testosterone) used five to eight animals per group.[247] Measuring circulating levels of male reproductive hormones can provide essential evidence to support a hormonally mediated mechanism of toxicity; however, the success of the study will depend on the hormonal study being adequately powered by enough animals. Based on the coefficients of variation of the different hormones, the following group sizes are recommended to obtain a reasonable (80%) chance of detecting a 50% change in hormone level: for testosterone in rats, n = 30/group; for FSH and LH in rats, n = 10–20/group; for testerosterone in dogs and monkeys, sample every 10–20 min over a 2–2.5 h period and pool the samples for an integrated value; otherwise, group sizes of n = 20 would be needed if only a single sample is taken.[248] When conducting hormonal measurements in any species, it is important to establish that the animals have attained sexual maturity and to control for factors such as diurnal variation and stress.

Many useful tests can aid in determining reproductive success, but such tests must be undertaken in both the male

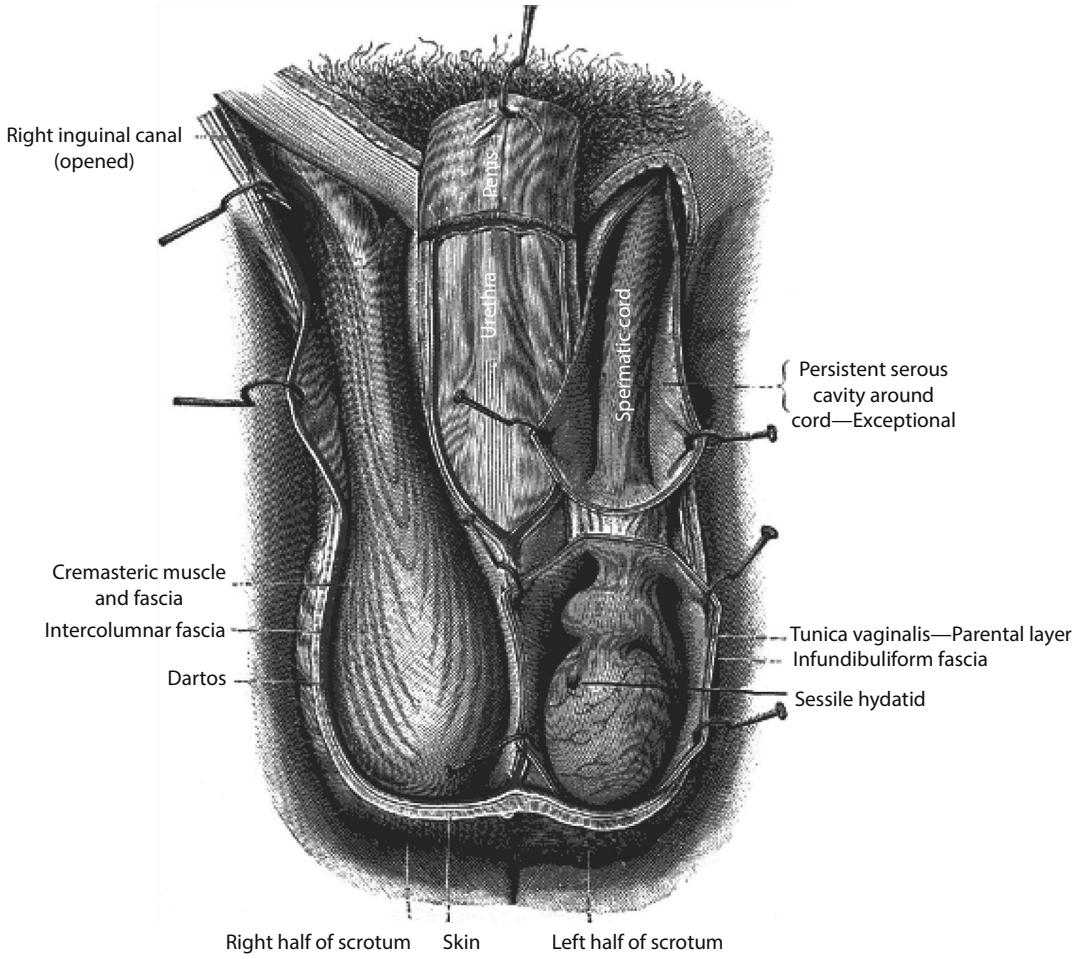

FIGURE 36.17 Male reproductive tract. (Courtesy of U.S. National Cancer Institute Surveillance, Epidemiology, and End Results [SEER] Program.)

(Table 36.23) and the female (Table 36.24). Although the measurement of fertility is imprecise, it does have the advantage of integrating all reproductive functions from both the male and the female.[249] Testicular histology is not quantitative but provides some information about gonadal physiology (or pathology).

Due to transgene technology, several animal models are now available for studying male sterility.[250] Knockout mouse models have been produced for the key components of the hypothalamo–pituitary–testicular axis, including the GnRH gene (hpg), FSH and LH receptors, FSH and LH subunits, ERs, and the aromatase enzyme. Germ cell transplantation models are based on the transplantation of spermatogonial stem cells from wild-type or factor-deficient animals into infertile hetero- or homozygous recipients.[251] Genetic aberrations are evident not only in the processes involved in spermatogenesis but also in anatomical defects observed in sex accessory organs. GnRH cell lines have been developed and can produce a transgenic mouse with hypothalamic hypogonadism[252]; hence, transgenic mouse models can be used to study the mechanisms of spermatogenic arrest as well as altered neuroendocrine systems that interfere with gonadotropin secretion.[253]

Female Sex Hormones (Estrogens)

Estrogens are a group of compounds important in the estrous and menstrual reproductive cycles of humans and other animals, being the primary female sex hormones. Animals that have estrous cycles resorb the endometrium if conception does not occur during that cycle, whereas animals that have menstrual cycles shed the endometrium through menstruation. Natural estrogens are steroid hormones, while some synthetic ones are nonsteroidal. The name comes from the Greek word for "gadfly" and the suffix "gen", meaning "producer of." Estrogens are synthesized in all vertebrates as well as some insects.[254,255] Estrogens are biosynthesized primarily by theca interna cells of the developing follicles in the ovaries, the corpus luteum, the placenta, and, in lesser amounts, the adrenal cortex. Secondary sources such as the breasts, liver, and adrenals are an important source of estrogens in postmenopausal women. Fat cells produce estrogen as well.[256]

The three major naturally occurring estrogens in women are estrone (E1), estradiol (E2), and estriol (E3). Estradiol is the predominant estrogen during reproductive years both in terms of absolute serum levels as well as in terms of

TABLE 36.23
Potentially Useful Tests of Male Reproductive Toxicity for Lab Animals and Man

Testis
- Size in situ
- Weight
- Spermatid reserves
- Gross and histologic evaluation
- Nonfunctional tubules (%)
- Tubules with lumen sperm (%)
- Tubule diameter
- Counts of leptotene spermatocytes

Sperm motility
- Time-exposure photography
- Multiple-exposure photography
- Cinemicrography
- Videomicrography
- Sperm membrane characteristics
- Evaluation of sperm metabolism
- Fluorescent Y bodies in spermatozoa
- Flow cytometry of spermatozoa
- Karyotyping human sperm proneuclei
- Cervical mucus penetration test

Endocrine
- LH
- FSH

Gonadotropin-releasing hormone

Epididymis
- Weight and histology
- Number of sperm in distal half
- Motility of sperm, distal end (%)
- Gross sperm morphology, distal end (%)
- Detailed sperm morphology, distal end (%)
- Biochemical assays

Fertility
- Ratio of exposed to pregnant females
- Number embryos or young per pregnant female
- Ratio of viable embryos to corpora lutea
- Number of 2–8-cell eggs
- Number of unfertilized eggs
- Sperm per ovum

In vitro
- Incubation of sperm in agent
- Hamster egg penetration test

Semen
- Total volume
- Gel-free volume
- Sperm concentration
- Total sperm/ejaculate
- Total sperm/day of abstinence
- Sperm motility, visual (%)
- Sperm motility, videotape (% and velocity)
- Gross sperm morphology
- Detailed sperm morphology spermatozoa

Accessory sex glands
- Histology
- Gravimetric

Other tests considered
- Tonometric measurement of testicular consistency
- Qualitative testicular histology
- Stage of cycle at which spermiation occurs
- Quantitative testicular histology

Source: Thomas, J.A., in *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 5th edn., Klaassen, C.D., ed., Pergamon Press, New York, pp. 547–581, 1996. With permission.

estrogenic activity. During menopause, estrone is the predominant circulating estrogen, and during pregnancy, estriol is the predominant circulating estrogen in terms of serum levels. Though estriol is the most plentiful of the three estrogens, it is also the weakest, whereas estradiol is the strongest with a potency of approximately 80× that of estriol.[257] Thus, estradiol is the most important estrogen in nonpregnant females who are between the menarche and menopause stages of life. However, during pregnancy, this role shifts to estriol, and in postmenopausal women, estrone becomes the primary form of estrogen in the body. Another type of estrogen called estetrol (E4) is produced only during pregnancy.

All of the different forms of estrogen are synthesized from androgens, specifically testosterone and AND, by the enzyme aromatase. Cholesterol is converted from AND to pregnenolone by the enzyme P450 side-chain cleavage; another enzyme, CYP17A, converts pregnenolone to 17α-hydroxypregnenolone and then to DHEA, which is converted to estrogens. Cholesterol, also synthesized to AND in the theca interna cells, is subsequently absorbed into the nearby granulosa cells of the ovary. In granulosa cells, AND is converted primarily to estradiol-17β. Evidence suggests that estrone is also secreted in the process and can be further metabolized to estradiol by hepatic enzymes (Figure 36.18). Of these three main naturally occurring estrogens, estradiol-17β is undoubtedly the most potent. All of these naturally occurring estrogens are steroids. Synthetic estrogens such as DES and dienestrol are not steroids. Although steroidal and nonsteroidal estrogens can be bioassayed using similar tests, their different molecular structures do not allow them to be measured by similar chemical methodologies. Testing guidelines for evaluating reproductive and developmental toxicity in the female are described on FDA, ICH, EPA, and OECD websites and are described in Hood and Parker.[258]

The two methods once commonly used to bioassay estrogenic hormones in rodents involve either histological

TABLE 36.24
Potentially Useful Tests of Female Reproductive Toxicity

Ovary
- Organ weight
- Histology
- Number of oocytes
- Rate of follicular atresia
- Follicular steroidogenesis
- Follicular maturation
- Oocyte maturation
- Ovulation
- Luteal function

Cervix/vulva/vagina
- Cytology
- Histology
- Mucus production
- Mucus quality (sperm penetration test)

Oviduct
- Histology
- Gamete transport
- Fertilization
- Transport of early embryo

In vitro
- In vitro fertilization of superovulated eggs, either exposed to chemical in culture or from treated females either exposed to chemical in culture or from treated females

Uterus
- Cytology and histology
- Luminal fluid analysis (xenobiotics, proteins)
- Decidual response
- Dysfunctional bleeding

Fertility
- Ratio of exposed to pregnant females
- Number of embryos or young per pregnant female
- Ratio of viable embryos to corpora lutea
- Number of 2–8-cell eggs
- Number of unfertilized eggs; abnormal eggs
- Number of corpora lutea

Hypothalamus
- Histology
- Altered synthesis and release of neurotransmitters, neuromodulators, and neurohormones

Pituitary
- Histology
- Altered synthesis and release of tropic hormones

Endocrine
- Gonadotropin
- Chorionic gonadotropin levels
- Estrogen and progesterone

Source: Thomas, J.A., in *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 5th edn., Klaassen, C.D., ed., Pergamon Press, New York, pp. 547–581, 1996. With permission.

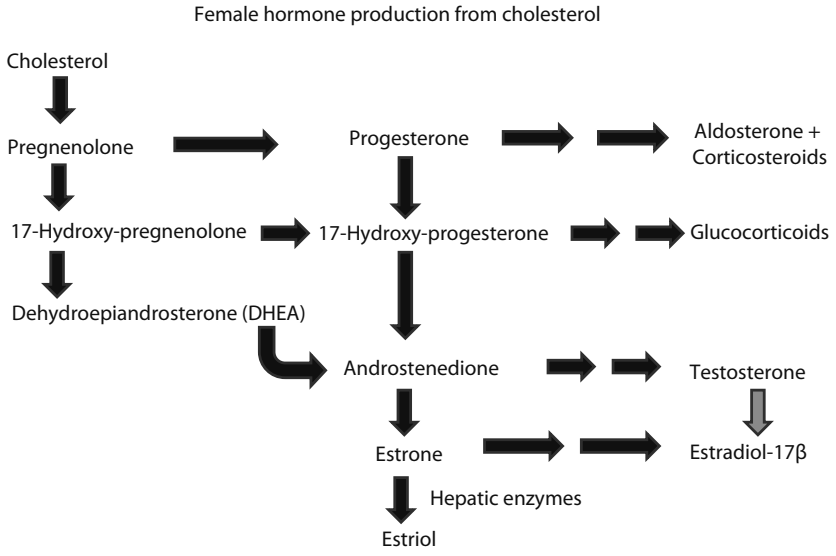

FIGURE 36.18 Biosynthesis of sex steroids.

changes in the vaginal epithelium or an increase in uterine weights. Both assays require the use of ovariectomized animals.

The mouse vaginal smear bioassay uses ovariectomized animals with different standard doses of estradiol as controls. To establish that the animals are responding, vaginal smears are characterized by nucleated epithelial cells or cornified cells. Vaginal responses characterized by epithelial cornification are considered positive responders to estradiol. Estrogen bioassays also include using an increase in

uterine weight in the ovariectomized rat or mouse. Uterine weight falls precipitously after ovariectomy. The reduced uterine weight can be restored by daily injections of estradiol. Ovariectomized mice are injected subcutaneously and killed, and the uteri are removed and weighed. Like the vaginal smear bioassay, at least two dilutions of the unknown substance are run concurrently with the estradiol dose–response curve.

These bioassays (i.e., vaginal cornification and uterine weights) are quite sensitive to estrogen but have largely been abandoned for newer and more expedient and accurate assays. Several useful tests can be utilized in determining reproductive toxicity in the female reproductive system (see Table 36.24). Estrogens can be measured colorimetrically, by RIA, monoclonal assays, and other immunotechniques. Estrogen and progesterone receptors can also be measured by mAb techniques that employ EIAs.[259] The evaluation of techniques for the detection of functional estrogenicity has received renewed attention.[260]

Female Infertility/Ovarian Toxicity

The ovaries are homologous to the testicles in the male. In humans, they are located internally on the posterior wall of the pelvis lateral to the position of the uterus called the ovarian fossa and are structurally supported by three ligaments: the suspensory ligament, the broad ligament, and the ovarian ligament. The ovaries are close to but not attached to the ovarian (fallopian) tubes, which transport the ovum as it is expelled from the ovary to the uterus (Figure 36.19). Usually, each ovary takes turns releasing eggs every month; however, in cases where one ovary is absent or dysfunctional, then the other ovary would continue providing eggs each month. Ovaries secrete both estrogen and progesterone. Estrogen is responsible for the appearance of secondary sex characteristics for females at puberty and for the maturation and maintenance of the reproductive organs in their mature functional state. Progesterone prepares the uterus for pregnancy and the mammary glands for lactation. Progesterone functions with estrogen by promoting menstrual cycle changes in the endometrium. Toxicants can affect reproductive outcomes at a number of points in the female reproductive process through their direct effects on the ovaries, ovulation, fertilization, implantation, and the developing fetus.

A paucity of research data exists regarding ovarian toxicants in humans leaving animal research, usually the rat, to provide much of the ovarian toxicity data. Direct effects on the ovary can produce a reduction in ovarian hormonal secretion, resulting in a change in the LH and FSH levels as a result of reduced negative feedback signals to the pituitary and hypothalamus. Direct ovarian toxicants could also hinder the production of follicles, which could result in early menopause or in a disruption of menses. Reproductive toxicants produce changes in ovarian functions at different levels. Some agents are known to be toxic to the ovaries, such as nitrogen mustards, chlorambucil, cyclophosphamide, busulfan, vinblastine, and polycyclic aromatic hydrocarbons[261]; hence, the ovary is clearly vulnerable to chemical injury.[262,263] A decrease in ovarian follicle count is usually considered a biomarker of an adverse reproductive event because no recovery is possible.[264] Both the granulosa cells and the thecal cells may be targets for chemical injury (Table 36.25). Chemicals that inhibit gonadotropin secretion, damage gonadotropin receptors, or uncouple the receptor from other molecules necessary for hormone action would be expected to adversely affect granulosa cells. Thecal

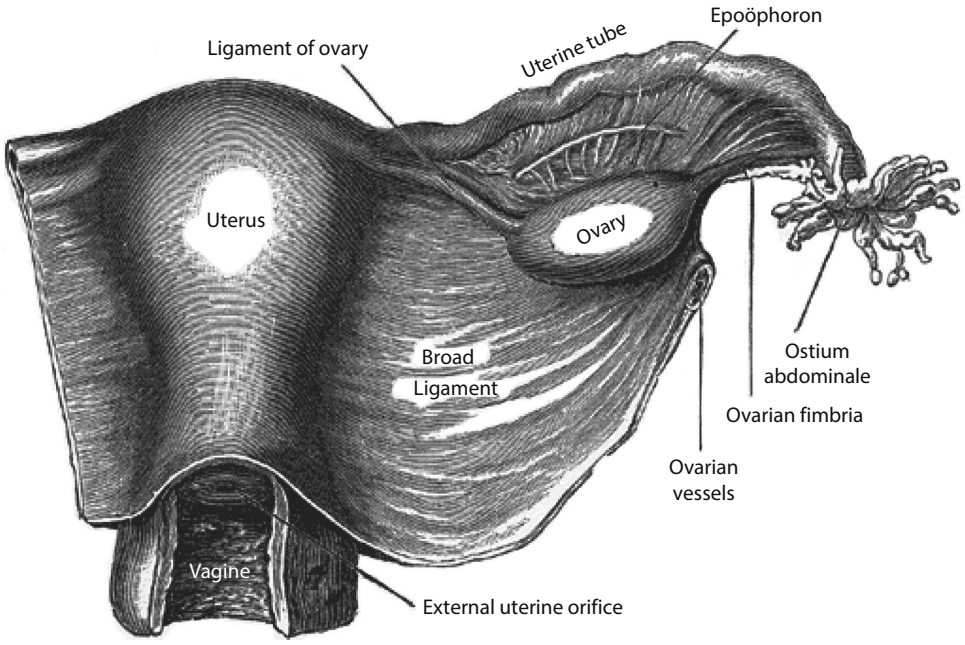

FIGURE 36.19 Female reproductive tract. (Courtesy of U.S. National Cancer Institute Surveillance, Epidemiology, and End Results [SEER] Program.)

TABLE 36.25
Ovarian Cells as Targets for Chemical Injury

Site of Action	Mechanism of Action (Outcome)
Granulosa cells	
FSH/LH receptors	Decreased receptor population
	Competition for receptors
	Uncoupling of receptors to secondary messenger
Steroidogenesis	Altered estrogen production (e.g., aromatase activity)
	Altered progesterone production (e.g., enzymatic inhibition)
	Insufficient androgens
	Inadequate luteinization (e.g., decreased progesterone)
Cell proliferation	Cytotoxicity and mitotic inhibitors
	Reduction of growth factors
Thecal cells	
LH receptors	Decreased receptor population
	Competition for receptors
	Uncoupling of receptors to secondary messengers
Steroidogenesis	Inhibition of enzymes (e.g., decreased androgens)
	Insufficient substrate for granulosa cells
Cell proliferation	Cytotoxicity and mitotic inhibitors
	Disrupted migration of stroma
	Reduction of growth factors

Sources: Mattison, D.R. et al., *Med. Clin. N. Am.*, 74, 391, 1990; Sanbuisso, A. et al., *J. Toxicol. Sci.*, 34(1), 1, 2009.

cells provide precursors for granulosa cell steroidogenesis. Xenobiotics can alter either granulosa cells or thecal cells. The oocytes themselves are also targets for chemical insult or injury. Alkylating agents, lead, and mercury can be destructive to the mammalian oocyte.[263]

PANCREAS

Pancreatic tissue is present in all vertebrate species, but its precise form and arrangement vary widely. There may be up to three separate pancreases, two of which arise from ventral buds and the other dorsally. In most species (including humans), these fuse in the adult; however, there are several exceptions. Even when a single pancreas is present, two or three pancreatic ducts may persist, each draining separately into the duodenum (or equivalent part of the foregut). Birds typically have three such ducts. In fish and a few other species (such as rabbits), there is no discrete pancreas at all, with pancreatic tissue being distributed diffusely across the mesentery and even within other nearby organs, such as the liver or spleen.[266]

In humans, the pancreas is a retroperitoneal lobulated organ located posterior to the stomach and in close association with the duodenum, approximately 1 in. in height and 5 in. in length and weighing up to about 100 g (Figure 36.20).[267] The pancreas has two primary functions: (1) The exocrine pancreas produces enzymes for digesting lipids and proteins that break down various categories of digestible foods from asini cells, and (2) the endocrine pancreas secretes hormones from clusters of glandular epithelial cells called *pancreatic islets* (islets of Langerhans) that produce the hormones glucagon, insulin, and somatostatin. Four main cell types exist in the islets. They are relatively difficult to distinguish using standard staining techniques, but they can be classified by their secretion: α cells secrete glucagon (increase glucose in blood), β cells secrete insulin (decrease glucose in blood), delta cells secrete somatostatin (regulates/stops α and β cells), and PP cells or gamma cells secrete pancreatic polypeptide (Figure 36.21).[268]

Under a microscope, stained sections of the pancreas reveal two different types of parenchymal tissue. Lightly staining clusters of cells are called islets of Langerhans, which produce hormones that underlie the endocrine functions of the pancreas. Darker-staining cells form acini connected to ducts. Acinar cells belong to the exocrine pancreas and secrete digestive enzymes into the gut via a system of ducts. The acini cell secretions collect in the acini ducts, which communicate directly with the intralobular ducts, eventually draining into the major pancreatic duct or the duct of Wirsung. This duct subsequently unites with the common bile duct, which enters the upper end of the duodenum in a duct common to both organs termed the *hepatopancreatic ampulla* or the *ampulla of Vater*. At this juncture, the exocrine pancreatic secretions enter the duodenum and exert their effects upon the intestinal chyme. The morphologic arrangement of the rat pancreas is depicted in Figure 36.20.

The endocrine pancreas secretes two hormones important in the regulation of carbohydrate metabolism: insulin and glucagon. Insulin is the primary regulator in carbohydrate homeostasis, as it controls blood glucose levels. Additionally, insulin regulates muscle tone, the uptake of amino acids

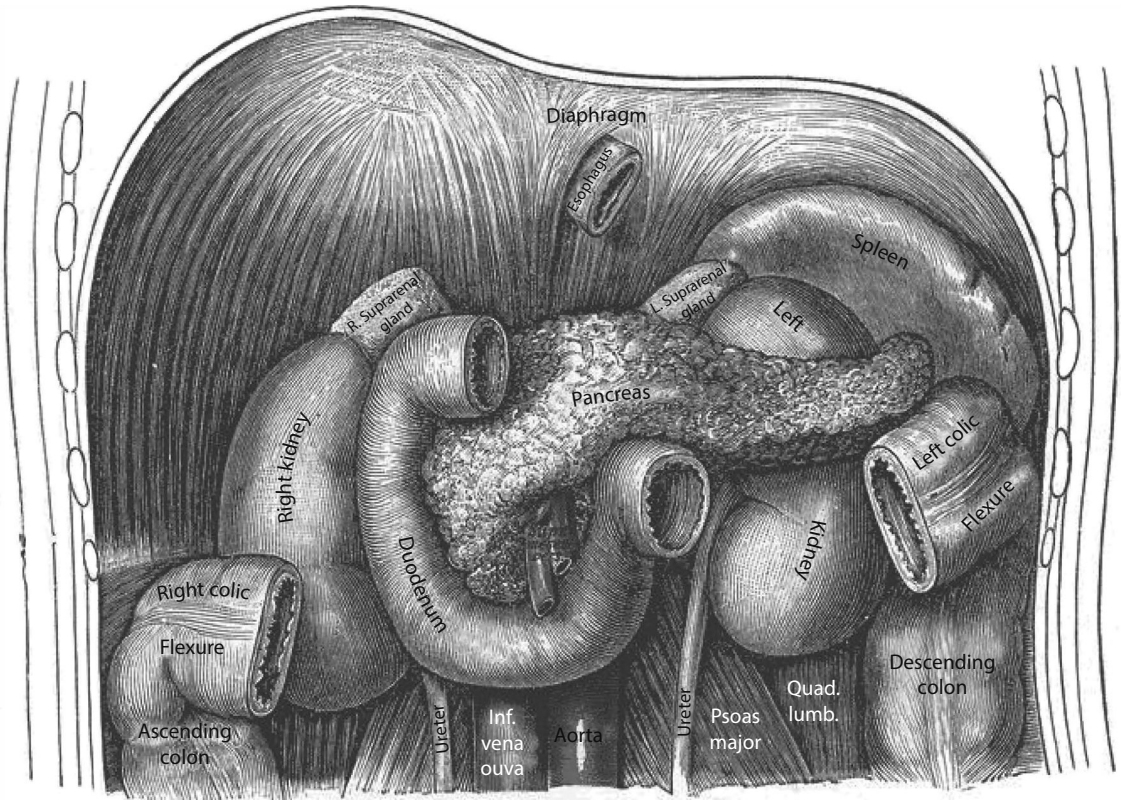

FIGURE 36.20 Anatomical relationship of pancreas to adjacent organs.

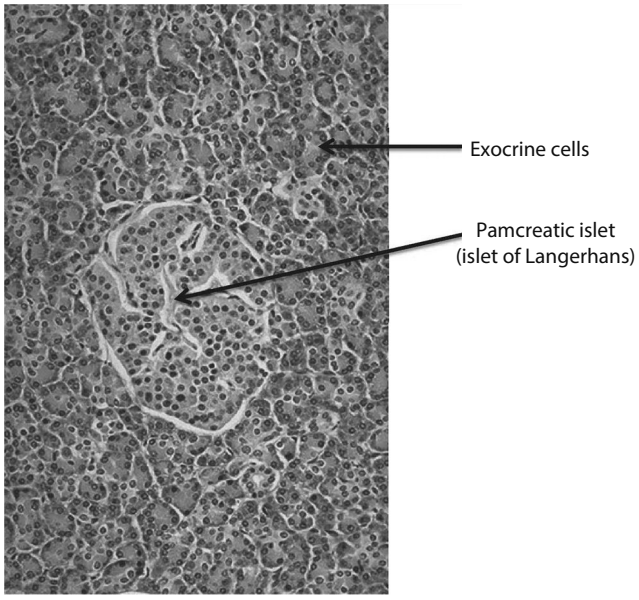

FIGURE 36.21 Histology of rat pancreas. Note the acini ducts (original magnification, 256×).

and electrolytes, and the release of triglycerides. By binding to specific hepatocyte receptors, glucagon also assists in the regulation of the level of glucose in the blood, resulting in (1) the release of stored glycogen as glucose into the bloodstream (termed *glycogenolysis*) and (2) the synthesis of additional glucose for release into the bloodstream (termed *gluconeogenesis*). Glucagon stimulates the release as well as the synthesis of glucose in response to hypoglycemia. These hormones are synthesized in the islets of Langerhans by α cells (glucagon) and β cells (insulin). Somatostatin is considered an inhibitory hormone that can retard the release of growth-hormone-releasing factor (GHRF), GH, TSH, insulin, and glycogen. It also suppresses the release of gastrointestinal hormones such as gastrin, CCK, secretin, motilin, gastric inhibitory polypeptide, and enteroglucagon and generally slows the rate of smooth muscle contractions and the flow of blood into the small intestine.

A concern when testing for toxicity is the potential for the agent to interfere with the normal functioning of the pancreatic β cells. The indicator that often alerts the toxicologist to a possible pancreatic side effect is hyperglycemia. Although this increased blood glucose level would usually be detected during routine clinical chemistry analyses, additional tests may be required to pinpoint specific pancreatic toxicity.

The interactions between insulin and carbohydrate, fat, and protein metabolism represent some of the complexities seen in diabetes mellitus. Hyperglycemia may result from impaired utilization of glucose by cells due principally to the insufficient production of insulin. The failure of glucose to penetrate adipose tissue mobilizes fat, producing a rise in the free fatty acid and triglyceride content of plasma and the triglyceride content of the liver. A diabetic fatty liver can occur from the absence of lipoprotein synthesis due to accelerated gluconeogenesis. If glucose oxidation is impaired, fatty acids form the major source of energy. This condition

generates an excess of intermediary metabolites, collectively described as ketone bodies (acetone, acetoacetic acid, and β-hydroxybutyric acid), which can lead to metabolic acidosis. Nitrosamine compounds, which are widely present in processed foods, and streptozotocin, a nitrosamine-related compound, have been shown to contribute to the pathogenesis of diabetic fatty liver.[269]

Hyperglycemia also can lead to the presence of glucose in the urine (glycosuria) when the blood glucose levels exceed the renal threshold of approximately 180 mg/dL. At lower levels, all the filtered glucose is normally reabsorbed by the renal tubules. Blood glucose levels increased to the point of glycosuria also can be caused by emotional stress and the concomitant release of glucose from liver glycogen in response to epinephrine. Several drugs can affect blood glucose levels and can either increase or antagonize glucose levels (Table 36.26). Glycosuria also can be the consequence of impaired renal tubular function caused by compounds such as the glycoside phlorizin.[270] Renal glycosuria can produce an osmotic diuretic effect that leads to dehydration and polydipsia. Glycogenolysis and gluconeogenesis are increased in diabetes mellitus, generating glucose, which in turn increases blood glucose levels.

Experimental diabetes mellitus can be produced by destroying β cell function with alloxan or streptozocin (Figure 36.22). In addition to chemically induced destruction of β cells by alloxan and streptozocin, these chemicals can be diabetogenic.[271,272] Alloxan, a cyclic urea analog, can produce permanent hyperglycemia in the rabbit. Streptozotocin, a methylnitroso–urea analog, has generally replaced alloxan to produce experimental insulin-dependent diabetes in laboratory animals. Both agents destroy pancreatic β cells, but

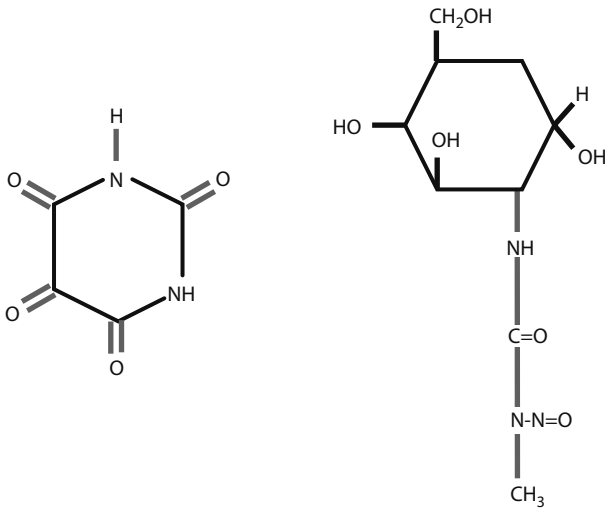

FIGURE 36.22 Agents that inhibit pancreatic insulin and glucagon secretion.

streptozotocin may involve the alkylation of critical cell components. Chlorozotocin, an analog of streptozocin, is lethal to β cells in culture. Vacor (pyriminil), cyproheptadine, and pentamidine are all capable of causing pancreatic dysfunction. Experimental animal models for diabetes mellitus may exploit genetic susceptibility (diabetes susceptible or diabetes resistant) (Table 36.27) or may involve the chemically induced destruction of β cells.[273]

Several inbred strains of mice are sensitive to *db* gene-induced diabetes, with sexual dimorphism in some inbred strains emphasizing the relationship between the obesity

TABLE 36.26
Interaction of Drugs with Oral Hypoglycemic Agents and Insulin

Enhanced Effect[a]	Antagonist Effect
Anabolic steroids	Acetazolamide (Acetamox and Acetazolam)
Chloramphenicol	Corticosteroids
Dihydroxycoumarin	Diuretics (e.g., chlorthalidone, ethracrynic acid, furosemide, thiazides, triamterene)
Ethanol	
Guanethidine	D-Thyroxine
K^+ salts	Epinephrine
MAO inhibitors	Marijuana
Oxytetracycline	Oral contraceptives (estrogen plus progestogen)
Phenylbutazone	Phenothiazines
Phenyramidol	Orinase
Probenecid	Diabinase
Propranolol	Barbiturates
Salicylates	Rifampin
Sulfinpyrazone	Laxatives (after protracted use)
Sulfonamides	Diazoxide

[a] Greater hypoglycemic action.

TABLE 36.27
Genetically Altered Rodents Used to Study Diabetes Mellitus

Diabetes-susceptible strains	
Mice	C57BLK/J
	DBA/2J
	SWR/J
	C3H.SW/SnJ
	C3HeB/FeCHp (males only)
	CBA/Lt (males only)
	NOD (IDDM)
Rats	BB(IDDM)
	BHE
	BHE/cdb
Diabetes-resistant strains	
Mice	C57BL/6J
	129/J
	Ma/MYJ

Sources: Thomas, 1996[229]; Etuk, E.U., *Agric. Biol. J. N. Am.*, 1(2), 130, 2010.

gene and sex. A major genetic regulator of inbred-strain diabetogenic sensitivity is gender related. In the rat, the Bureau of Home Economics (BHE) strain is an excellent animal model for the study of noninsulin-dependent diabetes mellitus (NIDDM). The Cdb:BHE stock is a subline of the parent BHE stock. The nonobese diabetic (NOD) mouse is an ideal animal model of insulin-dependent diabetes mellitus.[274] Spontaneous diabetes in the biobreeding (BB) rat, like human Type 1 diabetes mellitus, results from the destruction of the pancreatic islets by autoreactive T lymphocytes recognizing β-cell-specific antigens.

Chemical insult is not unique to the endocrine pancreas; indeed, many agents can cause acute pancreatitis (Table 36.28). Side effects resulting from a number of cytotoxic agents (e.g., colchicine), antibiotics (e.g., tetracyclines), antibacterial drugs (e.g., sulfonamides), and diuretics (e.g., thiazides) can produce irritation and inflammation of pancreatic cells, resulting in symptoms of severe epigastric pain radiating to the back, nausea, vomiting, diarrhea and loss of appetite, fever/chills, hemodynamic instability, which include shock, tachycardia, respiratory distress, and peritonitis.[276]

TABLE 36.28
Drugs Implicated in Acute Pancreatitis

Definite	Possible
Acetaminophen	Anticholinesterase
Azathiorine	Bumetanide
Cisplatin	Carbamazepine
Chlorthiazide	Chlorthalidone
Colaspase (L-asparaginase)	Chlorthalidone
Clozapine	Clonidine
Corticosteroids	Colchicine
Furosemide	Co-trimoxaz
Lisinopril	Cyclosporin
Sulfonamides	Cytarabine (cytosine arabinoside)
	Diazoxide
Tamoxifen	Enalapril
Tetracycline	ERCP contrast media
Thiazides	Ergotamine
Probable	Ethacrynic acid
Cimetidine	Isoniazid
Diazinon	Isotretinoin (13-*cis*-retinoic acid)
Estrogens	Mercaptopurine
Indomethacin	Methyldopa
Fonofos	Metronidazole
Mefenamic acid	Nitrofurantoin
Opiates	Oxphenbutazone
Pentamidine	Piroxicam
Phenformin	Procainamide
Valproic acid	Rifampicin
	Salicylates
	Sulindac

Sources: Banerjee, A.K. et al., *Med. Toxicol. Adverse Drug Exp.*, 4, 186, 1989; Badalov, N. et al., *Clin. Gastroenterol. Hepatol.*, 5, 648, 2007.

Measurement of Insulin

Although the RIA techniques that are available are similar with regard to the interaction between antigen and antibody, numerous variations exist in the methods for separating free insulin from antibody-bound insulin: gel filtration, salt precipitation, alcohol precipitation, precipitation with anti-gamma-globulin serum, and absorption on anion-exchange resin, cellulose, dextran-coated charcoal, antibody-coated tubes, or Sephadex-coupled antibodies. A radioreceptor assay has been developed for quantitation of plasma insulin levels.

Insulin in the specimen competes with a fixed amount of ^{125}I-labeled insulin for the binding sites of the specific insulin antibodies. Bound and free insulin are separated by adding a second antibody, centrifuging, and decanting. The radioactivity in the pellet is then measured. The radioactivity is inversely proportional to the quantity of insulin in the specimen.[277] This test is used to measure insulin levels in the bloodstream and is also useful in determining pancreatic β-cell activity.

Insulin plasma levels may also be determined by ELISA. The kit is a solid phase (ELISA) based on the sandwich principle. The microtiter wells are coated with an mAb directed towards a unique antigenic site on the insulin molecule. An aliquot of sample containing endogenous insulin is incubated in the coated well with enzyme conjugate, which is an anti-insulin antibody conjugated with biotin. The amount of bound complex is proportional to the concentration of insulin in the sample. Generally, ELISA has a number of drawbacks that preclude its routine use for the measurement of insulin, including a lower sensitivity than is seen with RIA. A hyperglycemic clamp can be used to assess insulin secretion and insulin sensitivity.[278] Insulin receptors can be isolated from a variety of sources, including placenta, rat liver plasma membranes, human lymphocytes, and guinea pig kidney.

Glucose Tolerance Tests

The oral glucose tolerance test (OGTT), useful for evaluating endocrine pancreatic function, is based on the compensatory regulation of blood glucose levels by insulin following the ingestion of a glucose load. A zero time (baseline) blood sample is collected. The glucose load is administered orally to a fasting patient; the intervals and number of blood samples vary according to the purpose of the test. If 50 g of glucose is taken orally by drink within a 5 min time frame, the blood glucose level rises rapidly for approximately 30–60 min and then falls rapidly to obtain fasting levels by 2–3 h. Fasting plasma glucose (measured before the OGTT begins) should be below 6.1 mmol/L (110 mg/dL). Fasting levels between 6.1 and 7.0 mmol/L (110 and 125 mg/dL) are borderline (impaired fasting glycemia), and fasting levels repeatedly at or above 7.0 mmol/L (126 mg/dL) are diagnostic of diabetes. The 2 h OGTT glucose level should be below 7.8 mmol/L (140 mg/dL). Levels between this and 11.1 mmol/L (200 mg/dL) indicate impaired glucose tolerance. Glucose levels above 11.1 mmol/L (200 mg/dL) at 2 h confirms a diagnosis of diabetes.[280]

In situations where the synthesis or release of insulin is insufficient, ingestion of the glucose load leads to an excessive rise in blood glucose levels, followed by a slow, gradual decline to preingestion levels. Abnormal glucose tolerance curves are evident in diabetes mellitus but may also be abnormal in other pathologic states. For example, elevated OGTT values can indicate any one of the following conditions or diseases: acromegaly, chronic renal failure, Cushing's syndrome, stress, hyperthyroidism, pancreatic cancer, pancreatitis, or the use of various drugs (e.g., tricyclic antidepressants, epinephrine, diuretics, and lithium). On the other hand, decreased OGTT response may indicate any one of the following conditions or diseases: adrenal insufficiency, liver disease, polycystic ovary syndrome, hypothyroidism, hypopituitarism, insulinomas, or the use of acetaminophen and anabolic steroids.[13] Glucose oxidase methods involving colorimetric reactions are routinely used to measure blood sugar. Capillary blood glucose can be used to monitor diabetes mellitus.[214]

Cytological Evaluation of Pancreatic Islet Cells

α and β cells can be differentiated using an aldehyde–fuchsin stain (Scott stain) following fixation of the tissue in Bouin's solution.[281] The α cells stain light, and the β cells stain dark (purple/black), permitting calculation of the ratio of α cell to β cells. Pseudoisocyanic staining permits direct demonstration of insulin in the β cells. The reaction involves the development of SO_2 groups formed by the oxidative splitting of the disulfide bridges of insulin with potassium permanganate. Organ culture techniques for studying pancreatic islets have been investigated.[282] The availability of suitably characterized dispersed islet cell preparations offers another in vitro test system to examine the effects of various drugs and chemicals on the pancreas.[283] mAb methods are also available to assay for islet cell antibodies.[284]

QUESTIONS

Essay

36.1 Briefly diagram and describe the HPG axis.

36.2 Outline the biosynthesis of thyroid hormones and the influence of TSH.

36.3 Describe factors that modulate secretions of the endocrine pancreas.

Multiple choice

36.4 Which serum protein(s) can specifically bind to hormones?
- a. SHBG
- b. CBG
- c. TBG
- d. All of the above

36.5 Which gonadal cell(s) produce testosterone?
- a. Germ cells
- b. Sertoli cells
- c. Leydig cells
- d. Endothelial cells

36.6 Suppression of ACTH occurs with
- a. Dexamethasone
- b. TSH
- c. Triiodothyronone
- d. None of the above

36.7 Destruction of pancreatic beta cells is most likely to occur following
- a. FSH administration
- b. Streptomycin
- c. PRL administration
- d. None of the above

36.8 Biosynthesis of adrenocortical steroids can be inhibited by
- a. Mitotane (*o*,*p*′-DDD)
- b. Aminogluthethimide
- c. Metyrapone
- d. All of the above

36.9 Castration can lead to the following endocrine modifications:
- a. Elevation of blood levels of FSH and LH
- b. Reduced levels of testosterone
- c. Atrophy of sex accessory organs
- d. All of the above

36.10 Infertility may be due to
- a. Failure to ovulate
- b. Reduced sperm count
- c. Absence of germinal epithelium
- d. All of the above

True/false

36.11 Monoclonal antibody techniques represent a very sensitive method for determining certain hormones.

36.12 Bioassays have largely been replaced by newer and more reliable hormone tests.

ACRONYMS AND ABBREVIATIONS

3β-HSD	3β-Hydroxysteroid Dehydrogenase
ASDN	1β-Androstenedione
ACTH	Adrenocorticotropic hormone (Corticotropin)
AhR	Aryl hydrocarbon receptor
AND	Androstenedione
AVP	Arginine vasopressin
CD molecules	Cluster of differentiation molecules
CG	Chorionic gonadotropin
CRH	Corticotropin-releasing hormone
CRH-BP	Corticotropin-releasing hormone binding protein
cRNA	Antisense RNA
DEST	Data entry spreadsheet template
DHEA	Dehhydroepiandrosterone
DHEAS	DHEA sulfate
DER	Data evaluation record
DES	Diethylstilbestrol
DIT	Diiodothyronine
E2	Estradiol

E3	Estriol
EAT	Estrogen, androgen, and thyroid
ED	Endocrine disruptor
EDC	Endocrine disrupting chemical
EDSP	Endocrine disruptor screening program
EDSTAC	ED Screening and Testing Advisory Committee
ELISPOT	Enzyme-linked immunosorbent spot assay
EMIT	Enzyme-multiplied immunoassay technique assay
EPA	U.S. Environmental Protection Agency
ER	Estrogen receptor
ERE	Estrogen-responsive element
FD&C	Federal Food, Drug, and Cosmetic Act
FISH	Fluorescence in situ hybridization
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
GC	Glucocorticoid
GHRH	Growth-hormone-releasing hormone
GH	Growth hormone
hCG	Human chorionic gonadotropin
HHPS	Hypothalamo-hypophysial portal system
HRP	Horseradish peroxidase
HPA	Hypothalamus–pituitary–adrenocortical
HPG	Hypothalamo–pituitary–gonadal
HPI	Hypothalamic–pituitary–interrenal
HPT	Hypothalamo–pituitary–thyroidal
IRMA	Immunoradiometric assay
LH	Luteinizing hormone
LHR	Leutinizing hormone receptor
mRNA	Messenger ribonucleic acid
MT TATA	Mouse metallothionein (MT) promoter TATA element
OCSPP	Office of Chemical Safety and Pollution Prevention
o,*p*-DDD	*o*,*p*′-Dichlorodiphenyldichloroethane
P4	Progesterone
PACAP	Pituitary adenylate cyclase–activating polypeptide
PCR	Polymerase chain reaction
PRL	Prolactin
PTH	Parathyroid hormone
PVDF	Polyvinylidenefluoride
qPCR	Quantitative polymerase chain reaction or real-time polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
RIA	Radioimmunoassay
rRNA	Ribosomal RNA
RNA-seq	RNA-sequencing
RRT-PCR	Real-time reverse transcription polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
SAGE Assay	Serial analysis of gene expression assay
SEP	Standard evaluation procedure
SHBP	Sex-hormone-binding protein
snRNA	Small nuclear RNA
SRIF	Somatotropin-release-inhibiting factor
STTA	Stably transfected transcription activation
T_3	Thyroid hormone (triiodothyronine)
T_4	Thyroid hormone (thyroxine)
TA	Transcription activation
TD	Thyroid disruptor
TG	Thyroglobulin
TGB	Thyroid-binding globulin
TR	Thyroid hormone receptor
TRH	Thyrotropin-releasing hormone
TRHR	Thyrotropin-releasing hormone receptor
tRNA	Transfer RNA
TSH	Human thyrotropin
V1aR	Vasopressin-1
WTSS	Whole transcriptome shotgun sequencing
ZF	*Zona fasciculata*
ZG	*Zona glomerulosa*
ZR	*Zona reticularis*

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37 Immunotoxicology
The Immune System Response to Toxic Insult

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CONTENTS

INTRODUCTION

The immune system is a complex multicellular organ system consisting of granulocytes, macrophages, lymphocytes, and dendritic cells with various functions and phenotypic characteristics, as well as various soluble mediators. These cells are of hemopoietic origin and, in adults, are found in the peripheral blood, lymphatic fluid, and organized lymphoid tissues, including bone marrow, spleen, thymus, lymph nodes, tonsils, and mucosa-associated lymphoid tissue (MALT). The immune system is in a constant state of self-renewal involving cell proliferation, differentiation, activation, and maturation. It exists to defend the body against invasion by infectious and opportunistic microorganisms and spontaneously arising neoplasia. This network of cells and soluble factors is highly regulated and interdependent, must discriminate self from nonself, and can react to nonself with many different (pleiotropic) defensive responses [1]. In addition, this immune system occasionally develops an unwanted response to a chemical or its metabolite that binds to or alters a host protein resulting in allergy or autoimmune disease. The immune system of experimental animals, while exhibiting some obvious differences from that of humans, is still sufficiently similar that data obtained from lower species are instructive of a potential human response [2].

IMMUNE MECHANISMS RESPONSIBLE FOR HOST DEFENSE

The host defense functions of the immune system are provided by two major arms: an innate arm that does not require prior sensitization with the inducing foreign agent to elicit a response and an adaptive arm directed against the eliciting agent to which the individual has been previously sensitized. Penetration of the skin or mucosal defense barriers by an invading material results in nonspecific reactions by phagocytic cells (granulocytes and macrophages [MØ]). If the microorganism is not controlled and persists, specific responses involving antibody production and the induction of effector lymphocytes follow. The effector lymphocytes respond through cytokine mediators to seek out and destroy the invading microorganism. Both antibody-producing lymphocyte responses (B-lymphocytes or B-cells) and thymus-dependent lymphocyte responses (T-lymphocytes or T-cells) are triggered by the presentation of foreign antigen to appropriate lymphocytes by dendritic cells, MØ, or other antigen-presenting cells (APCs). Following antigen-induced activation, B-cells proliferate and differentiate into plasma cells (PCs), with the support of T-helper 2 (Th2) cells, and produce large quantities of antigen-specific immunoglobulins (antibodies). Antibodies enter the plasma where they bind the foreign material and either neutralize, lyse, or facilitate phagocytosis of the agent. Antibody–antigen interactions are expanded by actions of the complement (C′) system and other inflammatory mediators (e.g., prostaglandins and leukotrienes). With the support of Th1 cells, another population of T-cells, referred to as cytotoxic T-cells, proliferate and recognize virally infected cells that they can destroy these cells before viral replication can occur.

Innate and Adaptive Mechanisms of Immunity

Two categories of phagocytic leukocyte, the polymorphonuclear phagocyte (PMN) or granulocyte and the mononuclear phagocyte or MØ, are involved with nonspecific mechanisms of host resistance. Both cell types originate from myeloid progenitor cells in the bone marrow and normally pass through several maturation stages before entering the bloodstream. PMN readily traverses blood vessels and provides the primary defense against infectious agents. The inflammation associated with a splinter is typical of a nonspecific PMN and MØ response. Both PMN and MØ exhibit phagocytic activity toward foreign material, especially MØ in the presence of specific opsonic antibodies and C′, and can destroy most microorganisms. MØ is recruited to the site in the event that PMN either cannot contain or is destroyed by the infectious agent, as is the case with certain bacteria (e.g., Listeria). MØ can be activated to a state of enhanced bactericidal or tumoricidal activity by soluble lymphocyte products (e.g., cytokines) produced by T-lymphocytes sensitized to the invading microbe.

The immune responses that characterize adaptive host defense represent a series of complex events that occur following the introduction of foreign antigenic material into the body. There are two major types of specific immune response: *cell-mediated immunity* (*CMI*), which is initiated by specifically sensitized T-cells and is generally associated with delayed-type hypersensitivity (DTH), rejection of

tumors or foreign grafts, and resistance to viral agents, and *humoral immunity* (*HI*), which involves the production of antibodies by PC following sensitization to a specific antigen and is important in resistance to extracellular pathogens.

Organization, Differentiation, and Function of Primary Lymphoid Tissue

The cellular elements of the immune system arise from *pluripotent stem cells*, a unique group of unspecialized cells that have self-renewal capacity. These cells are found in the blood islands of the embryonic yolk sac and in the liver of the fetus during fetal development and later in the bone marrow. The pluripotent stem cell differentiates along several pathways, giving rise to erythrocytes, myeloid series cells (i.e., MØ and PMN), megakaryocytes (platelets), or lymphocytes. Maturation generally occurs within the bone marrow, although lymphoid progenitor cells are disseminated through the blood and lymphatic vessels to the primary lymphoid organs where they undergo further differentiation under the influence of the humoral microenvironment of these organs (Figure 37.1).

The *primary lymphoid organs* include the *thymus* in all vertebrates and the bursa of Fabricius (in birds) or *bursa-equivalent tissue* in other vertebrates; the latter is believed to be bone marrow and gut-associated lymphoid tissue (GALT) in mammals (Table 37.1). Primary lymphoid organs are lymphoepithelial in origin and are derived from ectoendodermal junctional tissue in association with gut epithelium. During the beginning of the second half of embryogenesis (days 12–13 in the mouse), stem cells migrate into the epithelia of the thymus and bursa-equivalent areas, where they differentiate independently of antigenic stimulation into immunocompetent T- and B-cells, respectively (Figure 37.1). The thymus, which is derived embryologically from the third and fourth pharyngeal pouches, is an organization of lymphoid tissue located in the chest, above the heart. Thymus development occurs during the 6th week of embryological development in humans and day 9 of gestation in the mouse. The thymus reaches its maximum size at birth or shortly thereafter in most mammals and then begins a slow involution that is complete between the ages of 5 and 15 years in humans.

Histologically, the thymus consists of multiple lobules, each lobule containing a cortex (outer) and a medulla (inner) (Figure 37.2). Lymphocyte precursors from bone marrow proliferate in the cortex of the lobules and then migrate to the medulla. In the medulla, they further differentiate, under the influence of the thymic epithelium and hormonal factors, into mature T-lymphocytes before emigrating to secondary lymphoid tissues. The neonatal/postnatal thymus has a significant endocrine function supported by nonlymphoid thymic epithelium cells. These cells produce a family of thymic hormones essential for T-lymphocyte maturation and differentiation. In contrast, B-cell differentiation occurs in the bursa of Fabricius in birds, a lymphoepithelial organ that develops from a diverticulum of the posterior wall of the cloacae. The thymus is divided into a medullary region, containing lymphoid follicles and a cortical region (Figure 37.2). The mammalian bursa equivalent is believed to be the fetal liver, neonatal spleen, GALT, and adult bone marrow, depending on age. Mature B-lymphocytes migrate from the bursa-equivalent tissue to populate the B-dependent areas of the secondary lymphoid tissues.

Neonatal removal or chemical destruction of primary lymphoid organs prior to the maturation of lymphocytes into T- or B-cells, or prior to their population of secondary peripheral lymphoid tissue, dramatically depresses the immunological capacity of the host. However, removal of these same organs in adults has little influence on immunological capacity. In addition, neonatal thymectomy in mammals dramatically impairs the development of CMI but does not generally influence the generation of immunoglobulin-producing cells involved in antibody-mediated immunity (unless they strictly require T-lymphocytes help for the induction of antibody production). In contrast to the removal of primary lymphoid organs, removal of secondary lymphoid organs does not inhibit the development of immune competence, although it may suppress the magnitude or alter the tissue location of the responsive cells.

Organization and Function of Secondary Lymphoid Tissue

The organization and function of secondary lymphoid organs is extremely important for immune competence and host defense (Table 37.1). The organized areas of secondary lymphoid tissue are the spleen, lymph nodes, GALT, and bronchus-associated lymphoid tissue (BALT). The anatomical organization of these tissues provides a microenvironment for functional development of lymphoid cells and vital immune responses.

Lymph nodes are discrete, organized secondary lymphoid organs that serve as filtering devices for lymphatic fluid [3]. Lymph nodes are divided structurally into three areas—cortex, paracortex, and medulla (Figure 37.3)—and are served by several afferent lymphatic vessels that collect lymphatic fluid (lymph) from distal tissue sites. Since lymph may contain foreign antigens, the efferent lymphatic vessel, which drains lymph from the node, contains antibodies, cytokines, and lymphocytes produced in response to foreign antigenic stimulation occurring within the node. The cortex, located underneath the subcapsular sinus, receives the afferent lymph and serves as the major site of B-lymphocyte localization. The cortex consists of a narrow rim of small lymphocytes in the absence of antigenic stimulation. Also located in the cortex are aggregations of small lymphocytes, termed lymphoid follicles, which contain dendritic reticulum cells capable of retaining antigens on their plasma membranes. When lymphocytes within the lymphoid follicles are stimulated by antigens, they proliferate, giving rise to dense aggregations of lymphocytes, termed *germinal centers*. These germinal centers serve as sites for differentiation of B-lymphocytes into PC capable of antibody production. Following antigenic stimulation, germinal centers are easily detectable as spherical or ovoid structures containing many large- and medium-sized lymphocytes, predominantly B-lymphocytes. The paracortex, lying between the cortex and the medulla, is composed predominantly of T-cells and

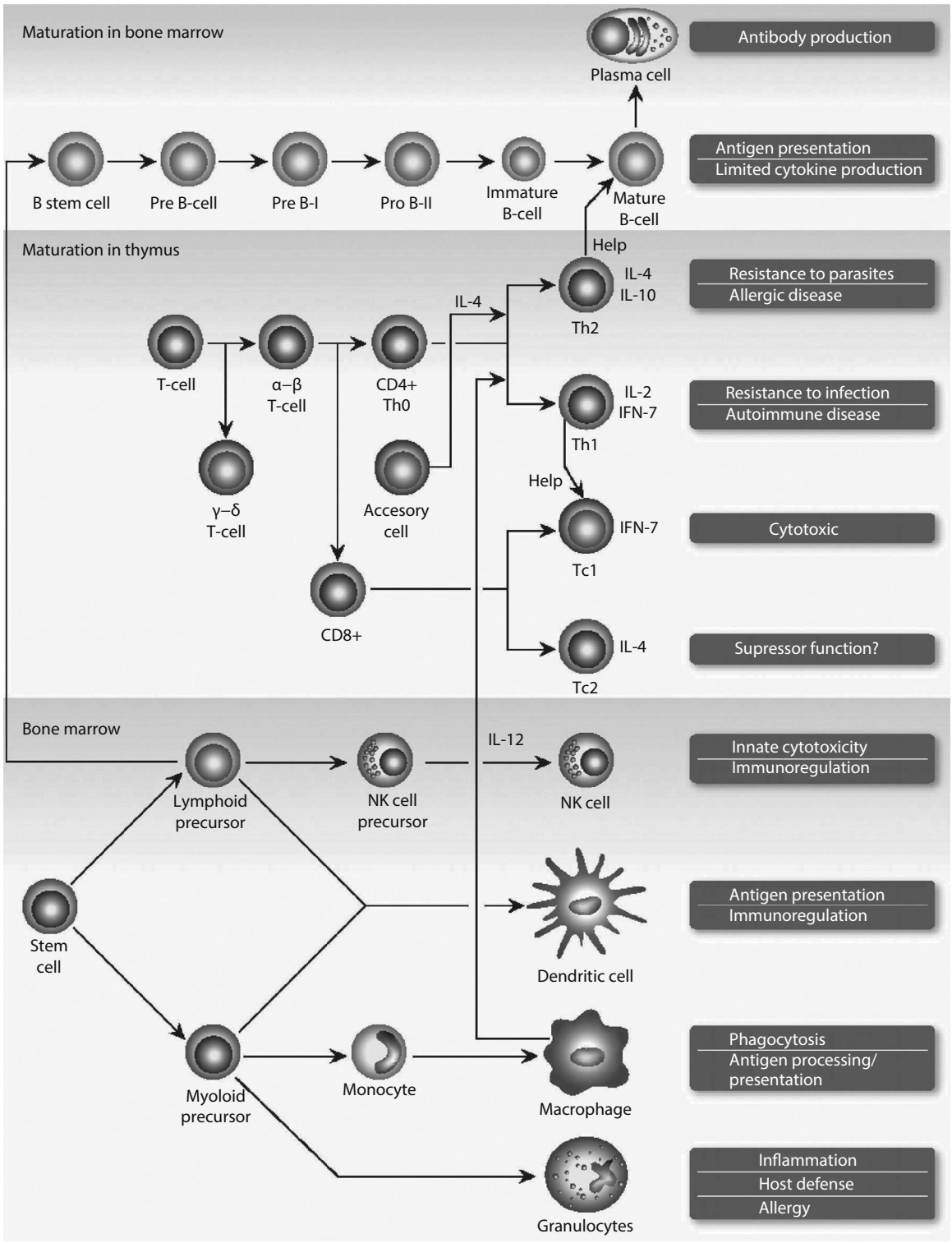

FIGURE 37.1 Origins and interactions of cells of the immune system.

is a major site of MØ and T-cell interaction. Neonatal thymectomy or lymphocyte depletion by cytolytic drugs reduces the production of paracortical lymphocytes, leading to depressed immune capacity. In addition, the paracortex contains a specialized blood vasculature, termed postcapillary venules, that serves as a point of entry for recirculating lymphocytes from the bloodstream. The medulla of the lymph node is composed primarily of networks of cords and sinuses; it serves as an effective filter for removing particulate material from lymphatic fluid. Following antigenic stimulation, the majority of antibody that is produced comes from the PC found within these medullary cords of the nodes.

TABLE 37.1
Organization and Characterization of Primary and Secondary Lymphoid Organs

Primary Lymphoid Organs	Secondary Lymphoid Organs
Thymus	Spleen
Bursa of Fabricius (avians)	Lymph nodes
Fetal liver (mammals)	GALT
Adult bone marrow	BALT
Origin: ectoendodermal junction	Origin: mesoderm
Thymus: days 9–10 in mouse; week 6 in man	
Bursa equivalent: days 10–13, mouse; week 10, man	
Independent of antigenic stimulation	Dependent on antigenic stimulation
Function: generation and maturation of cells	Function: expansion of cells after antigenic stimulation
Stem cells only	Differentiated lymphocytes
Effect of depletion: persistent effect if stem cells targeted	Effect of depletion: likely to regenerate

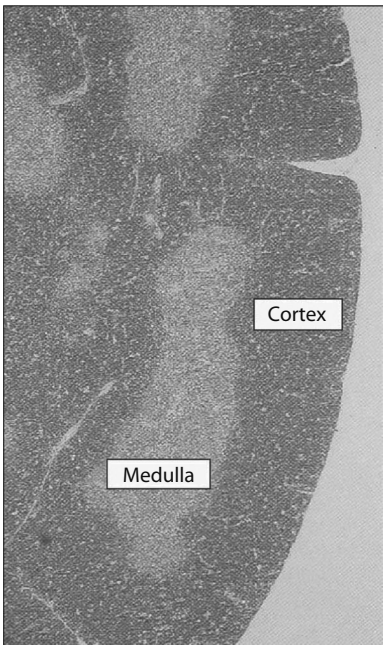

FIGURE 37.2 Histology of the thymus. Photomicrograph of a rat thymus with a densely populated cortex and a less densely populated medulla (H&E-stained section).

The spleen is the major filter of blood-borne antigens and the site of immunological response to these antigens [4]. In addition, the spleen is a site of extramedullary hematopoiesis (nonbone marrow–red blood cell production) and responsible for the removal of damaged blood cells. There are two major histological regions within the spleen: the red pulp and the white pulp containing IgM-staining B-cells and T-cells (Figure 37.4a and b). These areas have been named for their

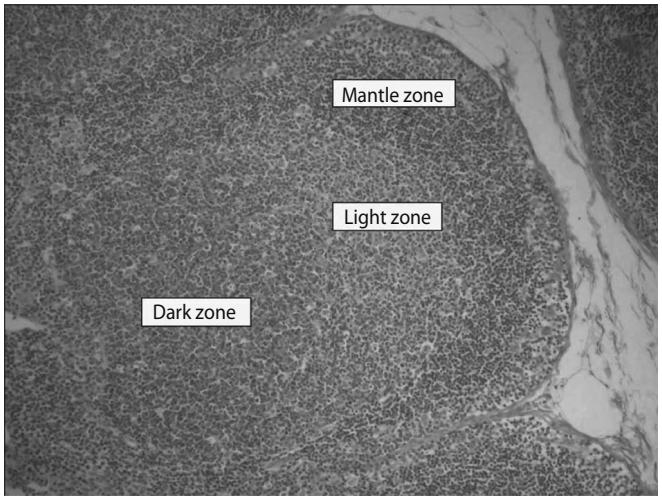

FIGURE 37.3 Histology of the lymph node. Photomicrograph of a rat lymph node showing a secondary lymphoid follicle including a mantle zone and a germinal center with a dark and light zones (H&E-stained section).

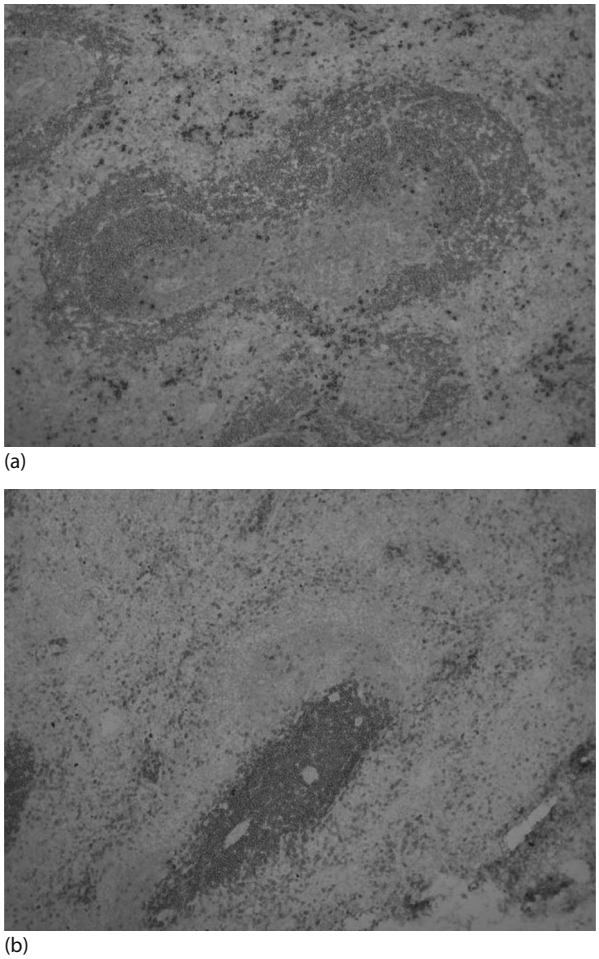

FIGURE 37.4 Histology of the spleen. (a) Photomicrograph of rat spleen showing IgM staining cells (original magnification, 50×). (b) Photomicrograph of rat spleen showing T-lymphocytes stained with immunoconjugated antibody (original magnification, 50×).

colors in a freshly cut spleen. The white pulp consists of numerous white blood cell aggregates and lymphoid follicles. The red pulp contains cords and venous sinuses analogous to the medullary region of lymph nodes. The spleen has no afferent lymphatic vessels; and thus, all antigenic material or cells enter the spleen through the blood vasculature. The marginal sinus in the spleen is structurally and functionally similar to the subcapsular sinus of the lymph node.

IMMUNE FUNCTION AND RESPONSES

Bone Marrow

The bone marrow functions as a primary lymphoid organ and serves as the principal source of uncommitted stem cells, including both myeloid and erythroid precursor cells. The bone marrow architecture is highly organized and complex, consisting of a matrix or cellular stroma derived from local mesenchymal cells, as well as cells of hemopoietic parenchyma that are descendants of circulating stem cells. The bone marrow matrix consists of reticular-dendritic cells, fibroblast-like cells, and immune cells within the bone marrow microenvironment.

Bone marrow stem and stromal cells have been shown to possess a significant capacity for metabolic activation because they contain cytochromes of the P450 and P448 families as well as peroxidases and can generate reactive oxygen species, which could also activate xenobiotics via oxidant-dependent mechanisms [5]. This metabolic activity is thought to contribute to the sensitivity of bone marrow elements to toxicants such as benzene, which is extensively metabolized within the bone marrow. In light of the cell proliferation and differentiation occurring within the marrow, this tissue is also one of the most sensitive tissues to drugs or chemicals affecting cell division. Dose-limiting bone marrow toxicities are a significant problem with antiproliferative drugs including cytotoxic agents, antifolates, AIDS therapeutics, and certain cytokines [6,7].

Mononuclear Phagocytic System

Whether an antigen induces CMI, antibody production, or both depends on the physical and chemical characteristics of the antigen, the mode of presentation of the antigen to lymphocytes, the pattern of antigen distribution within lymphoid tissue, and the molecular configuration of the antigen. In many instances, antigen is initially phagocytized and processed by APC. Antigenic peptides are transported to the cell surface, where they are presented to lymphocytes through cell surface interactions via specific surface proteins (e.g., class II molecule antigens).

Cells of the MØ/monocyte lineage are found in many tissues, including liver (Kupffer cells), lung (alveolar and interstitial MØs), skin (Langerhans cells), and brain (astrocytes and microglia). These cells, because of their proximity to portals of entry, are often the first cells to interact with drugs, chemicals, and physical agents entering the organism via air, food, or blood. The capacity of cells of the mononuclear phagocytic system (formerly known as the reticuloendothelial system) to carry out these functions is associated with their state of activation, which in turn is a function of both endogenous (e.g., interferon gamma [IFN-γ]) and exogenous (e.g., bacterial lipopolysaccharide [LPS]) stimuli. Responsive MØ obtained from the peritoneal cavity is relatively quiescent and requires extracellular signals or *priming*, followed by a second signal induced by triggers such as LPS to be fully activated.

Although the mononuclear phagocyte system is designed to protect the host, once a xenobiotic has gained entry, extensive and/or persistent tissue damage can result mediated, in part, by MØ products. Silicosis and asbestosis are two examples of diseases where MØ mediators are involved in the pathology [8]. In this condition, tissue damage from an environmental or infectious agent results in the influx of phagocytic cells mainly PMNs. In most instances, these cells effectively eliminate the agents by digesting them in internal vacuoles. However, if the foreign material persists (as, e.g., silica crystals or asbestos fibers), a chronic inflammatory process ensues in which PMNs are replaced by monocytes/MØ as the predominant effector cells. These cells release a variety of active molecules (cytokines, nitric oxide, amines, lipid mediators, etc.) that damage tissue, as well as recruiting other inflammatory cells into the local environment. This sometimes leads to the development of a granuloma, a collection of inflammatory cells surrounded by fibrotic tissue [9].

Humoral Immunity

The principal function of B-lymphocytes is production of specific antibody in response to antigenic stimulation. B-cells recognize antigen via a specific receptor, comprising membrane immunoglobulins associated with accessory proteins either directly or in the presence of an APC. Binding of the receptor with its cognate antigen triggers transmembrane signaling leading to activation of the B-cell. The antigen is subsequently internalized, where it is processed and associated with class II major histocompatibility complex (MHC) molecules. Antigen-derived peptides, along with MHC proteins, are then transferred to the cell surface where they are free to interact with helper T-cells.

Within 3–5 days following antigen exposure, this T-/B-cell interaction results in the B-lymphocytes differentiating into blast cells, then into immature PC, and finally into antibody-secreting PC. The establishment of HI is characterized by an early rise in IgM antibody titer in the serum, followed several days later by the appearance of IgG antibodies. During this differentiation process, some of the lymphocytes develop into long-lived or memory cells (sensitized but nonblast cells), so that subsequent antigen encounters result in an enhanced (secondary) response. This secondary response is characterized by a shorter latency for antibody appearance, as well as an increased affinity and synthesis of IgG antibodies. Antibody molecules react with specific antigenic determinants (epitopes) on their target, facilitating its removal (e.g., lysis or enhanced phagocytosis).

TABLE 37.2
Biological Properties of Mammalian Immunoglobulin Classes

Class	Serum Concentration (mg/dL)	Molecular Weight	Placental Transfer	Half-Life (Days)	Biological Function	Abnormalities
IgG	670 ± 33	150,000	+	23	Primarily synthesized during secondary immune response. Readily diffuses into extra vascular tissue. Fixes complement	Increased in liver disease chronic infection. Reduced in B-cell depression
IgM	61 ± 5	890,000	—	5	Produced early in immune response. Isoagglutinins. Fixes complement	Increased in infection. Reduced in B-cell depression
IgA	40 ± 4	170,000	—	6	Major Ig in seromucous secretions	Increased in liver disease. Increased or decreased in sinopulmonary infection
IgD	—	150,000	—	2.8	Lymphocyte receptor	Decreased following thymectomy
IgE	0.02	196,000	—	1.5	Mediator of allergic reactions and atopic diseases	Increased in parasitic and allergic diseases, homocytotropic

Based on chemical structure and biological function, the five classes of antibody molecules in mammals are IgM, IgG, IgA, IgD, and IgE; some of the physical and biological characteristics of each of these classes are listed in Table 37.2. Antibodies operate via several mechanisms to protect the host from infectious agents. Some of these mechanisms include virus neutralization, in which antibodies bind and prevent virus particles from infecting target cells; opsonization, the process by which antibody molecules react with infectious agents and thus enhance their phagocytosis; and antibody-dependent cellular cytotoxicity, the process whereby antibody-coated target cells are killed by Fc receptor–bearing lymphocytes.

Of increasing interest is the concept that naturally occurring IgM antibodies (i.e., antibodies that are secreted in the absence of antigen stimulation) may play an important role in immune surveillance against neoplasia [10,11]. This concept has not as yet been explored in the context of immunotoxicology but may be a contributory factor in decreased resistance to tumors following immunotoxic insult.

Cell-Mediated Immunity

CMI, often referred to as T-CMI, refers broadly to any host resistance mechanism in which cellular elements play a direct role and are part of the acquired arm of immunity. This is in comparison to HI in which there are certainly cellular interactions but in which the final host resistance products are soluble factors such as an antibody. There are a number of host defenses mediated directly by cells including MØ-mediated cytolysis, antibody-dependent cellular cytotoxicity, and natural killer (NK) cell cytotoxicity although cytotoxic T-lymphocytes (CTLs) usually predominate, particularly in the destruction of virus-infected cells.

Functions associated with CMI are commonly considered the province of T-lymphocytes, although immune cells (e.g., B-cells and MØ) as well as nonimmune cells (e.g., fibroblasts and dendritic cells) contribute to the development of CMI. As the primary effector cell in CMI, the T-cell represents one of the most complex and multifunctional immune cells. Antigens that generally elicit CMI include tissue-associated antigens, chemicals, and drugs that covalently bind to autologous proteins and antigenic determinants on persistent intracellular microorganisms. The route of exposure also plays a major role in the type of response generated. For example, sheep erythrocytes elicit antibody production (but not CMI) when injected intravenously in humans but elicit both when injected intracutaneously. The induction of CMI proceeds when small lymphocytes differentiate into large pyroninophilic cells and ultimately divide, giving rise to cells responsible for effector function as well as immunological memory. In contrast to HI, which is more effective against extracellular pathogens, CMI helps protect against intracellular bacteria, viruses, and neoplasia and is responsible for graft rejection.

T-cells can differentiate into populations responsible for either regulatory or effector function. For example, regulatory and inducer T-cell functions are provided by CD3/CD4 positive T-helper cells. T-helper function facilitates antibody responses by B-cells and assists in other T-cell responses. For most antigens, B-cells require assistance from T-cells for differentiation into PC. T-helper cells are integral in the B-cell response by participating in two distinct mechanisms: major histocompatibility locus–restricted B-/T-cell collaborations and cytokine-mediated differentiation. Helper function is a result of interactions between surface molecules on T-helper cells and B-cells, as well as the production and secretion of immunoregulatory cytokines.

Effector functions take the form of cytotoxic activity (CD3/CD8 phenotype), manifested by CTL. These cells are able to specifically lyse target cells via the release of various bioactive molecules. Another effector function is the ability of T-cells to mediate suppressor activity for both

T- and B-cell responses. Suppressor activity is also mediated by cells bearing the CD3/CD8 phenotype, although recent studies suggest that this activity may be the result, at least in part, of differential cytokine production by this population (Figure 37.1). This responsibility for both helper and suppressor activities indicates the crucial role of T-cells in normal immune function.

T-Helper 1/T-Helper 2 Cell Paradigm

An important conceptual breakthrough in immunology was the finding that two major populations of T-helper cells exist that have different, sometimes opposing, functions. Mosmann et al. [12] first established the concept by demonstrating that cloned murine T-cells exhibited differential patterns of cytokine production. One population, designated T-helper-1 (Th1)-type cells, was found to produce interleukin-2 (IL-2), IFN-γ, and lymphotoxin. The second major population (designated Th2 cells) produces IL-4, IL-5, IL-10, and IL-13. Both populations of T-cells produce IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor (TNF). Later, a third population, Th0, was described and was found to exhibit an intermediate pattern of cytokine production. These cells are less well defined but may be an early precursor of Th1 and Th2, or alternatively, they may represent an intermediate stage in development of the other two populations.

Although there were initial doubts that human T-cells followed this paradigm, it is now known that a similar paradigm exists for human T-cells [13]. The major differences appear to be in the profile of cytokine production, cytokine response (e.g., human Th1 and Th2 proliferate in response to IL-4, while only Th2 cells proliferate in the presence of IL-4 in rodents), and cytolytic potential. Despite these differences, the human and rodent systems are similar enough to make experimental rodent models meaningful for understanding the human immune response.

Recent studies suggest that Th1 and Th2 cells may not necessarily represent distinct lineages descending from a common precursor but rather may be seen as points in a continuum. For example, development of each population is influenced by type, location, and concentration of eliciting antigen. More important may be the cytokine milieu (Figure 37.1). For example, the cytokines IL-12 and IFN-γ-inducing factor (from MØ) and IFN-γ (from NK cells) drive the development of Th1 cells, whereas IL-4 (from the ill-defined *T-accessory* cell, mast cells, or other sources) drives the development of Th2 cells [14]. Interestingly, IL-4-driven development of Th2 appears to take precedence over IL-12-induced Th1 production; this may have ramifications in the etiology of some disease states.

The Th1/Th2 paradigm is important for immunotoxicology in that certain immunopathology has been associated with the predominance of one helper cell type over another, particularly in human disease states. For example, Th1 polarization has been associated with organ-specific autoimmune diseases such as multiple sclerosis and Hashimoto's thyroiditis, whereas systemic autoimmune conditions such as rheumatoid arthritis and Sjogren's syndrome lack a clear T-cell polarization [15]. On the other hand, strong Th2-type responses appear to result in many hypersensitivity disorders, including asthma. The extent to which cytokine polarization contributes to these pathologies, as opposed to being a sequel of other mechanisms, remains to be elucidated. It is possible, however, that assignation of Th1/Th2 patterns may eventually become much more important when designing and performing mechanistic immunotoxicology studies [16].

Natural Killer Cells

NK cells are a population of non-B-, non-T-lymphocytes that exhibit cytotoxicity toward a variety of target cells, including tumor cells and virally infected cells. NK cells express a unique panel of cell surface markers (e.g., asialo GM1) and are morphologically distinct, being larger than other lymphocytes. In addition, they contain numerous granules, leading to their designation as large granular lymphocytes (LGLs) [17,18]. Unlike CMI, NK cell–mediated cellular cytotoxicity is MHC-unrestricted and does not require prior exposure to the target.

NK cells have been seen principally as mediators of so-called immune surveillance [19], resulting in the concept of a constant removal of spontaneously arising neoplastic cells [20,21]. In fact, the standard methodology for assessing NK cell function relies upon the in vitro lyses of tumor target cells. However, NK cells are more likely to play a role in resisting the progression and metastatic spread of tumors once they develop, rather than preventing initiation [22].

In contrast to previous models in which NK cells were considered independent of the acquired immune response, recent studies have revealed an important role for these cells in the induction and regulation of acquired immunity [23–25]. NK cells respond to, and produce, key immunoregulatory cytokines and thus play an important role in the normal immune response. In fact, studies of individuals with NK cell deficiency states, most of which are associated with single-gene mutations, have helped identify a role for NK cells in the defense against human infectious disease. A resounding theme of NK cell deficiencies is susceptibility to herpes viruses [26–28].

Related to NK cells are the NKT cells, a population of CD1d-positive lymphocytes that exhibit properties of both NK cells and T-cells [29,30]. These cells appear to have primarily immunoregulatory functions and serve in the coordination of functions between the innate and adaptive immune responses [31]. To date, this cell population has not been a target of immunotoxicology assessment, although it is conceivable that these cells would be as likely a target as NK cells. Moreover, NKT cells have been associated with immunotoxicologically important endpoints including resistance to infection and autoimmunity [32,33].

Other Immunoregulatory Circuits

Cytokines

Cytokines are glycoproteins that are generally produced in response to cellular activation. Most cytokines studied have multiple and overlapping actions, and they frequently function via cascading mechanisms referred to as the cytokine network, interacting with each other both synergistically and antagonistically. Two important features of cytokines are that they usually act at a local level and they are rapidly cleared from the circulation. This combination of features helps ensure that cytokines remain compartmentalized, undoubtedly an important consideration given the potent bioactivity of these molecules [34,35].

Cytokines serve as immune system mediators and regulators. They are produced predominantly by T-helper lymphocytes but are not exclusive to the immune system. In fact, some cytokines are phylogenetically ancient and highly conserved. Furthermore, both IL-1 and TNF are intrinsically involved in apoptosis and cellular proliferation, both fundamental biological processes. Thus, cytokines should be recognized for their role as conveyers of bioinformation, rather than as simple effector molecules involved in a single physiological process such as immunity and host resistance. For convenience, cytokines may be grouped into several classes (Table 37.3). These classifications are necessarily arbitrary due to the overlapping activity of these molecules.

Chemokines

Another related group of molecules is the chemokines. Chemokines are small peptide molecules that, like cytokines, were originally associated with the immune system but which now are recognized as being produced by almost all cells of the body and involved in a multitude of biological functions (Table 37.3). Chemokines play many roles, including modulation of the Th1/Th2 balance associated with autoimmunity and hypersensitivity [36], as mediators of allergic inflammation [37], and modulating the function of leukocytes in disease states such as rheumatoid arthritis and asthma [38].

Immune/Nervous/Endocrine System Axis

It has been recognized for some time that the nervous, immune, and endocrine systems, rather than being separate in function and structure, share many features and appear to cross regulate each other's function [39–43]. The ramification for immunotoxicology of the existence of this nervous–immune–endocrine axis is that xenobiotics can affect the immune response indirectly by affecting other organ systems; conversely, modulation of the immune system may have secondary effects on other organ systems. To date, these interactions have not been extensively studied as they relate to immunotoxicology.

TABLE 37.3
Major Classes of Cytokines and Chemokines

Class	Members	Functions
Interleukins (ILs)	IL-1 (α and β), IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, and IL-9 through IL-30	Primarily immunoregulatory, act on immune system cells (generally lymphocytes) in either stimulatory or inhibitory fashion.
Colony-stimulating factors (CSFs)	Granulocyte (G-CSF) Macrophage (M-CSF) Granulocyte/macrophage (GM-CSF), IL-3, MEG-CSF	Involved in the proliferation of leukocyte progenitors. GM-CSF and IL-3 share certain immunoregulatory functions.
Interferon (IFN)		
• Type I	IFN-α, IFN-β, IFN-δ, IFN-ω	Primarily antiviral activity, some immunoregulatory functions.
• Type II	IFN-γ	Primarily immunoregulatory.
TNF ligand superfamily	TNF-α, TNF-β, TNF-related apoptosis-inducing ligand (TRAIL). CD27 ligand, CD30 ligand, CD40 ligand, CD95 ligand, FAS ligand	Immunoregulatory activities; antitumor effector functions; apoptosis growth regulation.
Hematopoietins	Stem cell factor, stem cell growth factor, erythropoietin, thrombopoietin	Involved in the regulation of bone marrow function and the production of hematopoietic cells.
Miscellaneous	Oncostatin M, leukemia inhibitory factor, transforming growth factor(s)	Various pleiotropic functions.
Chemokines		
C	Lymphotactin	Chemotactic for lymphocytes.
CC	C10, eotaxin, I-309, leukotactin-1, MARC, MCP, MIP-1, MIP-3, MPIF-1, PARC, RANTES, TARC, TECK	Primarily active on monocytes/macrophages.
CXC	IL-8, 6Ckine (Exodus), BLC, CINC-1, CINC-2, CRG, ENA-78, gro, KC, MIG, MIP-2, NAP-2	Primarily active on neutrophils and T-cells.
CX3C	Fractalkine	Modulates calcium flux; involved in cell adhesion.

An updated listing of all known cytokines may be found at http://www.copewithcytokines.de/cope.cgi.

CONCEPTS AND APPROACHES FOR DETECTING IMMUNOTOXICITY IN EXPERIMENTAL ANIMALS

Immunosuppression

Basic Considerations in Study Design

For the most part, adverse effects observed in the immune system are due to the general properties of the chemical (e.g., interaction with specific macromolecules) but are amplified by the complex nature of the immune system. Dean et al. [44] suggested a *tier* approach with the idea that each subsequent tier provides identification of a more defined effect on the immune system. Tiered screening panels have been the basis for several risk assessment guidelines [45], and most regulatory agencies in the United States, European Union (EU), and Japan have established or are developing requirements or guidelines [46–48]. Although the configurations of these testing panels vary by laboratory and species, they generally include measures for (a) altered lymphoid organ weights and histomorphology; (b) quantitative changes in cellularity of lymphoid tissue, peripheral blood leukocytes (PBLs), and bone marrow; (c) impairment of cell function at the effector or regulatory level; and (d) increased susceptibility to experimental challenge with infectious agents or transplantable tumors cells. While it is generally agreed that functional tests provide the greatest level of sensitivity, some guidelines do not suggest functional tests until Tier II. Instead, these guidelines suggest reliance on careful histological and hematological evaluation, particularly inclusion of extended histopathology endpoints [49,50].

There are a number of advantages and limitations to using such test panels in animals or humans. For example, although the sensitivity of these methods to detect immune system changes in animal models is probably high, it is difficult to extrapolate the clinical significance of subtle immune changes on the development of neoplasia or infectious diseases in humans. Furthermore, some of the tests require invasive procedures such as immunization and either require additional animals in standard 28 day toxicology studies or are not feasible or ethical for inclusion in human studies.

A variety of factors must be considered when evaluating the potential of an environmental agent or drug to adversely influence the immune system. Assessment requires validation of the endpoints to be measured (quality control and biological relevance) as well as knowledgeable selection of animal models, exposure parameters, and consideration of general toxicological parameters, including metabolism, distribution, and toxicokinetics. The treatment protocol should take into account the potential route and level of exposure expected in humans, the biophysical properties of the agent (e.g., protein binding, bioavailability, and toxicokinetics of the agent), as well as information on the agent's mechanism of action. Dose selected should attempt to establish a clear dose–response curve as well as a no-observable-effect level (NOEL), up to or near the maximum tolerated dose (MTD). While in some instances it might be beneficial to include a dose level that induces overt toxicity, any immune change observed at such a dose should be interpreted cautiously, as either severe stress or malnutrition can impair immune responsiveness. It is often recommended that the highest dose used be considerably lower than a dose producing severe weight loss. Although laboratories routinely employ three dose levels, dose range–finding studies are recommended prior to a full-scale immunotoxicology evaluation. The selected exposure route should parallel the most probable route of human exposure, which is most frequently oral, respiratory, or dermal. Since these major routes of exposure are also associated with local immunity, attention may need to be directed to assessment of local immune responses.

Selection of the most appropriate animal model for immunotoxicology studies has been a matter of some discussion. Ideally, toxicity testing should be performed in a species that will elicit chemical-related pharmacology and toxicities similar to those anticipated in humans. For most immunosuppressive therapeutics, results from rodent studies have been predictive of clinical observations [51,52] although differences exist (e.g., glucocorticoids are lympholytic in rodents, but not in primates). While for the most part mice and rat results have also compared well, exceptions have been shown to occasionally occur in their immune response to an agent [53].

The quantitative and qualitative response of an experimental animal to an immunotoxic agent can be influenced by their genetic composition (genotype), indicating a need to consider not only species but in some cases also strain. Rao et al. [54] described two approaches to the selection of genotypes for rodent toxicity studies. The first approach is to select a strain where the genotype is representative of the animal species, in the hope that the choice will also exhibit sensitivities similar to humans. This can best be accomplished by using randomly bred rodents. However, due to the variability in immune responses associated with outbred animals, it may be necessary to use a greater number of animals to identify a sensitive population. A second approach attempts to identify genotypes that are uniquely suitable for evaluation of a specific class of chemicals. This requires considerable knowledge of the mechanisms of toxicity for the particular compound. One compromise would be to use F1 hybrids that contain the stability, phenotypic uniformity, and background information of an inbred animal and yet have heterozygosis.

At present, it is impossible to determine how applicable these conclusions will be for immunotoxic compounds with different immune profiles. However, as more comparative analyses become available, the ability to accurately estimate the potential clinical effects from immunological tests in animals should increase.

Introduction and Fundamental Concepts

Based on the preceding discussion of the important role that the immune system plays in protection of the host from infectious organisms and incipient neoplasia, it is logical to expect that disruption of this system following exposure to xenobiotics would have serious consequences. A large body

TABLE 37.4
Examples of Drugs and Chemicals Associated with Immunosuppression

Pharmaceuticals	Cytoreductive agents	Transplantation drugs
	Opiates	Therapeutic immunosuppressants
	Antibiotics	AIDS therapeutics
Industrial chemicals	Organic solvents	Halogenated aromatic hydrocarbons
	Polychlorinated biphenyls	Polycyclic aromatic hydrocarbons
	Glycol ethers	
Environmental agents	Heavy metals	Air pollutants
	Ultraviolet light	Dusts (silica, asbestos)
	Pesticides	
Recreational adjuncts	Ethanol	Tobacco (smoke)
	Cannabinoids	Opiates
	Cocaine	

TABLE 37.5
Assays Commonly Employed to Assess Immunosuppression in Laboratory Animals

	Rodent	Nonhuman Primate
Initial assessment (*Tier I*)	Hematology	Hematology
	Bone marrow histomorphology	—
	Lymphoid organ weight and histomorphology	—
	Primary antibody response	Serum Ig level
	NK cell activity	NK cell activity
	Surface marker analysis	Surface marker analysis
Advanced assessment (*Tier II*)	CTL or DTH	—
	MØ function	MØ function
	Apoptosis	Apoptosis
	Cytokine analysis	Cytokine analysis
	Host resistance assays	—

Notes: —, not routinely performed; Ig, immunoglobulin; NK, natural killer; CTL, cytotoxic T-lymphocyte; DTH, delayed-type hypersensitivity.

of information has developed demonstrating that xenobiotic exposure can produce immune suppression and altered host resistance in experimental animals (Table 37.4) following acute or chronic exposure. Since the number of reports documenting immune dysfunction following human exposure to xenobiotics is limited, animal studies form the basis for human risk assessment [55].

Given the complexity of the immune system (both natural and acquired) and the many potential target cells and molecules, it is impractical to enumerate all of the potential targets of immunosuppressive agents. For this reason, a number of immune function assays have been developed and validated for evaluating immunotoxicity. These techniques and approaches are discussed in the following sections.

Techniques for Assessing Immunosuppression

The basic approach to immunotoxicity testing, as it is currently practiced, is based on the work of Luster et al. [56–58]. This early work validated the concept of the *tier* approach in which test materials are evaluated for effects on the immune system using a biphasic system of descriptive and functional assays. A fairly comprehensive evaluation of immune structure and function is shown in Table 37.5 and is based upon the work of Luster et al. [56–58]. Tier I includes tests that are incorporated into a standard 28-day safety study and include routine hematology, selected organ weights (spleen, thymus), and histology of lymphoid organs. The inclusion of the T-dependent antibody response (TDAR) using either sheep red blood cell (SRBC) or keyhole limpet hemocyanin (KLH) requires the use of additional animals and specialized technical training. Also, sometimes included in an assessment are measures of NK cell activity and/or immunophenotype profile, neither of which requires additional sets of animals.

The following tests are commonly performed using the B6C3F1 mouse or the Fischer 344 or Sprague Dawley rats, although they are readily applicable to other rodent strains. Many of these are now well developed for use in nonhuman primates [59], while fewer of the tests are validated for use with canines [60].

Routine Tests and Extended Immunopathology

A hemogram (complete blood count [CBC] and differential) and examination of lymphoid organ weights, including the spleen, thymus, and lymph node, are useful for assessing the immunotoxic activity of a material [61–63]. Because of the structural division of the spleen and lymph nodes into thymus-dependent and thymus-independent compartments, immunocytochemical staining may indicate preferential effects for T- or B-cells, although this is seldom conducted. Likewise, microscopic examination of the thymus may reveal a compound that affects thymocyte viability, although careful measurement of thymus weight is easier and provides equal, if not more, sensitivity. As indicated previously, extended histopathology provides considerably more information than routine histopathology and does not involve any specialized stains. This involves a semiquantitative assessment to estimate histological changes within different anatomical compartments of the spleen, thymus, and lymph node and considers changes in cell density and anatomical compartment size [49,50]. Five endpoints are examined in the spleen: cellularity of periarteriolar lymphoid sheaths (PALSs), lymphoid follicles, marginal zone, red pulp, and the number of germinal centers. Three endpoints are examined in the thymus: cortex cellularity, medullary cellularity, and the corticomedullary ratio. Four endpoints are evaluated in the lymph node: grade of cellularity in the follicles, paracortical areas, medullary cords, and sinuses.

T-Cell-Dependent Antibody Response

Within a few days following in vivo injection of a foreign antigen, antibody molecules of the IgM class are produced. The antibody-forming cell (AFC) assay (alternatively referred to as the plaque-forming cell [PFC] assay) quantitates the production of a specific antibody through the enumeration of antibody-producing cells in the spleen following a primary antigenic stimulus such as SRBCs [64]. The plaque assay has been, for the most part, replaced by ELISA techniques in which antibody levels are determined directly from serum [65]. In addition, KLH is often used in lieu of SRBCs as the immunizing antigen as it is more amenable to laboratory quality control [66,67]. Although the TDAR is, strictly speaking, a measure of B-cell function rather than T-cell function, it is an excellent functional parameter to examine, as this response requires cognate cell interaction and regulation by MØ, T-cells, and soluble regulatory molecules such as cytokines. The steps involved in this assay are illustrated in Figure 37.5.

IgM Plaque Assay

Materials and Reagents Required:

- Earle's balanced salt solution (EBSS) supplemented with 25 mM HEPES buffer
- SRBC in Alsever's solution
- Guinea pig complement (GPC′)
- Dulbecco's phosphate-buffered saline (DPBS)
- DEAE-dextran, 30 mg/mL in saline, pH 6.9
- Bacto-agar
- Petri dishes and cover slips

Procedure:

1. Four days prior to assay, immunize animals with an intravenous injection of washed SRBC in sterile saline. Recommended inocula are approximately 1×10^8 SRBC for mice and approximately 2×10^8 SRBC for rats.

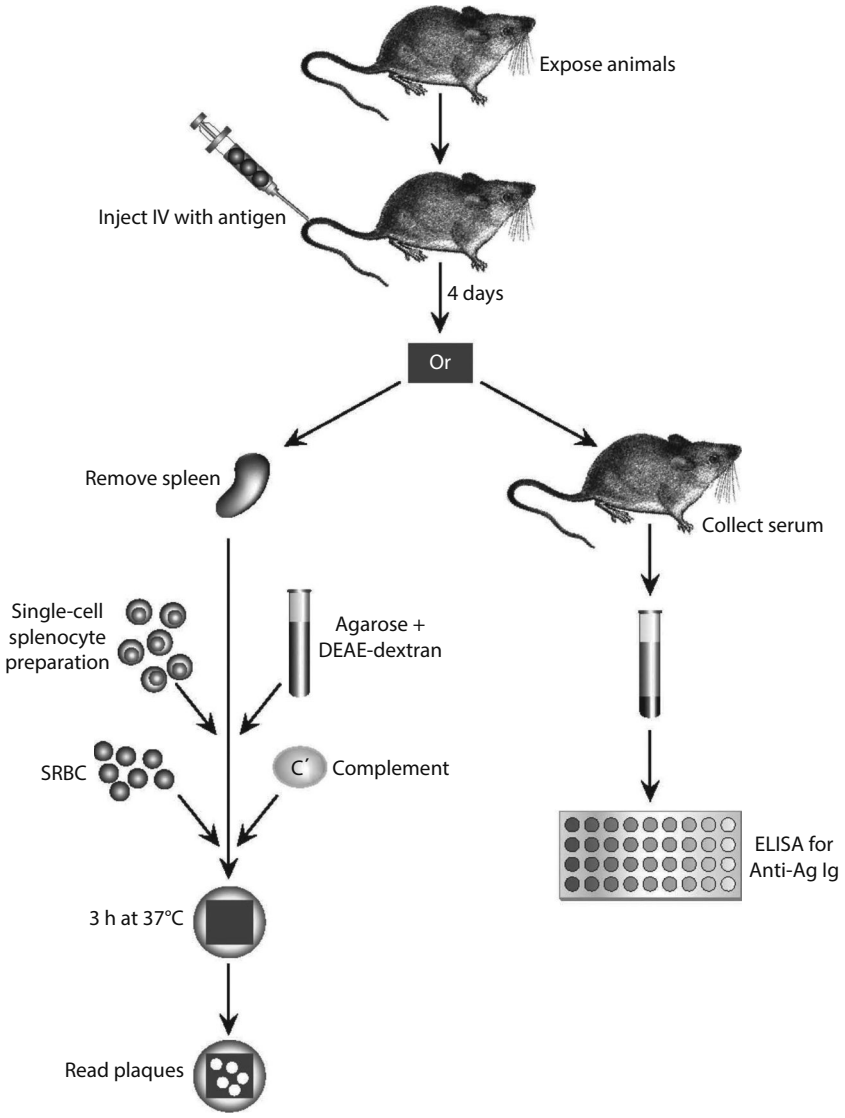

FIGURE 37.5 TDAR assay.

2. Euthanize the animals, remove the spleens, and prepare a single-cell splenocyte suspension in EBSS. Prepare two dilutions of the cell suspension in EBSS.
3. Wash SRBC three times by centrifugation. After the final wash, retain approximately 100 µL of SRBC, and then adjust the remaining cells to a final density of 10% in EBSS. Add GPC' to the reserved SRBC, mix well, and hold on ice until needed.
4. Prepare a solution containing 0.5% agar in DPBS, add DEAE-dextran (1.6 mL stock solution per 100 mL agar), and mix. Dispense the agar in 0.35 mL aliquots into polypropylene culture tubes, and maintain these tubes at 45°C.
5. For the assay, each tube contains 0.35 mL agar solution, 100 µL cell dilution(s), and 25 µL GPC'. Add SRBC first and then the cell suspension, and immediately remove the tube from the water bath. Add the GPC' and mix the contents of the tube. Dispense the contents into a Petri dish, and then drop the cover slip so that an even layer of fluid forms underneath.
6. Incubate the plates at 37°C for approximately 3 h, and enumerate the plaques. While the plates are incubating, determine the cell number and viability of the original splenocyte suspensions.
7. Calculate the results as follows:
   a. Plaques counted under each cover slip × 10 × dilution factor = PFC/mL of the original cell suspension (since 0.1 mL of the cell dilution is counted)
   b. PFC/mL × volume of original cell suspension = PFC/spleen
   c. PFC/mL/number of viable cells/mL = PFC/10^6 viable splenocytes

Anti-SRBC IgM ELISA
Materials and Reagents Required:

- SRBC in Alsever's solution
- Horseradish peroxidase (HRP)–conjugated, affinity-purified goat antimouse/antirat IgM antibody
- Peroxidase substrate (2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid [ABTS])
- ABTS buffer (phosphate–urea–hydrogen peroxide)
- Phosphate-buffered saline (PBS)
- 96-Well microplates
- General reagents and supplies for ELISA

Procedure:

1. Immunize mice or rats with SRBC as for the plaque assay. Five days (mice) or 6 days (rats) later, obtain serum from both immunized and naive animals. Pool each as appropriate to use as standards or controls and freeze at –20°C.
2. Treat mice or rats with test material and vehicle (and a positive control, if necessary). On day 5 or 6 posttreatment (respectively), obtain serum from animals. *Note*: If serum is collected via the retro orbital sinus, additional samples may be collected later for time-course studies.
3. Prepare SRBC membrane antigens by lyses and solubilization [64]. This antigen serves as the capture reagent in the ELISA.
4. Obtain anti-SRBC IgM monoclonal antibodies to use as standards. *Note*: Anti-SRBC must be of the appropriate species depending on the test animal (i.e., mouse or rat).
5. Dilute membrane antigen to 1.0 µg/mL in PBS and coat the wells of the microplates at approximately 4°C using 125 µL/well of the antigen preparation.
6. On the day of assay, wash the plates three times with 0.01% Tween-20 in water. Block any unbound sites on the plates by incubating the plates with 200 µL/well of PBS/0.05% Tween-20, 3% bovine serum albumin, or 3% powdered milk.
7. Prepare serial twofold dilutions of test sera and antibody standards. Add to the plates and incubate for at least 1 h at room temperature.
8. Wash the plates three times, then add HRP-conjugated secondary antibody. Incubate for at least 1 h at room temperature, and then wash the plates three times.
9. Add peroxidase substrate (ABTS) and incubate the plates at room temperature for 45 min. Stop the reaction by adding 3% oxalic acid to all wells.
10. Read the plates at 405 nm and calculate the results based on curves prepared using the antibody standards.

Positive Control:
Cyclophosphamide is routinely used as a positive immunosuppression control for the AFC assay (either plaque or ELISA format). For mice, cyclophosphamide is administered intraperitoneally at 80 mg/kg once approximately 24 h prior to euthanasia. For rats, it is given intraperitoneally at 20–25 mg/kg daily for 4–5 days prior to euthanasia.

Notes:

1. The AFC response varies depending on the day of analysis following immunization. Each species and strain should also be evaluated for the optimum response, although for intravenous injection, the optimum assay period is usually 4 days following immunization.
2. The dose and route of antigen exposure alters the peak AFC response. Intravenous injections shift the optimum response to an earlier time, whereas an intraperitoneal injection delays the peak response.
3. Each new test lot of complement should be tested and titrated prior to use.
4. The day of antibody induction relative to the last dose of chemical exposure should be considered when designing a study.
5. SRBC membrane antigens are generally prepared individually by a laboratory. Monoclonal and

polyclonal antibodies specific for SRBC are commercially available from a variety of sources.
6. The direct comparability of results between the plaque assay and the ELISA is the source of some discussion. The AFC assay measures antibody production in one organ (spleen) only, whereas the ELISA is a measure of systemic antibody production that may have a different time course.
7. The current trend in TDAR assessment favors the use of an ELISA against KLH, a widely used T-dependent antigen. Methodology for standardizing this technique and applying it to immunotoxicology assessment has been published [66–69].

Anti-KLH IgM ELISA (for Rats)
Reagents:

- KLH purified protein
- Positive controls: Mouse and rat anti-KLH and anti-KLH IgG (commercially available)
- HRP-conjugated, affinity-purified goat antimouse/antirat IgM antibody
- Peroxidase substrate (ABTS)
- ABTS buffer (phosphate–urea–hydrogen peroxide)
- PBS
- 96-Well microplates
- General reagents and supplies for ELISA

Materials and Reagents Required:

- SRBC in Alsever's solution
- HRP-conjugated, affinity-purified goat antimouse/antirat IgM antibody
- Peroxidase substrate (ABTS)
- ABTS buffer (phosphate–urea–hydrogen peroxide)
- PBS
- 96-Well microplates
- General reagents and supplies for ELISA

Procedure:

1. Immunize rats with 1000 mg/animal KLH in 0.4 mL PBS by the IP route following test agent treatment.
2. On day 5 following KLH administration (IgM) and day 17 (IgG), obtain blood samples and collect serum.
3. Store in aliquots and freeze at –70°C until testing.
4. Prepare QC and test samples by diluting 1:200 (IgM) or 1:50 (IgG) in blocking buffer.
5. The day before the test, coat microtiter plates with 2.5 mg/mL of KLH, 100 mL/well.
6. Seal the plates and incubate overnight at 4°C.
7. On the day of assay, wash the plates three times with 0.01% Tween-20 in water. Block any unbound sites on the plates by incubating the plates with 200 µL/well of PBS/0.05% Tween-20, 3% bovine serum albumin, or 3% powdered milk.
8. Prepare serial twofold dilutions of test sera and antibody standards. Add to the plates and incubate for at least 1 h at room temperature.
9. Wash the plates three times, and then add HRP-conjugated secondary antibody. Incubate for at least 1 h at room temperature, and then wash the plates three times.
10. Add peroxidase substrate (ABTS) and incubate the plates at room temperature for 45 min. Stop the reaction by adding 3% oxalic acid to all wells.
11. Read the plates at 490 nm and calculate the results based on curves prepared using the antibody standards.

Notes:

1. The AFC response varies depending on the day of analysis following immunization. Each species and strain should also be evaluated for the optimum response, although for intravenous injection, the optimum assay period is usually 4 days following immunization.
2. The dose and route of antigen exposure alters the peak AFC response. Intravenous injections shift the optimum response to an earlier time, whereas an intraperitoneal injection delays the peak response.
3. Each new test lot of complement should be tested and titrated prior to use.
4. The day of antibody induction relative to the last dose of chemical exposure should be considered when designing a study.
5. SRBC membrane antigens are generally prepared individually by a laboratory. Monoclonal and polyclonal antibodies specific for SRBC are commercially available from a variety of sources.
6. The direct comparability of results between the plaque assay and the ELISA is the source of some discussion. The AFC assay measures antibody production in one organ (spleen) only, whereas the ELISA is a measure of systemic antibody production that may have a different time course.
7. The current trend in TDAR assessment favors the use of an ELISA against KLH, a widely used T-dependent antigen. Methodology for standardizing this technique and applying it to immunotoxicology assessment has been published [65,67].

Natural Killer Cell Assay

NK cell activity is measured in vitro by culturing single-cell suspensions of lymphoid cells with a tumor cell line known to be sensitive to NK-mediated cytotoxicity. The target cells are radiolabeled prior to the assay; thus, any cells that have been lysed will release their radioactivity into the culture medium, where it can subsequently be quantitated. The procedure described in the following is modified from the microculture method described by Reynolds and Herberman [70] and is

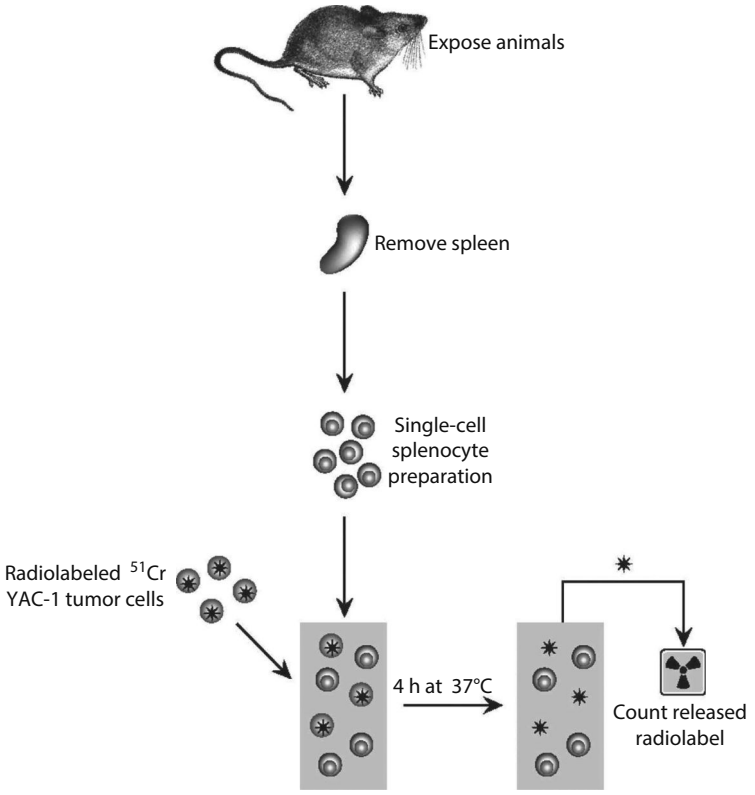

FIGURE 37.6 The NK cell assay.

the standard approach for immunotoxicity assessment. The procedure for this assay is illustrated in Figure 37.6.

Materials and Reagents Required:

- RPMI-1640 culture medium supplemented with 25 mM HEPES buffer, 10% FBS, 2 mM L-glutamine, and 50 μg/mL gentamicin
- Fetal bovine serum (FBS)
- DPBS
- Wash solution (DPBS/1% FBS)
- YAC-1 cell line (for rodent NK evaluation, ATCC #TIB 160) or K562 cell line (for primate NK evaluation, ATCC #243) maintained in log-phase growth in the culture medium described previously
- 96-Well round-bottom microculture plates
- 0.1% solution of Triton X-100 in distilled H_2O
- ^{51}Cr as sodium chromate in sterile saline; specific activity of 200–500 mCi/mg
- Supernatant collection system

Procedure:

1. Prepare a single-cell suspension of the effector spleen cells, and adjust to a density of 5×10^6 viable cells/mL in culture medium.
2. Prepare two serial 1:3 dilutions of the cell suspension in culture medium. Dispense 100 μL of each dilution in quadruplicate wells of 96-well, round-bottom microculture plates.
3. Centrifuge a log-phase culture of target cells and suspend the cell pellet in 0.5 mL FBS. Add 200 μL ^{51}Cr to the cells, mix well, and incubate at 37°C for 1 h. Wash the cells three times.
4. Suspend the target cells in culture medium, determine cell number and viability, and adjust the cells to a final density of 5×10^4 viable cells/mL in culture medium. Add the target cells to all wells in a volume of 100 μL/well. Include a row containing 100 μL target cell suspension and 100 μL culture medium/well (spontaneous release) and one row consisting of 100 μL target cell suspension and 100 μL 0.1% Triton X-100/well (total release).
5. Incubate the plates at 37°C, 5% CO_2 for 4 h. Harvest all wells with a supernatant collection system, and determine radiolabel release in a gamma counter.
6. Harvest supernatant fractions either manually or by using a semiautomatic harvesting system. Quantitate radiolabel released into the supernatant fractions in a gamma counter, and determine percent cytolysis using the following formula:

$$\text{Percent cytolysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100$$

Positive Controls:
Immunosuppression control: Unless the laboratory has extensive experience, a positive suppression control of the

NK response should be included. Approximately 24–78 h prior to euthanasia, a separate group of animals is injected intravenously with an optimum concentration of anti-asialo GM1 antibody. The exact amount to be given will vary from lot to lot and between suppliers. Treatment with an optimum dose of anti-asialo GM1 will result in an essentially complete abrogation of the NK response in rodents [71].

Immunostimulation control: In some cases, it may be useful to include a positive control for NK cell augmentation. Although cytokines (IL-2 and IFN-γ) can enhance this response both in vivo and in vitro, an equally efficient, and more economical/reproducible, option is the use of interferon inducers such as polyinosinic/polycytidylic acid (poly I/C) [23]. Poly I/C is administered intraperitoneally at a concentration of 100 μg/mouse or 500–1000 μg/rat approximately 24 h prior to assay.

Notes:

1. NK activity is highest in young mice, declining after 12 weeks of age. Basal NK activity may be highly variable or undetectable in mice over 20 weeks old.
2. The target cells must be in log-phase growth to achieve adequate labeling with ^{51}Cr. In addition, the target cell lines should be assessed for *Mycoplasma* contamination at periodic intervals.
3. The assessment of NK cell activity has been utilized most extensively in rodents and primates. In instances in which evaluation of canine NK cell function would be useful, modified techniques have been published [72].
4. For laboratories unable or unwilling to use radioisotopes, flow cytometry serves as a useful alternative [73]. A full comparison has not been made between these alternative methodologies and the standard chromium release assay, although a study by Motzer et al. [74] found little advantage in flow cytometry in comparison to the standard radiolabel assays.

Phenotypic Analysis of Cell Surface Markers by Flow Cytometry

There are both pros and cons for including immunophenotypic analysis as part of an immunotoxicology screen [74]. It offers a rapid, sensitive, and quantitative measure of a heterogeneous cell population in blood or cell suspensions prepared from lymphoid organs. The major concern is that the test is not well validated in rodents, particularly with respect to sensitivity or how minor changes relate to disease. For mechanistic studies, however, they are of unquestionable relevance especially when considering the vast array of well-defined surface markers now available for rodent and human immune cells as well as nonhuman primates (75). The technique involves treating cells with monoclonal antibodies covalently bound to different fluorochromes. These antibodies recognize surface antigens, referred to as *cluster of differentiation* (CD), unique to different cell types. The availability of fluorochromes, which emit light at different wavelengths following excitation, combined with flow cytometers that are capable of performing multiple color analysis, provides a rapid and effective method of analyzing cell types. The most commonly examined CDs in the mouse are those that recognize pan T-cells (CD90 and T-cell receptor [TCR] complex), T-helper cells (CD4), T-suppressor cells (CD8), and pan B-cells (CD45R/B220 or CD19).

Materials and Reagents Required:

1. Prepare single-cell suspensions from the spleen (Ficoll-separated and whole blood have both been used occasionally). For washing and staining, use DPBS (0.01 M).
2. Centrifuge conjugated reagents at $15 \times 10^3 \times g$ to remove aggregates.
3. Pipette desired concentration of antibody or control sera in 50 μL volumes to a small test tube or 96-well microtiter plates. For two-color analysis, both conjugated antibodies can be added together.
4. Add 10^6 viable cells in a volume of 50 μL to the test tube or well containing the antibody.
5. Incubate 30 min on ice in the presence of 0.1% sodium azide.
6. Wash with 2.0 mL if in tubes or two 100 μL washes if in wells.
7. Suspend to a volume of $1–2 \times 10^6$ cells/mL in cold PBS containing 0.1% sodium azide and perform analysis.
8. Cell fluorescence and integrity can be preserved for up to 5 days by rapidly suspending the cell pellet in 50 μL cold PBS containing 1% paraformaldehyde.

Mechanistic Immunotoxicology Assays

From its beginnings, the discipline of immunotoxicology has constantly evolved and incorporated new techniques and paradigms to understand the nature of immunomodulation. The assays described previously in this section allow one to make a first-pass evaluation of drugs and chemical agents for generalized immunotoxicity. That is, the assays will indicate that the immune system had been perturbed, although the cellular and molecular mechanisms involved will not necessarily be obvious. These assays are valuable for quick and relatively accurate identification of toxic agents. On the other hand, the tools and concepts of immunotoxicology are increasingly being utilized as research tools to understand the function of the immune system. For example, it may be useful to know not only whether or not an agent modulates the immune response but why. This is especially important in the discovery and development of pharmaceutical agents, where therapeutic manipulation of the immune system may be a desirable goal. In response to these novel applications of immunotoxicology, assays are needed that will allow us to determine the precise mechanism of immunomodulation.

The methodology for Tier II-type assays such as T-cell-mediated immune function (CTL/DTH), MØ function, IgG antibody cell forming response, and host resistance models has been reviewed in detail elsewhere and will not be

reiterated here [76–79]. These assays are still valuable tools for understanding the mechanistic basis of immunotoxicity. In addition to these assays, several other methodologies are now being included in the immunotoxicology armamentarium.

Assessment of apoptosis: Apoptosis (programmed cell death) is increasingly recognized as a fundamental process in both health and disease states, including the response to toxic insult [80]. Apoptosis plays a vital role in the immune response, regulating the number and action of immune cells such as lymphocytes [81,82]. Given the important role apoptosis plays in normal regulation of the immune system, as well as its implication in immune-related disease, it is a logical and potentially valuable endpoint for mechanistic immunotoxicology evaluation [83]. Apoptosis has been found to play an important role in the immunotoxicity of a number of compounds including organotins [84], polychlorinated biphenyls [85], methylmercury [86], and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [87].

Numerous techniques are available for assessing apoptosis, including analysis of DNA degradation, flow cytometry, morphological analysis, 3′-OH end labeling, and endonuclease analysis [88]. More recently, a number of ELISAs have become available for assessing apoptosis; these ELISAs are based on the detection of Bcl-2 or histone-associated DNA fragments. The ELISA format offers a number of benefits over the other techniques including rapidity, simplicity, and cost-effectiveness.

Cytokine analysis: As described previously, cytokines represent an important mechanism not only for regulating the function of the immune system but also for linking the immune system with other organ systems. Early studies employing cytokine analysis in immunotoxicology studies were more descriptive [89,90]. However, as the intricacies of the cytokine/chemokine network become better understood, these assays are allowing us to assess the mechanisms responsible for a variety of immunomodulatory effects. As an example, a variety of nonbiological agents have been described that either specifically or nonspecifically alter cytokine production. These agents act via myriad mechanisms including direct toxicity to cytokine-producing cells (cyclophosphamide), inhibition of cytokine production (cyclosporin, FK506, pentoxifylline), inhibition of cytokine release (pentamidine), induction of immunosuppressive factors (leflunomide), alterations in cellular homeostasis (tenidap), alterations in cellular activational or transcriptional mechanisms (thalidomide), alteration of cell cycle progression (Rapamune), and miscellaneous or undefined mechanisms (glucocorticoids, phosphodiesterase isozyme inhibitors, metalloproteinase inhibitors, and p38 kinase inhibitors) [91].

There are currently four major types of cytokine assays used: bioassays, immunoassays, mRNA gene expression, and flow cytometry. Sometimes used is what may be termed the *hybrid assay*, employing elements of two or more of the main assay categories, and molecular biology assays to examine cytokines and cytokine receptors [92]. Each of these assay types exhibits advantages and disadvantages, and no one type of assay is best suited for all applications. The type of assay chosen is subjective and will depend on the capabilities of the laboratory, as well as the type of information to be gained.

One recently developed and not yet validated, a cytokine profiling test based upon procedures initially developed by RBM Myriad (Austin, TX) and Meso Scale Discovery (Gaithersburg, MD) may be useful as an immunotoxicology screening test. In this assay, spleen cells from treated animals are collected and stimulated in vitro with a cocktail that stimulates the release of Th1, Th2, and monocyte cytokines. The levels of released cytokines are then measured in relation to baseline (no stimulation). Since the stimulation is performed ex vivo, there is no need for an additional group of animals. Specific changes in profiles likely coincide with various aberrant immune responses. Cytokines play key roles in the initiation, amplification, and specificity of immune responses as well as inflammation. Aberrant production of cytokines can lead to modulation of the immune response such that the host may be more susceptible to infectious agents or development of allergic or autoimmune response to agents.

Allergy and Autoimmune Reactions to Xenobiotics

Introduction and Fundamental Concepts

Immune reactions to xenobiotics (e.g., industrial and naturally occurring chemicals, metals, and drugs) can give rise to allergy and autoimmunity, and these reactions are frequent and can encompass a broad spectrum of diseases and organs. To decrease the health risks associated with exposure to these agents, it is important to understand the pathogenic mechanisms involved and to identify human subjects at risk of developing these reactions prior to serious clinical manifestations.

Allergic or hypersensitivity reactions, which include allergic asthma, food and environmental allergies, and allergic contact dermatitis, are one of the most common and costly health problems in the United States afflicting a large part of the population. Individuals with potential occupational exposure (e.g., agricultural or manufacturing workers) are at a higher risk than the general public for the development of respiratory and cutaneous contact hypersensitivity to chemicals. The indirect costs, such as wages lost because of illness, are estimated to be in excess of $1 billion annually for asthma alone, with more than 35 million workdays lost to sickness each year [93]. Food allergy affects 5%–7.5% of all children and 1%–2% of adults. Industrial processes utilize many materials capable of inducing occupational immunological lung disease or contact hypersensitivity in workers and thus must be rigorously controlled to ensure worker safety. Studies in the metal-refining industry, for example, suggested that many workers regularly exposed to the complex salts of platinum develop disorders of the respiratory tract. A study of workers exposed to toluene diisocyanate (TDI), a substance used in the manufacture of polyurethane, revealed that 5% of those surveyed developed occupational asthma to TDI.

Drug allergy is also a significant problem and among the most common causes for new pharmaceuticals being

withdrawn from the market after they are released. This adverse immune reactivity is not well predicted from the current battery of preclinical safety assessment methods [52]. A distinction should be made between immunologically mediated adverse drug reactions that represent true drug allergy reactions and those that are autoimmune in nature. In drug allergy, the adverse reaction occurs as a consequence of an immune response directed against the drug. Examples of drug allergy include immediate anaphylactic reactions mediated by IgE antibody or cell-mediated reactions. Drugs are unique in that they can provoke allergic and autoimmune reactions against blood cells including erythrocytes and platelets, as well as a variety of other antigens including the haptenated drug. Usually the reaction occurs to the drug or drug metabolites, in which case it is necessary for both the drug and the antibody to be present to produce the allergic or autoimmune reaction. In addition to producing allergic manifestations or pathology under certain conditions, drug-specific antibodies can also alter the pharmacokinetics and clearance of the drug in plasma. Thus, drug-induced allergy reactions can come in many forms producing either allergic or autoimmune phenomena.

Allergy (acute hypersensitivity) is a pathological state resulting from prior sensitization to a specific molecule or structurally related compound. Because of the clinical and pathological similarities between xenobiotic-induced allergy, autoimmunity, and GVH reactions, there is considerable evidence that these reactions are initiated and maintained by sensitized T-cells [94]. The major difficulty in trying to study these immune reactions is the fact that the ultimate neoantigen formed by the chemical or drug is often unknown. Based on our understanding, it is assumed that allergic reactions to most xenobiotics involve the formation of protein adducts (i.e., hapten–carrier conjugates). Metal ions react by oxidizing proteins or forming stable protein–metal chelates through multiple binding points with several amino acid side chains in the protein. A second mechanism for xenobiotics not reactive enough to bind covalently to proteins is through the direct antigen recognition by γ–δ and α–β T-cells. This hypothesis is supported by evidence of human gamma–delta T-cells specific for the drug lidocaine [95] and human CD8+ alpha–beta T-cells that recognize pollen-derived carbohydrate antigen [96].

Thus, there appear to be multiple mechanisms by which xenobiotics can become sensitizers. Highly reactive organic compounds most often act as electrophiles and react with protein nucleophilic groups such as thiol, amino, and hydroxyl groups to form hapten–protein conjugates (reviewed by Griem et al. [97]). Examples of reactive, haptenic chemicals that frequently lead to sensitization after dermal or inhalation exposure are TDI, trimellitic anhydride, phthalic anhydride, benzoquinone, formaldehyde, hexyl cinnamic aldehyde, ethylene oxide, dinitrochlorobenzene (DNCB), picryl chloride, penicillins, and D-penicillamine. In contrast, most xenobiotics are unable to bind to proteins until they are converted to reactive metabolites and are thus considered prohaptens.

The liver is the main organ for metabolic conversion of chemicals and drugs, and in spite of this, immune reactions to liver or its constituents are relatively rare. However, such reactions, although rare, have been observed as seen with autoimmune hepatitis that results from chronic treatment with the diuretic tienilic acid and are believed to result from the production of autoantibodies against the cytochrome P450 isozyme 2C9, the enzyme that converts this prohapten [98]. Two other examples of drug-induced autoimmune hepatitis involving a similar mechanism where autoantibodies are raised against the enzyme converting the prohapten to a reactive metabolite are produced by the anesthetic halothane (anti-CYPE1 [99]) and dihydralazine (anti-CYP1A2 [100]).

Extrahepatic metabolism appears to play a more critical role in hypersensitivity reactions in prohapten conversion, and the skin is a good example of an organ with metabolic conversion potential via dendritic Langerhans cells (isoenzyme CYP1A) or inflammatory leukocytes (myeloperoxidase [MPO], prostaglandin H synthase, and CYPs). Examples of prohaptens converted in the skin include the polyaromatic hydrocarbon dimetylbenz-[*a*]-anthracene that is converted to a potent haptenic sensitizer [101] and urushiol, a mixture of allergenic 2-alkyl and 3-alkenyl catechols from poison ivy and poison oak that are oxidized to reactive quinones that elicit specific T-cell responses [102].

Exposure to any of a number of industrial chemicals and drugs has been associated with the development of allergic or hypersensitivity reactions (Table 37.6). In addition, in some cases, the reactive metabolite of the xenobiotics acts as a hapten, and producing the adverse immune response has been identified in rodents, man, or both (Table 37.7).

What is not well understood is why only a few of the hapten–protein conjugates formed in the body induce clinical

TABLE 37.6
Materials Associated with Contact, Food, or Respiratory Allergy

Pharmaceuticals	Phenylglycine acid chloride	Ampicillin
	Piperazine	Spiramycin
	Amprolium hydrochloride	Antibiotic dust
	Antihistamines	Quinidine
	Anesthetics	Plasma substitutes
Foodstuffs	Castor bean	Pancreatic extracts
	Green coffee bean	Grain and flour
	Papain	Molds
	Tree nuts	Shellfish
	Peanuts	
Industrial chemicals	Ethylenediamine	Phthalic anhydride
	Diisocyanates (TMI, HDI, MDI)	Trimellitic anhydride
	Metallic salts	
Miscellaneous	Wood dusts	Animal products
	Latex proteins	Fragrance components
	Flour	Detergent enzymes (subtilisin)

TABLE 37.7
Examples of Allergic Reactions to Xenobiotics That Involve Reactive Metabolites

Parent Compound	Allergic Reaction Observed	Candidate Metabolite
Dihydralazine	Drug-induced lupus, autoimmune hepatitis	Hydralazine radical
Gold thiomalate	Dermatitis, glomerulonephritis	Gold (III)
Halothane	Autoimmune hepatitis	Trifluoroacetyl chloride
Practolol epoxide	Oculomucocutaneous syndrome	Practolol
Procainamide	Drug-induced lupus	*N*-Hydroxyprocainamide
Propylthiouracil	Vasculitis, drug-induced lupus	Propyluracilsulfonic acid
p-Phenylenediamine	Contact dermatitis	Bandrowski's base
Tienilic acid	Autoimmune hepatitis	Thiophene sulfoxide
Urushiol	Contact dermatitis	3-Pentadecyl-*o*-quinone

allergy or autoimmunity. Administrative route, dose, and genetic background clearly all play an important role in this process. Cutaneous sensitization of mice to the hapten dinitrofluorobenzene (DNFB) does not occur if the animals are orally pretreated with DNFB [103]. This is believed due to the induction of both hapten-specific CD8+ T-cells and CD4+ suppressor T-cells. In the case of dose, subsensitizing doses of oxazolone induce tolerance, while higher doses sensitize. Genetic background further complicates our ability to predict since gold thiomalate induces a Th2-like response in brown Norway rats, but not in Lewis rats. This induction of autoimmunity in brown Norway rats is explained by a polymorphism in a putative enhancer element in the second intron of the IL-4 gene [104].

Autoimmunity is a multifactorial pathological process comprised of at least two processes. Initially, an immune response is initiated to normal components of the host, and second, a pathological condition may ensue in which the response causes structural or functional damage (e.g., pathology). An autoimmune response does not necessarily reflect disease, although it is a prerequisite for disease to occur. The autoimmune response induced can be cellular in nature, mediated by CD4 and/or CD8 T-cells, or more often, it arises from antibody, mediated by specific B-cells. The most common autoimmune diseases are rheumatoid arthritis and those associated with the thyroid, such as Graves' disease. In total, they represent a significant and chronic morbidity problem with recent estimates indicating that 1 in 31 individuals in the United States are affected, with women at 2.7 times greater risk than men [105].

What is the underlying problem in autoimmunity and autoimmune disease? It is well understood that the immune system is able to recognize and produce a response against foreign material but is tolerant toward the body's own constituents. Unfortunately, immunological tolerance to self is not absolute and autoantibodies can be produced experimentally and are seen in a variety of human autoimmunity diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus (SLE), and myasthenia gravis). The mechanism(s) responsible for conversion from an autoimmune response to autoimmune disease is not clear. It is believed that the failure of any one of several immune processes can lead to the development of autoimmune disease. However, the key process involves the loss of self-tolerance, such as the missed deletion or activation of autoreactive lymphocyte precursors. This process may be exacerbated by altered immunoregulation, such as overexpression of the immunoregulatory cytokine IL-4 or underexpression of IFN-γ. Autoimmunity may also occur in the absence of an aberration in the immune system, such as when microbial agents express cryptic determinants [106]. Although autoimmunity is a disease of the immune system, nonimmunological genetic and epigenetic factors play a major role in disease development. For example, autoimmunity is influenced strongly by infectious agents, stress and diet (epigenetic), as well as polymorphisms in the TCR and drug metabolizing phenotypes (genetic). The association of autoimmune diseases with certain MHC haplotypes, such as HLA-DR3 in systemic lupus, is striking. A detailed description of the potential mechanisms and the influential factors leading to autoimmune disease is beyond the scope of this section, and the reader is referred to several excellent reviews [107–110].

In drug-induced autoimmunity, the adverse reaction results from an immune response directed against the body's own tissue, subcellular or cellular components. A variety of drugs, with a molecular weight of less than 1000 Da, from a variety of different chemical classes, have been shown to induce autoimmunity. These include aromatic amines (procainamide, practolol), hydrazines (hydralazine), hydantoins (phenytoin, mephenytoin, ethotoin, nitrofurantoin), thioureylenes (methimazole, propylthiouracil), oxazolidinediones (trimethadione, paramethadione), succinimides (ethosuximide, methsuximide, phensuximide), dibenzazepines (carbamazepine), phenothiazines (chlorpromazine), sulfonamides (sulfasalazine, sulfadiazine), pyrazolones (phenylbutazone), amino acids (D-penicillamine, captopril, methyldopa), allylamines (zimeldine, halothane), and certain metal salts (mercuric chloride, gold salts). It is important to note that drug-induced autoimmune disease is different than the classical spontaneous counterpart. For example, in drug-induced autoimmune disease, the disease is usually milder, there is minimal organ involvement, autoantibodies to native DNA are seldom observed in the circulation, and disease remission occurs following cessation of drug treatment. The latter phenomenon may be unique to compounds that induce autoimmunity via the haptenization of native proteins. Nonetheless, the health significance of this adverse effect can be significant.

The most common examples of drugs that have produced autoimmune disease are those that cause hematological disorders such as neutropenia, thrombocytopenia, and immune hemolysis and include a variety of antibiotics as well as anticonvulsants such as phenytoin (Table 37.8). Approximately 10%–20% of patients receiving procainamide and 5%–20%

TABLE 37.8
Examples of Drugs and Chemicals Implicated in Autoimmune Disease

Pathology	Agent	
SLE	Hydralazine	Heavy metals
Immune complex glomerulonephritis	Penicillamine	Isoniazid
	Chlorpromazine	Organic solvents
	Anticonvulsants	Procainamide
	Alfalfa sprouts (L Canavanine)	
Hemolytic anemia	Methyldopa	Diphenylhydantoin
	Penicillin	Interferon α
	Mefenamic acid	Sulfa
Thrombocytopenia	Acetazolamide	*p*-Aminosalicylic acid
	Chlorothiazide	Rifampin
	Gold salts	Quinidine
Scleroderma-like disease	Vinyl chloride	L-Tryptophan
	Silica	
Pemphigus	Penicillamine	
Thyroiditis	PCBs	Lithium
	Iodine	IL-2

receiving hydralazine develop drug-induced SLE. More recently, unexpected hematologic toxicity was related to treatment with a biotherapeutic treatment [111].

Autoimmunity can also be induced by substances found in food or the environment. Regarding food consumption, strong associations have been found to exist between the consumption of iodine and autoimmune thyroiditis, L-5-hydroxytryptophan and scleroderma, and alfalfa seeds and SLE. Exposure to occupational agents has also been linked to autoimmune diseases. Epidemiological studies suggest that exposure to silica-containing mineral dusts is associated with elevated risk for a number of systemic autoimmune diseases, including rheumatoid arthritis (known as Caplan's syndrome), systemic sclerosis, SLE, and antineutrophil cytoplasmic antibody (ANCA)-related vasculitis/nephritis. Although some authors have considered these adjuvant effects, silica may influence circulating immunocompetent cells, particularly Treg and Tresp cells. Epidemiological studies also have suggested a higher than expected risk of systemic autoimmune disease among asbestos-exposed populations [112]. Scleroderma-like skin diseases can result from exposure to vinyl chloride or aniline derivatives; the latter were presumably the active agent resulting in the *toxic oil syndrome* [113]. Agents such as heavy metals, nitrofurantoin, and organic solvents such as trichloroethylene are associated with SLE or glomerulonephritis, the latter being supported by a number of epidemiological studies. Like their idiopathic counterparts, xenobiotic-induced autoimmune diseases are also associated with certain genetic backgrounds. Individuals with the low acetylator phenotypes are associated with drug-induced SLE. The relative risk for developing autoimmunity from gold salts increases 32-fold in individuals who possess the HLA-DR3 allele. Likewise, experimental studies of mercury-induced autoimmunity in the brown Norway rat and B.10 mice suggested that the same genetic influences apply in animals as in humans [114].

Techniques for Assessing Contact and Respiratory Allergy

Given the potential economic and medical importance of hypersensitivity, the importance of sensitive and reliable assays for the detection of sensitizing potential for drugs and chemicals is obvious. For over 100 years, the guinea pig has served as the principal model for allergic reactions in humans since they demonstrate many similarities in their response to pulmonary hypersensitivity (response to histamine, demonstration of immediate and delayed allergic reactions, etc.), as well as dermal hypersensitivity. In addition, the lightly pigmented skin of albino guinea pigs and their relatively small size and docile nature make them manageable model animals. Thus, they have traditionally been used for assessing the human safety of drugs, as well as other chemicals, for contact and respiratory sensitization. Based on the specific experimental needs at hand, a variety of modifications have been described. In this section, we will discuss only two, that is, the Buehler assay and the guinea pig maximization test, which are probably the two most widely used guinea pig tests for risk evaluation of contact sensitization [115].

The mouse has been developed as an alternative model to the guinea pig. The impetus for this development has been the mouse's small size and reduced cost, more thoroughly understood immune system, and the perceived need of a more quantitative endpoint than the subjective degree of erythema that is the hallmark of most guinea pig assays. Of the various mouse models developed including the mouse ear swelling test (MEST) [116], the murine local lymph node assay (LLNA) first described by Kimber and Weisenberger [117] has undergone extensive validation studies [118] and is rapidly replacing guinea pigs tests as the method of choice for identifying contact sensitizers and, thus, will be described in detail in this section.

Considerable efforts have been devoted to establishing relatively simple tests to identify respiratory and food allergens. Guinea pig models have been used historically for the assessment of potential respiratory sensitizers [119,120]. One is directed to a number of excellent reviews on the use of these assays in risk assessment and drug development [121–123]. In addition, murine models for assessment of respiratory sensitizing potential, such as cytokine fingerprinting and the mouse IgE test, have been examined for their utility [124–126]. Likewise, a number of models are being developed to identify food allergens including the development of IgE antibody in brown Norway rats or BALB/c mice and a transgenic mouse strain engineered to produce class II HLA molecules (reviewed by Tryphonas et al. [127]).

Buehler Assay

The Buehler assay [128] was originally developed to evaluate strong and moderate contact sensitizers, leaving only negative or weakly positive compounds for testing in humans.

The hallmark of the Buehler assay was the use of an occlusive patch to enhance or exaggerate exposure to test materials. This method also has the advantage of using an exposure method similar to that encountered in human exposure.

The specific technique for performing this assay is involved and is only summarized in the following. A detailed description of the assay has been provided by Buehler [128,129].

Materials and Reagents Required:

- Young adult albino guinea pigs (Dunkin–Hartley strain)
- Guinea pig restrainers
- Patch delivery system (e.g., Hilltop chambers, Webril patch, PMP patch)
- Dental dam

Procedure:

1. On the day before induction exposure, clip the guinea pig's fur. Expose the skin to the selected test dose using a patch delivery system and then restrain the animal using a combination of guinea pig restrainer and dental dam. Duration of exposure, number of inductions, and induction regimens may vary but are generally three 6 h induction exposures with an interval of 5–9 days.
2. Approximately 2 weeks after the last induction exposure, the animals are exposed to the test material again but at a different skin site that has not been previously exposed. Again, the timing and duration of exposure may vary.
3. If necessary, an animal may be rechallenged with test material between 6 and 14 days following the primary challenge.
4. The day after the challenge or rechallenge, depilate the animals using a commercial depilatory. Two hours later, the animals are ready to be scored.
5. Results are scored as 0 (no reaction), ± (slight patchy erythema), 1 (slight but confluent erythema), 2 (moderate erythema), and 3 (severe erythema, with or without edema). Scoring should be performed at 24 and 48 h after the challenge or rechallenge.
6. Scores of 1 or greater in the test group usually indicate that the test material is a sensitizer, if the scores in control animals are less than 1. Results of the challenge and rechallenge should be expressed as both incidence and severity.

Positive Control:
DNCB has been the traditional positive control for this assay. Suggested test concentrations are 0.3% DNCB in ethanol for induction and two concentrations of DNCB in acetone (0.05% and 0.01%) for challenge. Inclusion of two different challenge doses provides for a range of response [129].

Notes:

1. An irritation screen is performed prior to the actual test. This assay requires an induction concentration that will not produce severe irritation or toxicity. The concentration used for challenge should produce only a slight degree of irritation.
2. Proper technique is essential to the success of this assay. In particular, animal restraint, test material occlusion, and consistency in scoring are all critical aspects. Whenever possible, it would be advisable to learn these techniques from a laboratory that has already demonstrated success with the assay.
3. Due to the relatively small number of animals and the nature of the readout, statistical analysis has not generally been practical for this assay. Rather, hazard assessment has been defined in terms of threshold levels.
4. It is important to maintain occlusion for the entire duration of the exposure in this assay. Proper use of the restraining devices is considered to be critical to obtaining consistent, meaningful results. Proper clipping and depilation of the animals are also important variables.

Guinea Pig Maximization Assay

The maximization assay was described by Magnusson and Kligman in 1964 [130] and was developed to *maximize* the sensitivity of guinea pig tests. This was accomplished by intradermal injections of test material, inclusion of Freund's complete adjuvant (FCA), and the use of a pretreatment to irritate the skin at the site of exposure. These treatments enhanced the chance of a test material to penetrate the skin and subsequently produce allergic contact dermatitis [131]. Perhaps even more so than the Buehler assay, the maximization assay is technically detailed, and only the basics of its performance are summarized in the following.

Materials and Reagents Required:

- Young adult albino guinea pigs (Dunkin–Hartley strain)
- FCA
- Hilltop chambers or PMP patches
- Hypoallergenic tape and elastic wrap
- Dental dam

Procedure:

1. Similar to the Buehler assay, a preliminary irritation/toxicity screen must be performed to determine the highest concentration of test material that can be tested. Both intradermal injection and occlusive patch tests are performed.
2. For the induction step, the animal's fur is clipped over the back on either side of the spine, and test material is injected intradermally in a volume of

0.1 mL. Each animal receives a total of six injections: two each of diluted adjuvant, two each of adjuvant containing test material, and two each of test material in vehicle. Control animals are treated similarly but do not receive test material. Let the animals rest for 6 days.

3. If the test material being used is not an irritant, the injection sites should be treated with 10% sodium lauryl sulfate in petrolatum under an occluded patch for 24 h. This step may be skipped if the test material is a known irritant.
4. Place 0.8 mL of test material on a PMP patch (booster patch) and place this patch over the injection sites. Cover with dental dam and wrap the animal with elastic tape. Control animals should be treated with vehicle only. Remove the booster patch after 48 h.
5. Challenge the animals 10 days later by exposing to test material (or vehicle) under an occlusive patch (PMP patch or Hilltop chamber). Wrap the animals with dental dam and elastic tape. *Note*: Animals must be clipped prior to challenge, since the fur will have grown back.
6. Remove the patches 24 h later. Approximately 21 h later, remove any remaining fur with depilatory. Grade the reactions approximately 24 and 48 h, after the challenge patches are removed.
7. Animals may be rechallenged with the same test material at the same or a different site within 2 weeks of the primary challenge. Naive test sites should be used on the animals.
8. The grading of this assay is similar to that of the Buehler assay.

Positive Control

The positive control material generally used in the maximization test is 1-chloro-2,4-dinitrobenzene, at a concentration of 0.1% in a vehicle of propylene glycol for the intradermal injections. The booster patch incorporates 0.1% (w/v) of test material in an 80:20 ethanol/water (vol/vol) vehicle.

Murine Local Lymph Node Assay

Guinea pig contact sensitization models have been used for assessing the potential of compounds to induce hypersensitivity reactions for some time, but they have certain drawbacks. The endpoints are subjective and require skilled technicians to evaluate the intensity of the reaction; in addition, this subjectivity precludes the use of statistical analysis. Moreover, the assays are relatively expensive and time-consuming, and there are animal welfare issues regarding the use of an adjuvant. Although none of these issues alone are major detriments, together they led to the search for an alternative method.

In 1989, Kimber and Weisenberger [117] reported the development of an alternative approach to assess potential contact hypersensitivity using the mouse as a model system referred to as the LLNA. In contrast to the guinea pig tests described previously, sensitizing activity is assessed as a function of events occurring in the induction, rather than the elicitation phase of sensitization and, thus, does not utilize a secondary (challenge) exposure to the test material. The test involves quantifying cell proliferation in the draining lymph node following topical application of the test material. The LLNA exhibits a number of advantages over guinea pig assays including more quantitative and objective endpoints, insensitivity to colored compounds, reduced turnaround time and cost, being independent of specialized reagents or materials (adjuvant, wrapping material), and benefits to animal welfare. Currently, the LLNA has become the preferred method for assessing skin sensitization hazard by most regulatory authorities.

The biological basis of the LLNA is simple. Test materials are applied epicutaneously to the dorsal surface of the pinnae (Figure 37.7). The test agent is transported from the skin by Langerhans cells to the draining (i.e., local) lymph node, where it is presented to T-lymphocytes. Contact sensitizers induce proliferation of these T-cells. By radiolabeling

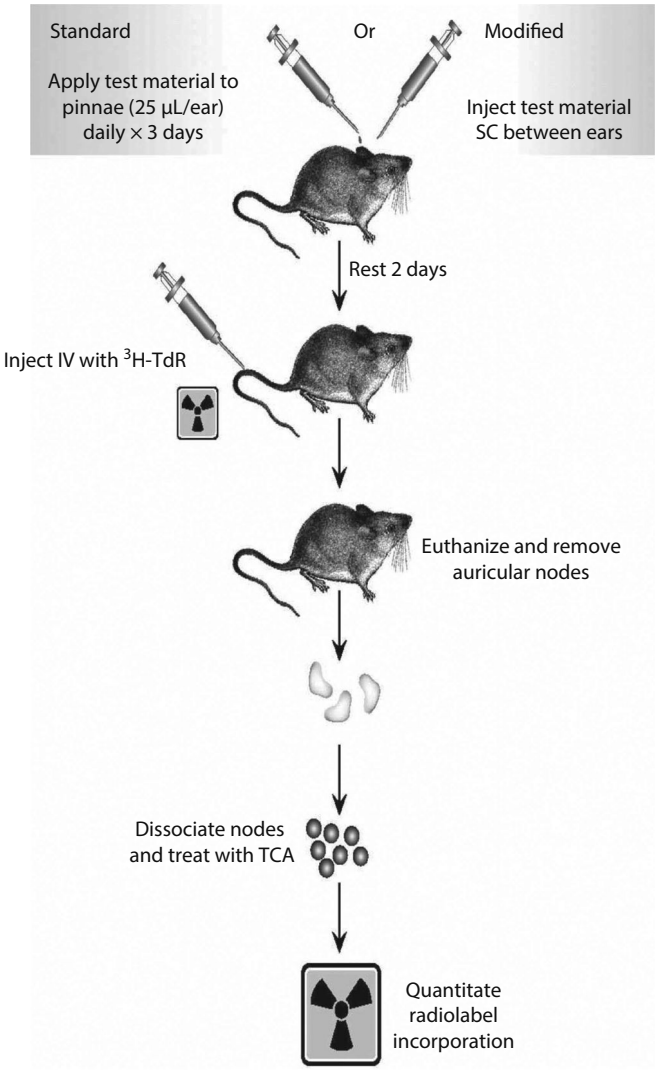

FIGURE 37.7 The murine LLNA.

these proliferating cells in situ using a radioactive tracer or a direct cell count, it is possible to determine the degree of proliferation induced. It is important to note that the LLNA does not utilize a secondary exposure (challenge) to the test material, as do the guinea pig assays. Thus, the LLNA differs fundamentally in that it evaluates only the induction phase of the hypersensitivity response. A chemical is classified as a skin sensitizer if at one or more test concentrations it induces a threefold or greater increase in draining lymph node cell proliferation compared with concurrent vehicle-treated controls (stimulation index [SI] ≥ 3). In the original LLNA, four mice per treatment group were used. Recently, the use of a reduced (r) LLNA has been approved by the International Coordinating Committee on the Validation of Alternative Methods (ICCVAM) under certain circumstances. Since the rLLNA does not provide dose–response information, its utility for risk assessment purposes is limited.

The LLNA has been the subject of numerous international validation studies, including a round of international validation studies employing a standardized protocol [132–134]. The LLNA validation data were evaluated by ICCVAM, and the assay was found to provide an equivalent prediction of the risk for human contact dermatitis when compared to guinea pig assays [135,136]; in addition, the method was robust, sensitive, and reproducible. More recently, efforts have been made to define how the LLNA can be used to measure the relative potency of contact allergens including a two-category system for the classification and labeling of sensitizers under the Globally Harmonized System of Classification and Labeling of Chemicals (high or low potency), which correlates well with findings in humans [137,138].

The following protocol is the standard procedure recommended by the ICCVAM Working Group (IWG) [139]:

Materials and Reagents Required

- Female CBA/J mice, 6–9 weeks old at initiation of assay
- Tritiated thymidine ([^{3}H]TdR), specific activity 5–10 Ci/mM
- PBS
- Nylon mesh (100 μm opening size)
- 15 mL conical capped polypropylene centrifuge tubes
- 5% (w/v) trichloroacetic acid (TCA)
- Scintillation vials and scintillation cocktail

Method:

1. Apply vehicle, test compound, or positive control compound to the dorsum of each pinna (25 μL/ear), ensuring that the vehicle is evenly distributed on the pinna. Dose the animals daily for 3 consecutive days.
2. Rest the mice for 2 days, and then inject each mouse intravenously with 20 μCi of [^{3}H]TdR in saline.
3. Five hours following [^{3}H]TdR injection, euthanize the mice by CO_2 and remove the lymph nodes draining the ear. Place the nodes from individual mice in culture tubes containing 4 mL of PBS.
4. Transfer the nodes from the culture tubes to Petri dishes containing a 1 in.^{2} of nylon mesh. Gently rub the lymph node cells through the mesh, then transfer the cell suspension back to the tube, and allow it to settle for approximately 5 min.
5. Transfer the cell suspension to a 15 mL centrifuge tube containing 6 mL of PBS, taking care not to transfer the sedimented debris. Centrifuge the tubes for 10 min at approximately 200 × *g*. Wash the cells a second time in PBS.
6. After the second wash, suspend the cell pellet in 3 mL of 5% TCA and incubate at approximately 4°C for approximately 18 h.
7. Centrifuge the cell suspensions at approximately 200 × *g* for 10 min, discard the supernatant, and suspend the pellet in 1 mL of 5% TCA. Transfer this suspension to a scintillation vial. Rinse the culture tubes with an additional 1 mL of TCA, and add this to the scintillation vial. Mix the contents of the vial thoroughly.
8. Count the samples in a scintillation counter for 5–10 min, and record the counts as disintegrations per minute (DPMs).
9. Using the results obtained with the vehicle controls as baseline, calculate the SI (i.e., mean experimental results divided by mean control results). Test compounds that induce an SI of 3 or greater at any concentration evaluated in this assay, and for which test DPM are statistically different from control DPM, are considered to be contact sensitizers.

Positive Control:

Hexylcinnamaldehyde (a contact sensitizer of moderate activity) in a solution of 20% serves as a useful positive control for this assay.

Notes:

1. The LLNA is technically straightforward and has been demonstrated to be forgiving of technical modifications. Perhaps the only difficulty a new investigator might have is in locating the lymph nodes draining the pinna. A relatively simple way to determine this is to inject the ears with a dye (e.g., Evans Blue) and subsequently identify the nodes incorporating the dye. In 2010, the Organization for European Cooperation and Development (OECD) adopted two modified skin sensitization LLNA (OECD 442A [*Skin Sensitization: Local Lymph Node Assay: DA*] and OECD 442B [*Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA*]) without the use of radioactive material.
2. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has developed standard performance for the LLNA [140].

Techniques for Assessing Autoimmunity

A number of experimental animal and in vitro models are available to study the mechanisms of autoimmune disease (Table 37.9). However, immunotoxicology screening studies to identify autoimmune-inducing agents have historically used autoimmune-prone rodents and monitored for the accelerated development of autoimmune disease as indicated by elevated levels of serum autoantibodies and/or Ig levels as well as histopathological changes in the target organ(s) or changes in serum chemistries. While a number of syndromes similar to those observed clinically in humans can be mimicked in animal models, the diversity of immune mechanisms and pathogenesis of autoimmune diseases limits the utility of any single model as a screening tool and validated tests do not exist. The primary reason for the lack of validated assays probably stems from the complexity of the disease. Autoimmune disease is not one disease but a group of over 25 diseases affecting distinct organs, often through different mechanisms. Unless a common early process is identified, a single test would be unlikely to provide an adequate degree of concordance to be useful for predictive risk assessment. Secondly, in humans, intrinsic factors (e.g., specific gene polymorphisms, sex-related hormones, and age) and extrinsic factors (e.g., lifestyle, infectious agents) are sometimes strongly associated with the induction, development, and exacerbation of autoimmunity. Lastly, when using animal models, there is some uncertainty regarding what actually constitutes autoimmunity. This is also reflected by a lack of well-defined diagnostic tests for identifying autoimmune disease in humans. Despite these challenges, attempts to develop predictive screening assays for detecting xenobiotic-induced autoimmunity have been undertaken in several laboratories. While screening approaches have most often used spontaneous autoimmune-prone rodents [135], other approaches have included the following: identifying immunoglobulin complexes or immunoglobulin deposits using immunohistological procedures, monitoring for increased levels of serum autoantibodies, and the use of the popliteal lymph node assay (PLNA) with reporter antigens [112,141].

The successful use of exacerbating disease by chemical exposure in autoimmune-prone rodent species has been illustrated by administering streptozotocin in diabetic mice [137] or $HgCl_2$ in glomerulonephritis-prone rodents [142]. Less studied has been the monitoring of Ig deposits or autoantibody production. In the PLNA model, the *autoimmunogenicity* of a chemical, believed to mimic the GVH reaction, is determined by its ability to stimulate specific IgG responses to TNP–Ficoll and TNP–ovalbumin in the popliteal lymph node (PLN). It is independent of the nature of the neoantigens and eliminates many of the potential genetic confounders. In this assay, the test compound is coinjected with 10 μg TNP–Ficoll or 10 μg TNP–ovalbumin subcutaneously into the right hind paw of BALB/c mice. The amount of test substance injected can be equimolar to a related compound or, if known, a concentration demonstrated to be stimulatory in the PLNA. Seven days following treatments, the thickness of the paw is measured using a micrometer and the draining PLN is isolated. Specific AFCs from the PLN are quantitated by any one of several methods such as ELISPOT. For memory responses, mice are similarly treated and then challenged in the right paw with 10 μg antigen, 4–5 weeks following the primary immunization, and antibody-producing cells from the PLN determined 6 days later. Serum samples can also be collected weekly following the primary immunization and serum antibodies determined using commercial procedures.

TABLE 37.9
Examples of Potential Experimental and Screening Models for Autoimmunity

Experimental models to study autoimmunity

Organ-specific autoimmunity
- Induced by immunization (EAE, AA)
- Spontaneous mice (NOD, transgenics)
- Toxicant induced (streptozotocin, Cd)

Systemic autoimmunity
- Allogeneic reactions
- Neonatal thymectomy
- Spontaneous mice (NZM)

Models to evaluate the potential of xenobiotics to induce autoimmunity
- PLNA with reporter antigens
- Increased titers of antibodies to self-constituents
- Examination of Ig complexes/deposits (immunohistochemical staining for immune complexes)
- Spontaneous animal models

Notes: PLNA, popliteal lymph node assay; Ig, immunoglobulin; EAE, experimental autoimmune encephalitis; AA, autoimmune arthritis; NOD, nonobese diabetic (develop immune diabetes); Cd, cadmium; NZM, New Zealand mixed (prone to develop lupus).

EVALUATION OF IMMUNOLOGICAL CHANGES IN HUMANS

Fundamental Concepts

Although infectious disease is the most obvious adverse consequence of immunotoxicity (immunosuppression), the etiology, progression, and/or severity of a much broader range of disorders including certain cancers, allergy, and autoimmune disease can result. Establishing the quantitative relationship between altered immune responses and frequency or severity of diseases in human populations is challenging, as many factors may contribute [143]. This can be summarized schematically in Figure 37.8, where the appearance, progression, and outcome of infectious disease are viewed as an interrelationship between the virulence of the organism, infectious dose (number of organisms required to produce illness), the integrity of the host's anatomical and functional barriers, and the overall immunocompetence of an individual. The latter, in turn, is affected by genetics as well as age, gender, use of certain medications, drug/alcohol use, smoking history,

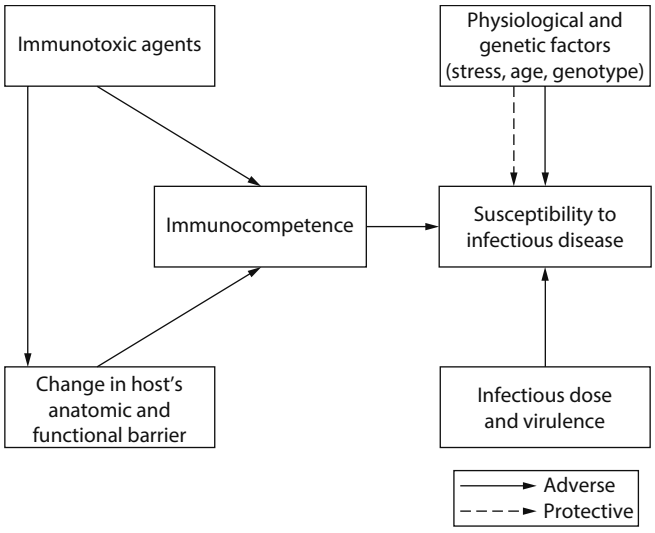

FIGURE 37.8 Relationship between altered immune responses and frequency or severity of human disease.

stress, and nutritional status. These factors probably account for most of the variability reported in mean immune values, which in some instances demonstrate two standard deviations.

Infectious agents associated with immunodeficiency disorders include community-acquired (common), opportunistic, and latent pathogens. Community-acquired pathogens, such as influenza virus, occur in the general population at frequencies associated with their infectious nature. The respiratory system is the most vulnerable target for common pathogens. Upper respiratory infections occur in all age groups but, due to their lack of immunocompetence, can be more severe in the very young and very old. Although influenza is responsible for more morbidity and mortality than any other infectious agent in recorded history [144], the low individual rates of infections in the general population (only one or two episodes in an individual per year), combined with underreporting, make changes in infection rates difficult to detect in prospective epidemiological studies. While infections with common pathogens occur in the healthy population, opportunistic infections, such as *Candida albicans*, *Mycobacterium avium* complex (MAC), and *Pneumocystis carinii*, are most often seen in patients with profound forms of immunosuppression such as HIV/AIDS or primary immunodeficiency diseases. A third group of infections that are observed in immunosuppressed individuals is reactivation of latent infections. The most common agents are from the herpes virus family, which include cytomegalovirus (CMV), herpes simplex virus (HSV), and Epstein–Barr virus (EBV). These viruses remain in the tissue in a latent form following primary infection for the duration of the host's life. In healthy individuals, the immune system usually maintains viral latency. When the cellular immune response is compromised, viral replication can ensue and clinical consequences ensue such as the appearance of cold sores although in some instances, severe complications can ensue. Preceding viral activation, a vigorous immune response to viral-specific antigens occurs in response to replication. Infections with these viruses can occur in individuals with secondary immunodeficiency disorders where mild-to-moderate immunosuppression exists (reviewed in [145]).

Immunodeficiency is also associated with an increased incidence of certain virally induced tumors, such as non-Hodgkin's lymphomas (NHLs) and tumors of the skin [146]. In contrast to cancers of internal organs, in particular those in the lung and liver that are often induced by chemical carcinogens, virus-induced cancers are more immunogenic and, thus, more likely influenced by immunological factors. Suppression of CMI has been associated with higher incidences of skin cancers, leukemias, and lymphoproliferative disorders in transplant patients, whereas Kaposi's sarcoma and EBV-associated B-cell lymphomas are associated with severe immunosuppression as seen in patients with AIDS.

General Testing Considerations and Approach

The design of human studies can range from controlled clinical trials to large, population-based observational studies. Clinical studies offer advantages in that exposure parameters of interest can often be controlled (e.g., clinical drug trials, chamber studies of inhaled toxicants, challenge studies of adenovirus infection) and outcomes can be prospectively monitored. However, there are also disadvantages as ethical considerations provide little opportunity for exposure with suspected toxic chemicals. Furthermore, studies with extensive biological monitoring and functional immune tests can be expensive, and exposures as well as outcomes of interest may be difficult to study in the available time frame as study participants are not typically available for long-term exposures or extended follow-up. For the purpose of obtaining data for immunotoxicological risk assessment, clinical studies are particularly useful as they provide an opportunity to collect data on the frequency of infections or the level of immune response to vaccines. Variations on this type of study design might include follow-up of patient populations administered immunosuppressive therapy (i.e., transplant patients) that, as described in the following, may also have many of the characteristics of observational studies. Other types of human studies that have been employed in immunotoxicology are typically classified as observational or epidemiological. Observational studies can be of varying size and be cross-sectional (one point in time), retrospective, or prospective in nature, each design having advantages and disadvantages. The initial means of control in observational studies is introduced through the study design, and the quality and validity of results can be greatly affected by the methods used to select the study sample and the rigor with which exposures and outcomes are measured. In addition to high costs, observational studies are challenging for many reasons, including potential confounding by host (age, gender, and lifestyle) and environmental (frequency of exposure to chemicals and infectious agents) factors. A secondary measure of control in observational

studies is through the use of multivariable analysis techniques (e.g., regression modeling), provided there is sufficient sample size and information on potential confounders. Overall, well-designed epidemiological studies (e.g., absence of selection bias, exposure or outcome misclassification, and control of confounding) can contribute valuable information to the assessment of risk due to immunotoxic exposures. Because of concern about individual variation, a confirmatory evaluation and a cross-sectional or longitudinal study design can be employed using randomized normal, nonexposed individuals. It is also imperative to obtain a careful medical history, that covers the clinical features of immune dysfunction and accurate exposure assessments.

Testing for primary immunodeficiency diseases is normally undertaken by a stepwise (tiered) approach [147] and is usually initiated because the patient has a history of excessive infectious disease episodes. Initial screening tests include measurements of general parameters, such as CBCs, serum immunoglobulin levels, chest radiograph of the thymus (for adolescents), and delayed hypersensitivity tests. In immunotoxicology, this screening paradigm may be appropriate for clinical studies or in individuals where significant immunosuppression is suspected. However, this approach is unlikely to detect subtle immune changes, and there are no currently validated tests for determining immunotoxic effects in human epidemiological studies. A systematic approach based on simple screening procedures followed by more specialized tests of immune function should provide the best overall assessment. This approach should include the functional evaluation of cellular immunity (T-cell), an antibody response (B-cell), and nonspecific resistance (e.g., PMN function). Recently, it has been suggested that immunization through vaccination, in order to elicit a primary immune response, may be the best criterion to establish immunotoxicity [148]. Most investigations have included one or more of the following: CBCs and differential, immunophenotypic analyses, quantification of serum immunoglobulin levels, and subject's recall of infectious disease frequency.

Tests Commonly Conducted in Humans

Complete Blood Count and Differential

Complete white blood cell counts (WBCs) and a cell differential on all individuals whose immune status is being evaluated are routinely incorporated into a Tier I panel (Table 37.10). The data should be expressed as absolute lymphocyte count for each cell type that is the total WBC multiplied by the differential percentage for that cell type. Higher absolute lymphocytes counts should be expected in children than adults and in certain ethnic groups. Lymphocyte counts consistently below 1500/mm^3 are indicative of lymphocytopenia and may signal a defect in the T-cell system. Lymphocytopenia can be associated with primary immune deficiency disease but also can occur secondary to viral infections, malnutrition, severe stress, autoimmune diseases, and hematopoietic malignancy. When lymphocytopenia is repeatedly observed, a bone marrow biopsy is recommended as an important adjunct for exclusion of other diseases and for identification of normal PCs, pre-B-cells, or diagnosis of bone marrow depression or dysplasia. Individuals with lymphocytopenia should be reevaluated and further assessed for changes in CMI. Lymphocytosis can be caused by chronic infections or allergic reactions. Monocytosis is often associated with stress, infections, and hematologic disorders. Eosinophilia can be caused by allergic reactions, parasitism, skin diseases, neoplasia, and adrenocortical dysfunction.

Immunoglobulin Concentrations

Serum concentration of the major immunoglobulin classes IgG, IgM, and IgA can be readily measured in epidemiological studies. There are several standardized laboratory methods and reagents available for measuring these major classes of immunoglobulin. These methods include single-radial diffusion, double diffusion in agar gel, immunoelectrodiffusion, radioimmunoassay, enzyme-linked immunoassay, and automated laser nephelometry. Serum Ig concentrations vary with age, ethnicity, geographical location, gender, and

TABLE 37.10
Classification of Immune Assessment Tests for Humans

I. Basic Tests	General Indicators	Procedures
Should be included with general health panels along with immune status questionnaire	• Assay methods are standardized among laboratories. • Results are clinically interpretable. • Reference ranges established.	1. CBC and differential 2. Acute phase proteins (CRP) 3. HI: serum IgG, IgA, and IgM levels 4. CMI: delayed-type skin test
II. Confirmatory Tests	**More Specific Immune Tests**	**Procedures**
Should be included when indicated by clinical findings or prior test results	• Assay methods are less standardized. • Results are difficult to interpret. • Reference ranges less well established.	1. Surface marker analysis: assessment of phenotypes for major lymphocyte subsets (CD3, CD4, CD8, CD2) 2. HI: primary antibody response to immunogen; total serum IgE; secondary Ab response to proteins and polysaccharide antigens 3. Nonspecific: autoantibodies (ANA, DNA, mitochondria, RA); granulocyte/leukocyte function (oxidative burst) 4. Bank serum sample for additional analysis

environment. Thus, appropriate norms must be used with any type of population assessment. Patients with primary immunodeficiency disease can manifest a profound decrease in all Ig classes or only in a single class or subclass and are associated with increases in infectious disease frequency. The adverse health effects associated with small changes in immunoglobulin levels are unclear.

Delayed-Type Skin Testing

Skin testing is a commonly used procedure (Basic Panel, Table 37.10) to assess cellular immune competence since delayed cutaneous hypersensitivity, a localized immunological skin response, depends on functional T-cells and the production of inflammatory cytokines. Antigens commonly employed to elicit a positive skin response include purified protein derivative (PPD), mumps, trichophyton, candida, tetanus, or diphtheria. These antigens usually are employed in a panel and are administered by intradermal injection at the appropriate dilution. Skin responses are read at 48 and 72 h for maximal diameter of erythema and induration. The test is not considered very sensitive unless very severe immunosuppression is suspected, which is unlikely to occur.

Specific Antibody Assessment

Recently, efforts have been made to quantify the response to vaccines, either by measurement of antibody titers (HI) or lymphocyte proliferation tests (CMI) to specific vaccine epitopes following vaccination as an indicator of immune function. To use this test in children, the study will need to be conducted in specified age groups and serum samples collected at specific times following vaccination to help limit variability. A number of investigations have examined associations between chronic psychological stress and immune function in adults using hepatitis B, influenza virus, influenza, or pneumococcal vaccine responses [149,150]. More recently, Sleijffers et al. [151] demonstrated a decreased response to hepatitis vaccination in a subgroup of students exposed to ultraviolet radiation in association with polymorphisms in genes that control inflammatory cytokines. An excellent review on the use of vaccination in immunotoxicity testing has recently been published [152].

Although quantifying vaccination responses for clinical diagnoses has not been validated, the ability to detect changes in populations with moderate degrees of immunosuppression, likely associated with the previous examples, suggests a level of sensitivity that would unlikely be achieved with more commonly employed clinical tests such as immunophenotyping or determination of immunoglobulin levels. Historical values for normal vaccine responses in children can be found. For example, Swartz et al. [153] monitored changes in antibody titers in a large group of children following diphtheria–tetanus–pertussis (DTP) vaccination from birth to 8 years of age. Antibody titers to diphtheria, while significantly elevated shortly after the primary and secondary (booster) immunizations, were almost undetectable at 3 years of age. These data are applicable to epidemiological studies as they provide an indication of the antibody decay rates and the variability in titers that may be expected to occur within a normal population.

Phenotypic Analysis by Flow Cytometry

Enumerating cell surface markers (CD) on lymphoid cells by flow cytometry has provided considerable information on the ontogeny and activation state of the human immune system in children and adults, as well as assisting in the clinical diagnosis for immunological and hematopoietic disorders [154]. Specific CD markers have been identified for almost all lymphoid cell populations and subpopulations, as well as for specific stages of cell differentiation and activation. In contrast to adults, children in the first few years of life have a much larger number of total lymphocytes, and both the percentage and numbers of leukocyte populations can vary significantly during critical periods of development. Age-related differences in immunophenotypic profiles in children were recently addressed by the Pediatric AIDS Clinical Trials Group, sponsored by the National Institute of Allergy and Infectious Diseases and National Institute of Child Health and Human Development [155]. In this study, lymphocyte subsets were phenotyped in 807 normal children ranging from birth to 18 years of age. Despite efforts to control for inter- and intralaboratory methodological differences, the variance within each age group was significant, often exceeding twofold, even when discarding the highest and lowest 10th percentile. Certainly, not all the variability in human immunophenotyping studies is related to technical variability, as both genetic and environmental influences play even more significant roles [156]. Nonetheless, this database may prove useful for epidemiological studies in developmental immunotoxicology as it not only provides extensive reference values but can assist in developing appropriate study designs.

Immunophenotyping is conducted using flow cytometers that have multiple photomultiplier tubes (four or more) and are capable of measuring three-color fluorescence, 90° light scatter, and forward light scatter. When highly specific fluorochrome labeled monoclonal antibodies are used in these instruments, very quantitative measurements can be made of B- and T-cell subsets. The most commonly used procedure for processing peripheral blood samples for immunofluorescence is first to stain an aliquot of whole blood with fluorescent-conjugated monoclonal antibodies and then to lyse the erythrocytes. The proportion of circulating T-cells is then determined by immunofluorescence with Fluor-labeled CD2 or CD3 monoclonal antibodies in a flow cytometer. Normally, T-cells constitute 55%–80% of peripheral blood lymphocytes. Normal values reported for absolute numbers of circulating T-cells are 590–3090/mm^3 for individuals greater than 18 months of age [157]. If an immune defect is suspected, the ratio of CD4 to CD8 T-cells can also be beneficial. Although this method is quite quantitative, the ability of this test method to detect subtle immune changes in populations of individuals has recently been challenged [154].

Nonspecific Measurements

Neutrophil Function

The measurement of nitroblue tetrazolium dye reduction by actively phagocytosing PMN is a method that has been historically utilized when a PMN defect is suspected. Flow cytometric methods are also available [158].

Autoantibodies

It is often stated that the immune system is established on a principle of self-/nonself-recognition. In some cases, tolerance to self-antigens breaks down and autoantibodies are produced, which can be manifested as autoimmune disease. Antibodies to cellular components and nuclear antigens (ANA, DNA, mitochondria) and to rheumatoid factor (RA) and their frequency in a population may reflect an immune alteration. Standardized diagnostic kits are available to detect the presence of these autoantibodies in sera. Finally, it is a good practice to establish a freezer bank of an aliquot of each test subject's sera for later evaluation when new research questions or test methods are developed.

RISK ASSESSMENT AND DATA INTERPRETATION

Immunotoxicology data used in risk assessment are derived primarily from animal toxicology studies. When adequate data are available, epidemiological or controlled clinical exposure studies take precedence. The results obtained from in vitro studies, quantitative structure–activity relationships (QSARs), or mechanistic investigations are used normally as supportive information. Mechanistic studies, however, are important, as they help determine plausibility and human relevance [159]. Interpretation of immuntoxicology data is dependent upon the immunopathology studied, that is, suppression/stimulation, hypersensitivity, or autoimmunity.

Immunosuppression

Animal studies for testing immunosuppression or stimulation are used primarily for semiquantitative hazard identification. Since tests for immunosuppression use multiple and, to some extent, overlapping endpoints, the data can also be evaluated in terms of establishing an immune profile. This may help allow to identify the *mode of action*, such as direct cytotoxicity, hematopoietic toxicity, altered immune regulation, or altered regulatory circuits. At the least, the test panel can determine which arm of the immune system is the primary target. Hazard identification for immunosuppression can also be described as a weight of evidence approach as recommended by recent guidance documents prepared by the IPCS/WHO [159]. This approach suggests addressing seven specific questions in which weights are given for various endpoints including in vitro studies, QSAR, human studies, and other animal studies. The strength of association will also take into account dose–response relationships, consistency of association, temporal association, biological plausibility, specificity, coherence, and analogy.

Hypersensitivity/Allergy

In cases where ethical considerations prevent the use of human patch testing to establish the potential of agents to induce allergic contact dermatitis, animal models, particularly the Buehler-occluded and Magnuson–Kligman maximization tests in guinea pigs, have been used as predictive tests. Several graded doses of antigen may be examined simultaneously and comparing skin reactions in individual animals can generate an entire dose–response curve. However, it is expensive to purchase and maintain guinea pigs, there are few inbred strains, and immunological reagents are not widely available. Furthermore, there is some suggestion, although never fully substantiated that these models are overly sensitive when compared to the experience in humans and produce a low frequency of false positives.

While the strengths and weaknesses of the LLNA have been discussed earlier (see "Murine Local Lymph Node Assay" section), it is important to note that the assay has undergone a series of examinations to provide *technical refinement* and assess *inter- and intralaboratory reproducibility*, as well as relative sensitivity and specificity, referred to as *concordance* [135,140,160]. Concordance for a new assay should be established to previously used test models as well as to available human data. Such data for the LLNA [130,131] indicated that the LLNA was highly comparable to guinea pig tests (concordance almost 90%), but only about 70% accurate when compared directly to human studies. As this is similar to results obtained when guinea pig tests are compared to human studies, in terms of risk assessment, the LLNA can be used in lieu of guinea pig tests, but an alternative assay that could provide higher concordance with humans would be desirable.

In contrast to predictive tests for allergic contact hypersensitivity, the identification of proteins and chemicals capable of inducing respiratory hypersensitivity is in its infancy. As these tests are difficult to undertake, often involving respiratory exposure and lung function tests, efforts to develop and validate new methods are limited. Although the guinea pig has significant immunological differences compared to humans (e.g., IgG1 vs. IgE reaginic antibodies), it appears to be a predictive model for humans given the limited comparative data available and has been used to test for high- and low-molecular-weight sensitizers. This test requires a systemic or inhalation sensitization phase and an aerosol challenge, and both immediate and delayed-onset responses are measured, although this does not distinguish between nonspecific pulmonary hyperreactivity and specific immune responses [161]. The latter can be established by examining sera for the presence of reaginic antibodies.

Like immunosuppression, LLNA data are used primarily for hazard identification. Since multiple doses are evaluated, the results can be interpreted in a semiquantitative manner, or at least in terms of relative potency, based upon the lowest concentration that elicits a response above an EC3. Two-category systems have been introduced for the classification and labeling of sensitizers under the Globally Harmonized

System of Classification and Labeling of Chemicals (high or low potency), which correlates well with humans. LLNA results should also be evaluated broadly in terms of a weight of evidence approach as recommended by IPCS/WHO [159], using all available data including QSAR, additional animal studies, and human data.

Autoimmunity

General agreement exists among the regulatory and pharmaceutical communities that predictive tests for autoimmunity or systemic allergy are in most need of development in order to improve risk assessment in immunotoxicology [112]. While many models exist to study autoimmune processes, they do not readily lend themselves to use in risk assessment, as they do not consider the multifactoral nature of the disease. To improve the risk assessment process, screening models need to be developed and validated that not only incorporate mechanistic information into the assessment process but also allow for the consideration of the genetic, physiological, and environmental influences that lead to the loss of self-tolerance, autoimmune disease, or systemic allergy. Despite the challenges in developing such screening tests, the considerable amount of data generated by immunologists and pharmacologists pertaining to basic mechanisms of chemical-induced autoimmune diseases has provided a conceptual framework, which allows the establishment of potential QSARs. These QSARs are by no means definitive, and as the database increases, no doubt some will not be supported while others will be added. In all cases, a basic understanding of immunological and pharmacological processes supports these relationships. For example, estrogens are known to be a major factor in classical autoimmune diseases presumably due to their ability to stimulate certain components of the immune system [162], and as such, agents with estrogenic activity may be of concern.

Laboratory studies have also shown that thymolytic chemicals, such as cyclophosphamide and cyclosporin A, can induce autoimmunity when given neonatally by altering normal patterns of autoreactive T-cell deletion [163]. In this respect, the thymus has been shown to be a target for many toxic chemicals. As in the case of halothane, chemicals that form protein adducts or damage tissue in such a way to allow expression of cryptic determinants would provide novel host antigens that could now be recognized by T-cells. Agents that have adjuvant activity, or biologicals that stimulate certain cytokines, may shift the balance of Th1 and Th2 cells and allow exacerbation of preexisting autoimmune disease [164]. Common features associated with many drugs that induce autoimmune diseases are that they serve as myeloperoxidase substrates and/or cause changes in methylation [165]. The explanation for the latter association is less clear but may require identification of the specific antigenic epitopes responsible for the autoimmune response. In the case of the association with myeloperoxidase substrates, it has been suggested that many of the chemicals require metabolism in proximity to immune cells in order to be antigenic and immune cells such as monocytes contain high levels of myeloperoxidase.

In the interpretation of autoimmunity studies, any effect and/or dose–response relationship should be evaluated relative to generalized overt toxicity as well as for consistency, specificity, and coherence. The data should also be interpreted in context of all the tests that were examined for autoimmune potential whether supportive or not, as recommended in IPCS/WHO [159]. Again, this implies a weight of evidence using all available data including QSAR, in vitro, human, and laboratory animal data that are relevant to either induction or exacerbation of autoimmunity whether supportive or not.

REGULATORY GUIDANCE FOR ASSESSMENT OF IMMUNOTOXICITY

Overview

Some of the earliest codified immunotoxicology test guidelines were developed to augment toxicological assessment of pesticides. In 1996, the Office of Prevention, Pesticides, and Toxic Substances (OPPTS) of the U.S. Environmental Protection Agency (EPA) published guidelines entitled *Biochemicals Test Guidelines: OPPTS 880.3550 Immunotoxicity* [166], which described the preferred study design for evaluating potential immunotoxicity in biochemical pesticides. The panel of tests included in this guideline includes standard toxicology tests as well as immune function tests. A second document published concurrently (*Biochemicals Test Guidelines: OPPTS 880.3800 Immune Response*) [167] provided rationale for testing, detailed explanations for testing strategies, and additional mechanistic tests including host resistance and bone marrow function.

Whereas immunotoxicity evaluation encompassed by the 880 series of guidelines would arguably detect any type of immunotoxicity, its breadth would probably render it tremendously expensive and time-consuming. In 1998, EPA followed up with the *Health Effects Test Guidelines: OPPTS 870.7800 Immunotoxicity* [168], which described immunotoxicology testing for nonbiochemical agents that would be regulated by EPA. This document provides descriptions of both why and how, with a much more abbreviated panel of testing to be performed. Whereas the 880 series of immunotoxicology guidelines are excessive, the testing approach mandated by 870.7800 has stood up well in intervening years and reflects the more limited, case-by-case approach currently favored. Most notably, the functional assessment is pared down to T-dependent antibody formation, NK cell function and quantitation of T- and B-cells.

In Europe, the OECD regulates testing of chemicals for toxicity. OECD Guideline 407 entitled *Repeated Dose 28-day Oral Toxicity Study I Rodents*, while not specific for immunotoxicology, includes a variety of toxicological endpoints that can provide early evidence of immune system alterations. Missing, however, are any functional assays to directly measure any immune deficit.

Pharmaceuticals

In the United States, safety testing of small-molecule pharmaceuticals is the purview of the U.S. Food and Drug Administration's Center for Drug Evaluation and Research (FDA CDER). In October of 2002, CDER released a long-awaited document entitled *Guidance for Industry: Immunotoxicology Evaluation of Investigational New Drugs* [169]. This document is arguably the most comprehensive of any published guidance, describing a diversity of adverse events including immunosuppression, immunogenicity, hypersensitivity, autoimmunity, and adverse immunostimulation. The document describes each of these types of immunotoxicity (more accurately, immunomodulation) in detail and not only provides approaches but also suggests methodology for evaluating each type. Like the CPMP document (described in the following), the FDA/CDER guidance advocates the use of information derived from standard repeat-dose toxicity studies to provide early evidence of immunotoxicity, with subsequent evaluations to be rationally designed to use a minimum of animals and resources while deriving the maximum amount of information. Subsequent to the publication of the FDA/CDER document, the primary author of the guidance published a manuscript describing the implications of the guidance [170].

In Europe, safety testing of pharmaceuticals is regulated by the Committee for Proprietary Medicinal Products (CPMP). In October of 2000, CPMP published *Note for Guidance on Repeated Dose Toxicity (CPMP/SWP/1042/99)* [171]; although the primary purpose of this particular document was to describe an overall approach to safety testing of pharmaceuticals, it was important as the first guidance document mandating specific immunotoxicology screening for pharmaceuticals. An appendix in this document describes a staged evaluation, emphasizing that information gained in standard toxicology evaluation can be useful as a primary indicator for immunotoxicity. Functional tests may be incorporated to gain additional information, first as an initial screen and then progressing to extended studies as indicated. The choice of assays to be used includes combinations of functional tests known to be predictive of immunotoxicity, as described in the early National Toxicology Program publications.

As the first published document requiring immunotoxicology evaluation, *CPMP/SWP/1042/99* predictably was met with a combination of resistance and confusion. Much of this was allayed in a Drug Information Association-sponsored workshop held in Noordwijk, the Netherlands in November of 2001. A summary of findings from this workshop has been published [172]. Differences in how the U.S. and EU guidance to immunotoxicology assessment is interpreted have led to intense discussion regarding the best approach for performing this assessment on a routine basis. Having reached step 4 of the International Conference on Harmonization (ICH) process at the ICH Steering Committee meeting on September 15, 2005, S8 ICH Guideline was recommended for adoption to the three regulatory bodies [173]. This document was finalized in 2006 by FDA and in entitled *Guidance for Industry: S8 Immunotoxicity Studies for Human Pharmaceuticals.*

Biologicals

Biologicals (therapeutics derived by biotechnology) present a unique challenge for immunotoxicity assessment. Some of these agents, such as cytokines and other immunomodulatory molecules, are intended to therapeutically modulate the immune response; therefore, it can be difficult to differentiate between the agent's efficacy and a truly adverse reaction. Second, because many of these agents are proteins or peptides, their introduction into a host often triggers an immune response directed against the molecule itself; this can lead to alterations in pharmacodynamics or to other adverse reactions. Thus, development of appropriate guidance on testing these agents is problematic. One approach is promulgated by the International Conference on Harmonisation, with the document *Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals S6* [174]. This document includes sections on immunogenicity (as described previously) as well as a brief mention of immunotoxicity studies. In short, the S6 document recognizes the inappropriateness of a structured *tier* approach, opting instead for careful design of screening studies, followed by mechanistic studies to clarify any potential evidence of immunotoxicity. Specific techniques and approaches are not described in ICH S6.

Safety evaluation of biological drugs is regulated in the United States by the FDA's Center for Biologics Evaluation and Research (CBER). To date, CBER has not promulgated any written guidance on immunotoxicology; the reason for this lack of written guidance is the extreme diversity of biological therapeutics, which makes it difficult to design a standardized testing approach. Rather, CBER's approach to addressing potential immunotoxicology has been case by case and followed suggestions provided in ICH S6.

Vaccines

Along with certain biologicals, vaccines present a challenge for immunotoxicological evaluation since they are specifically designed to induce an immune response, a situation deemed undesirable (or potentially so) for most of the other agents described in this review. Since methodology is well established to evaluate the desirable immunomodulation produced by vaccine, the concern of regulatory agencies is the potential of these agents to produce an undesired or deleterious effect on the immune system.

European regulation of vaccines is described in *Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines* published by the CPMP [175]. In this document, immunotoxicology is to be considered during toxicology testing. In particular, vaccines should be considered for their immunological effect on toxicity, such as antibody complex formation, release of cytokines, induction of hypersensitivity reactions (either directly or indirectly), and association with autoimmunity. No specific methods or approaches were described; each vaccine is to be evaluated on a case-by-case basis.

FDA/CBER is tasked with regulating vaccines in the United States. One of the primary documents describing vaccine studies is *Guidance for Industry for the Evaluation of Combination Vaccines for Preventable Diseases: Production, Testing and Clinical Studies* [176]. Animal immunogenicity is covered in detail in the document, although immunotoxicity is not specified as an area of concern. On the other hand, CBER's *Considerations for Reproductive Toxicity Studies for Preventive Vaccines for Infectious Disease Indications* [177], although intended primarily to assess effects of vaccination on reproductive function (including generalized toxicity such as fetal malformations), acknowledges the potential immunological reactions resulting from the vaccination process to exert unintended consequences. No specific guidance is provided on methods or approaches to be used in this evaluation.

Devices and Radiological Agents

It has been recognized by the FDA that immunotoxicity may result not only from chemical or biological agents that dynamically interact with humans' physiology such as small-molecule drugs or biological agents but also from medical devices that contact the body externally (via skin or mucosa), internally (implantable devices), or by external communication to the blood or tissue. Thus, the FDA Center for Devices and Radiological Health published the guidance entitled *Guidance for Industry and FDA Reviewers: Immunotoxicology Testing Guidance* in May 1999 [178] that addresses testing for medical devices. This guidance is based on the General Program Memorandum G95-1, an FDA-modified version of International Standard ISO-10993, *Biological Evaluation of Medical Devices Part 1: Evaluation and Testing*. The Immunotoxicology Testing Guidance provides detailed guidance for determining when immunotoxicity testing should be performed (including a flowchart and numerous tables) but does not provide details on which methods should be employed or for overall study design. Anderson and Langone [179] provided explanatory details on the use of this guidance.

Hypersensitivity

Although much attention is paid to immunosuppression (low immune response) in the majority of guidance documents, it is hypersensitivity (hyperactive immune response) that is the most common type of immunomodulation resulting from exposure to xenobiotics. Due to the acknowledged frequency of this occurrence, as well as the multiplicity of testing methods that have been developed, a complete coverage of this condition will not be included here. However, one method for assessing hypersensitivity has taken priority in assessing contact hypersensitivity, namely, the murine LLNA. Detailed explanations of this assay and its use are covered in the OECD 429, 442A, and 442B guidelines [180].

FRONTIER TECHNOLOGIES IN EXPERIMENTAL IMMUNOTOXICOLOGY

Biotherapeutics

The biopharmaceutical industry is already aware that biotherapeutics can be immunogenic, and the resulting production of antidrug antibodies (ADAs) can significantly alter their clearance safety and efficacy. Testing recommendations for predicting ADA have been suggested and are being validated [181]. Of particular concern, however, is the safety of biotherapeutic monoclonal antibodies, particularly those that are immunomodulatory. While generally proven to be safe, on occasion, they present exaggerated pharmacology that was not predicted based on an understanding of the intended function or results from nonclinical studies [182]. This first became evident following the well-publicized adverse events observed with an immunomodulatory anti-CD28 super agonist mAb (TGN-1412) during a clinical trial in the United Kingdom [183].

Nanotechnology

Another potential safety concern is the emergence of successful implementation of nanotechnology, the manufacture of materials and devices at the molecular scale. It is unknown at present whether these new materials will exhibit unanticipated toxicology, although several groups have already begun raising potential concerns [184]. One of the most immediate concerns is the effects on respiratory function following inhalation of nanoscale particles [185], although immunotoxicity has been often reported [186]. The fact that these materials can intercalate into DNA as well as cell organelles opens up the question of whether current immunotoxicity testing methods are adequate.

Interface between Innate and Adaptive Immunity

Although the innate immune system is often described as *primitive*, it is increasingly being recognized that the innate and adaptive systems represent a continuum of highly interactive and often complementary protective mechanisms. There are a number of effectors that form the bridge between these systems, including the Toll-like receptors [187], γ–δ T-cells [188], NK cells, dendritic cells [189,190], T-regulatory cells (TREG) [191], and Fas signals [192] to name but a few. As a better understanding of these complex regulatory circuits develops, it is becoming apparent that this system is responsible for immunomodulation of both normal [193,194] and pathologic processes such as autoimmune disease [195].

Alternative Methods

Alternative methods to replace the use of animals are a requisite for immunotoxicant screenings in the twenty-first century and the use of laboratory animals for such tests are generally prohibited in Europe. At present, alternative methods to screen and rank the sensitizing potential of chemicals

are being developed and under validation at the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) [196].

Developmental Immunotoxicology

There is an increasing link between early-life exposure to chemicals, developmental immunotoxicity, and the development of postnatal immune function. This concept introduces the need to identify chemical hazards to the developing immune system [197].

CONCLUSIONS

The discipline of immunotoxicology has grown in importance in toxicology since its inception in the mid-1970s. It has progressed from the early identification of chemicals that may cause immunosuppression/modulation and allergic contact dermatitis, through the validation of sensitive and quantitative assays that serve as biomarkers of immune system alterations in animals and man. More recently, academic, industrial, and government scientists have taken a more mechanistic approach to define how therapeutic and environmental agents alter immune function at a cellular and molecular levels as well as efforts to establish predictive tests to assess agents that may induce autoimmune disease and chronic inflammation. Immunotoxicity data derived from experimental and human immunosuppression and hypersensitivity studies play an increasing role in establishing health standards and defining permissible levels of toxic chemical exposure in humans.

What is still needed is better correlation between animal data with known immunotoxicants and epidemiological or clinical studies in order to ascertain the predictive value of the immune evaluation methods for human populations that may be occupationally or environmentally exposed. Well-controlled studies are still needed in human subjects exposed to environmental chemicals to establish concretely the relationship between documented exposure and immune-mediated effects. With pharmaceuticals where exposure is well documented, the correlations of the prediction value of animal studies for immunoalterations (e.g., immunosuppression or allergy) in man are more clearly defined.

QUESTIONS

37.1 The immune system exists to protect the body against the following:
- a. Specific invading pathogens and microorganisms
- b. Neoplastic cells
- c. Nonself-antigens
- d. Transplanted foreign antigen
- e. All of the above
- f. a, b, and d

Answer: e

37.2 Which of the following statements is false?
- a. Macrophages and leukocytes are types of phagocytic cells derived from the bone marrow
- b. Cell-mediated immunity represents a type of nonspecific immune response
- c. The two major mechanisms of immunity are nonspecific and specific
- d. Humoral immunity is associated with the production of antibody
- e. Pluripotent stem cells are found in the bone marrow and give rise to megakaryocytes and lymphocytes

Answer: b

37.3 Which of the following statements are true?
- a. The primary lymphoid organs are represented by the thymus and bursa-equivalent tissues
- b. Lymphoid tissue is derived from ectoendodermal junctional tissue

Answer: a

37.4 Autoimmunity is best defined as follows:
- a. It is an immune response to normal components of the host
- b. It is mediated by IgE
- c. It can best be measured in guinea pigs
- d. It reflects a single organ

Answer: a

37.5 Immunotoxicity assessment is most often conducted using the following:
- a. Epidemiology studies
- b. In vitro studies
- c. Animal studies
- d. Combinations of SAR and clinical trials

Answer: c

37.6 Chemical- or drug-induced autoimmunities differ from their idiopathic counterparts in that they
- a. Usually remit when the drug is withdrawn
- b. Only target the kidney
- c. Only target blood elements
- d. Are more common in females

Answer: a

37.7 Validation of animal models for immunotoxicology studies requires
- a. Laboratory validation
- b. Establishment of specificity
- c. Establishment of sensitivity
- d. Reproducibility
- e. All of the above

Answer: e

37.8 The most appropriate animal model for evaluating immunotoxicity appears to be
- a. Rodents
- b. Minipigs

c. Guinea pigs
d. Nonhuman primates

Answer: a

37.9 Allergic reactions to drugs may result from
a. Direct antigenicity of the drug moiety
b. Activation of complement proteins
c. Haptenation of self-proteins by the drug or a metabolite
d. Bone marrow ablation

Answer: a and c

37.10 Macrophages are an important potential target of immunotoxicants because of the following reasons:
a. They are capable of metabolizing xenobiotics
b. They are potent immunoregulatory cells
c. They secrete large quantities of inflammatory antibodies
d. a and b
e. b and c

Answer: e

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38 Assessment of Behavioral Toxicity

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CONTENTS

INTRODUCTION

To survive, organisms must be sensitive to events occurring in their environments and respond appropriately. At the most elementary level, organisms must avoid hazards such as predators and other threats, must secure food, and, for species survival, must pursue reproduction. The nervous system is the site at which such transactions with the environment are processed. The nervous system also governs endogenous transactions such as controlling neuroendocrine secretions and carries on commerce with the immune system, but such functions are processed in the background, so to speak. The integrity of the nervous system, paramount to both individual and species survival, is reflected predominantly by the integrity of behavior.

This chapter describes methods and issues related to the assessment of behavioral toxicity. Its complexities are significant because of the multiple dimensions and behavioral domains that comprise the human behavioral repertoire. Primary among these are motoric, sensory, and cognitive (e.g., learning, memory, attention) domains, each of which can range from simple to highly complex levels of function. Further, human behavior requires integration across these various domains. It is for this reason that in vitro methods are unlikely to ever provide sufficient alternative approaches for behavioral toxicology,[1] particularly when it is clear that even subtle functional differences can significantly influence neurochemical and neurophysiological outcomes.

Often, the first clues of toxicity to humans may be subjective disturbances such as nervousness (chlordecone) or personality changes (manganese), succeeded by more overt signs such as tremor (chlordecone) or akinesia (manganese). Regulatory standards aim for exposure levels, providing enough of a margin to preclude even these preliminary, nonspecific symptoms.[2] In contrast, early animal laboratory investigations tended to adopt exposure levels likely to evoke a clearly visible toxic response, a strategy requisite to develop and validate methods responsive to neurotoxic agents and to acquire corresponding information about mechanisms of toxicity applicable to lower exposure levels. Since then, animal studies have expanded to focus on lower-level and cumulative exposures and associated behavioral mechanisms, and both human and experimental studies now address issues such as fetal basis of adult disease and long-term effects of toxicants as well as reversibility of adverse effects.

In addition to providing a measure of functional competence, behavioral outcomes often give guidance to the underlying neurochemical, neurobiological, and histopathological substrates, an understanding continually increased by parallel advances in the fields of psychology and neuroscience. For example, elevated motor activity may indicate actions on specific neurotransmitter systems; an inability to distinguish geometric form would point to the visual cortex as a possible site of damage.

Given that behavioral functions can range from simple to highly complex, the assessment of behavioral toxicity often proceeds in stages. The first stage often examines dose–response relationships based on systematic observations of responses such as those included in functional observation batteries that generally evaluate a range of simple and innate behaviors. While alerting to potential behavioral toxicity, such evaluations typically provide little information about the specificity of a functional deficit,[3] which requires more complex assays that provide greater precision, thus yielding more specific and quantitative information to permit better prediction of possible adverse effects in humans.

The chapter proceeds from descriptions of the simpler behavioral techniques generally applied in the earliest steps of hazard identification to more complex approaches designed to clarify specific deficits and to understand both behavioral and neurobiological mechanisms. It addresses both human and animal behavioral testing. The techniques and paradigms described include those utilized in an experimental context, many of which are appropriate across species, including humans, with appropriate parametric modifications, a strategy that should facilitate risk assessment. In fact, some approaches were originally implemented in human subjects and subsequently modified for experimental animal use. The chapter aims to provide enough familiarity with these techniques to allow a more than elementary understanding of behavioral toxicology. Two early reports[4,5] aimed at the impact of neurobehavioral toxicants on public health demonstrate how behavior serves as the sentinel for less accessible endpoints.

Origins of Behavior

The behavioral repertoire varies along two dimensions: origin and modifiability. At one end of such a continuum are behaviors designated as innate or hardwired that include unconditioned reflexes, some fixed-action patterns, and instinctive behaviors that do not require learning. The components of such innate behaviors may be unique to a particular species and may be insensitive to modification by the environment. For some such behaviors, once evoked, the responses continue to completion even in the absence of the appropriate environmental substrates. On the other extreme are learned or acquired behaviors that are subject to modification by the environment. Such behaviors are based on voluntary emitted responses, which are increased or decreased in strength and altered or refined in a dynamic manner across time by environmental consequences through operant or respondent conditioning. Falling between these two extremes are instinctive behaviors such as some fixed-action patterns that are modifiable by environmental circumstances. The extent to which the behavioral repertoire of an organism is comprised of learned versus unlearned behaviors is dependent on the species, with a tendency for learned behaviors predominating at the higher end of the phylogenetic continuum.

Respondent Conditioning

In respondent conditioning, as shown in Figure 38.1 (left column), an *unconditioned reflex* elicited (evoked) by an unconditioned stimulus is the basis for a new conditioned respondent. The term *unconditioned* signifies the innate characteristics of the stimulus and response comprising the reflex. When the unconditioned stimulus is repeatedly paired with a neutral stimulus (*procedure*), the neutral stimulus comes to acquire eliciting properties similar to those of the unconditioned stimulus, that is, the neutral stimulus becomes a conditioned stimulus that can evoke a *conditioned reflex* that typically, though not uniformly, resembles the unconditioned response. Figure 38.1 uses the classic example from the Russian physiologist I.P. Pavlov, who used meat powder, an unconditioned stimulus, to evoke salivation, an unconditioned response, in dogs. Tones repeatedly paired with the meat powder eventually came to function as conditioned stimuli that elicited conditioned salivation. In respondent conditioning, pairing of the conditioned and unconditioned stimuli must occur at least intermittently for the conditioned stimulus to retain its ability to elicit a conditioned reflex.

Operant Conditioning

Operant conditioning (Figure 38.1, right column) is based on emitted or *voluntary responses* (not evoked responses such as in reflexes) that occur at some baseline level in the absence of any environmental influences. For example, newborn babies engage in voluntary skeletal muscle motions even at birth that can be modified by the environmental consequences that follow them (*procedure*) and thus serve as the basis of later, more coordinated movements and precise motor functions (operant behaviors). Operant conditioning is the process by

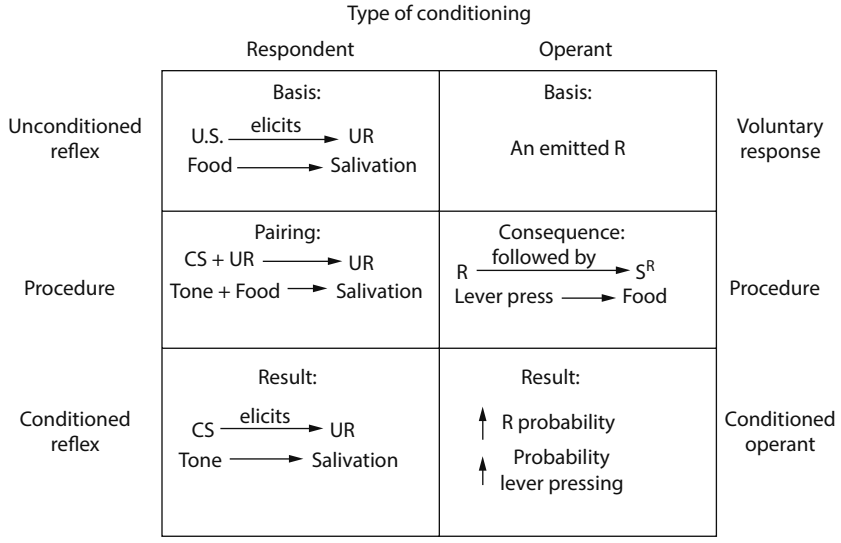

FIGURE 38.1 The two types of behavioral conditioning are illustrated. Respondent conditioning (left column) is based on unconditioned (innate) reflexes. In respondent conditioning, an unconditioned stimulus elicits an unconditioned response. When an initially neutral stimulus is paired with this unconditioned stimulus (procedure), it acquires eliciting properties; that is, it becomes a conditioned stimulus capable of eliciting a conditioned reflex. In the example, elicitation of salivation by food is an unconditioned reflex. When a tone is appropriately paired with the food (procedure), its subsequent presentation alone will come to elicit salivation. Operant conditioning (right column) is based on voluntary responses emitted by the organism. If these responses are followed by a reinforcing stimulus (procedure), the frequency of the response will subsequently increase (result). In the example, pressing of a lever by an organism results in food reward (procedure), thereby increasing the frequency of lever pressing. If reinforcement is withheld (extinction) or a punishing stimulus is presented, response frequency will decline.

which the frequency or strength of an operant (voluntary) response is modified by the consequences of that behavior. Figure 38.1 presents the example of a lever press response by a slightly hungry rat resulting in food as a reward that increases the future probability of the lever press.

Reinforcement

Reinforcement is the presentation of a stimulus contingent upon a voluntary response that results in an *increase* in the future frequency of that response (Figure 38.1). Reinforcers may be either positive or negative, a distinction that is purely procedural (Table 38.1). Specifically, a reinforcer strengthens a response; a *positive* reinforcer increases probability of the response by its *presentation*, such as the delivery of food to a hungry organism or the presentation of money for work performed. With *negative* reinforcement, a response is strengthened by the *removal* or diminution of a stimulus event or the prevention of its onset. Here, common examples used experimentally include cessation of electric shock or loud noise contingent upon a response; a human example might be the prevention of a parent's scolding by cleaning one's room. Shock avoidance and escape procedures fit into this category.

Reinforcers cannot be classified intuitively or categorically but only on the basis of the change in behavior subsequent to their presentation. Food may not serve as a positive reinforcer for someone with the stomach flu; attention and affection often fail to function as positive reinforcers for autistic children. Given the appropriate behavioral training conditions, electric shock presentation can actually maintain rather than suppress responding.[6] As these examples indicate, a stimulus that serves as a reinforcer for one person may not so function for another. The sum total of an individual's interaction with the environment and reinforcement contingencies is deemed its *behavioral history*. Because all individuals could never simultaneously be in the same place, make the same responses, earn the same reinforcers, and so forth, each person's behavioral history is unique. This becomes particularly important because differences in behavioral history can modify the response to drugs and, thus, perhaps to toxicants as well, an important possibility that as yet has received little experimental attention.

Reinforcers also are defined as primary (unlearned, S^R) or secondary (conditioned, learned, S^r). Primary reinforcers function as effective reinforcers without any prior experience or conditioning. Examples include food, water, and the opportunity to engage in sexual behavior. Conditioned reinforcers are stimuli that *acquire* their reinforcing efficacy through pairing with other established reinforcers. Money serves as an extremely effective generalized conditioned reinforcer because of its pairing with many other reinforcers, both primary and conditioned. Conditioned reinforcers frequently are generated in an experimental setting by repeatedly pairing an initially neutral stimulus, such as a light flash or tone, with a primary reinforcer, such as food delivery. After repeated pairings, the light stimulus acquires conditioned reinforcing properties of its own and can thereby maintain substantial operant responding itself. To retain its reinforcing efficacy, however, the conditioned reinforcer must be paired at least occasionally with the primary reinforcer.

TABLE 38.1
Consequences of Responding

Stimulus	Change in Response Strength	
Conditions	**Increased**	**Decreased**
Presentation	Positive reinforcement	Punishment
Withdrawal	Negative reinforcement	Extinction

Punishment and Extinction

Two operant procedures that *decrease* the frequency of learned responses are punishment and extinction. Table 38.1 summarizes these terms, with respect both to the procedures involved and to the subsequent behavioral outcome. In *punishment*, a stimulus presented after a response decreases the frequency of that response. Scolding a child who has drawn on the wall may constitute an example. Again, the stimulus must be classified only on the basis of the subsequent change in response frequency; if a promptly delivered scolding fails to decrease the frequency of writing on the wall, then by definition, it was not an effective punishing stimulus. In a clinical context, the presentation of ammonia to the nose contingent on self-abusive behavior often decreases the frequency of this self-injurious response sometimes observed in autistic children. In *extinction*, the reinforcer for a response is withheld and the frequency of the response eventually (usually after an initial increase) declines. Withdrawing social attention from a child who has thrown a temper tantrum will in many cases decrease the future incidence of tantrums, albeit after an initial increase in frequency.

Discriminative Stimuli

A stimulus in the presence of which an operant response is repeatedly reinforced comes to acquire *stimulus control* over the response; in the presence of this and related stimuli, the probability of the response is increased. This stimulus that defines the occasion on which an operant response is followed by reinforcement is called a *discriminative stimulus* (S^D). For example, the sight or smell of freshly baked cookies may be an S^D that sets the occasion for the response of reaching into the cookie jar to be reinforced with a cookie, whereas an empty cookie jar would be unlikely to occasion such a response. Similarly, a red light and a stop sign are both discriminative stimuli that control the responses involved in stopping a vehicle. Braking at other times (e.g., at a green light) may have disastrous consequences. It is important to remember that discriminative stimuli do not elicit or evoke responses as do unconditioned and conditioned stimuli in respondent conditioning procedures; they merely, but importantly, indicate the likelihood or probability of reinforcement for a given operant response and thereby influence response probability.

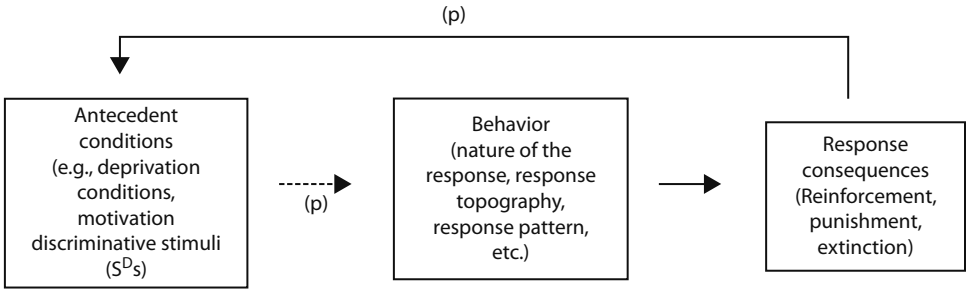

FIGURE 38.2 The three-term, contingency-describing operant behavior. In the presence of antecedent conditions, operant responses are followed by consequences. The nature of the consequence determines the future probability (frequency) of the response and will also alter the strength of the antecedent stimuli (discriminative stimuli) controlling the response. A toxicant may act by altering the antecedent conditions (e.g., functional deprivation level or motivation of the subject) or the efficacy of relevant discriminative stimuli. A toxicant may act by altering the characteristics of the response itself (e.g., its topography or duration). A toxicant may act to alter the response consequences (e.g., change the perceived magnitude of reward or punishment). Understanding how a toxicant interacts with these factors defines the behavioral mechanism of effect.

The response of an organism may be explicitly reinforced in the presence of one stimulus (S^+), such as a red light, while reinforcement is explicitly withheld in the presence of another stimulus, the S^- (or $S\Delta$), such as a green light. Using such a *discrimination procedure*, the organism soon comes to respond in a certain way only when the red light is on (i.e., in the presence of the S^+), such that the behavior is said to be under *stimulus control*, as noted earlier. When responding generalizes to other stimuli of the same or related stimulus classes, such as when a young child refers to all grown males as *daddy*, responding has *generalized* across the stimulus class *adult male*, that is, *stimulus generalization* has occurred. These processes of stimulus control and stimulus generalization are important aspects of such behavioral functions as concept formation and learning to learn.

In summary, a three-term contingency describes operant conditioning, as illustrated in Figure 38.2.[7] In the presence of antecedent conditions that include discriminative stimuli, operant behaviors occur and are followed by consequent events (response consequence) that alter the future probability or frequency of that response and the control by the antecedent S^D over the response. It should be noted that discriminative and reinforcing stimuli need not be external or visible/audible environmental events; internal stimuli, whether internal physiological changes such as headaches or our own nonvocal verbal behavior (thinking), can acquire important behavioral functions, including serving as both discriminative stimuli and conditioned reinforcers.

Precise and operational definitions are critical in behavior because a toxic agent, like a brain lesion, may affect any stage in this three-term contingency. A goal of behavioral toxicology is to understand how toxic agents alter function or, more precisely, the behavioral mechanisms of action of the compound. As discussed by Thompson and Schuster[8] and Thompson and Boren[9] in the context of behavioral pharmacology, attaining such a goal depends on a thorough knowledge of the variables that control behavior, just as the biologist must understand a biological system. Behavior is influenced by multiple factors: by antecedent factors, such as deprivation or motivational state, by the nature of the response and response parameters (i.e., its topography or physical characteristics), and by the consequences that serve to maintain the behavior. A toxicant may change behavior through its interaction with any or all such variables; that is, it may alter antecedent conditions such as functional deprivation levels (e.g., induce nausea), or it may interfere with the ability of the organism to discriminate among stimuli. Alternatively, it could modify the topography of the response, such as by evoking incoordination, or it could interfere with the ability to associate the S^D with the contingency, and so forth. Understanding the exact components of behavioral processes affected by toxic agents assists in understanding the underlying behavioral mechanisms of action and offers guidance as to the associated neurobiological substrates of effect.

SCREENING BATTERIES FOR ANIMAL STUDIES

Screening batteries are used for evaluating the potential neurotoxicity of new or existing chemicals. In animal studies, these have typically included two behavioral components: a functional observational battery (FOB) and motor activity. The behavioral tests utilized for such purposes are often referred to as *apical tests* because they require the integrated function of several organ systems, including the nervous system. Often, a distinction is made between what are deemed naturalistic behaviors, such as those scored on FOBs, and complex learned behaviors. But, all behaviors are naturalistic; none exceeds the bounds of biological possibility. For many naturalistic behaviors, there is simply a lack of understanding of the controlling variables rather than an absence of their sensitivity to environmental control. Even as apparently natural and spontaneous a behavior as self-grooming by monkeys can be brought under experimental control by reinforcing (rewarding) it with food.[10]

From the standpoint of screening and hazard identification, two points related to the interpretation of FOB

and motor activity studies deserve mention. First, effects observed in response to toxicant exposure in such batteries can either be the result of a direct effect of the toxicant on the nervous system or be secondary to changes in other systems, as such apical tests rely on the functional integrity of multiple systems. Under some circumstances, the fact that the toxic effect is ultimately expressed in behavior may minimize the importance of the source of the effect. A second point is that the concurrent presence of body weight loss or decline in food or water intake does not necessarily indicate that behavioral changes observed in an FOB or in locomotor activity are the result of malaise or sickness, as these measures may change independently of each other. Certain agents, such as volatile organic solvents, tend to enhance motor activity at low concentrations, so the problem of confounding with malaise is negligible with such results.[11]

Functional Observational Batteries

Figure 38.3 depicts the component tests of the FOB developed by Moser[3] which include an array of measures of both unconditioned operant and respondent behaviors. Such batteries have been shown to exhibit utility for screening potential neurotoxicity (i.e., hazard identification and elaboration). As an example of validation, it has been shown that components of the FOB directed toward cholinergic functions exhibited sensitivity to the effects of the anticholinesterase carbaryl, whereas few such signs of cholinergic disturbances were evident in the presence of the nonanticholinesterase pesticide chlordimeform. Further discussion of FOB measures by domain is provided by Moser et al.,[3] Baird et al.,[12] and Boucard et al.[13]

- Home-cage and handling
  - Posture
  - Ease of handling
  - Ease of removal
  - Piloerection
  - Vocalizations
- Open field
  - Time to first step
  - Urination, defecation
  - Gait
  - Bizarre behavior
  - Rearing behavior
- Reflex and physiological
  - Approach response
  - Touch response
  - Finger snap response
  - Righting reflex
  - Grip strength
  - Catalepsy
  - Forelimb grip strength

FIGURE 38.3 Typical measures used in many FOBs include aspects of home cage and handling, behavior in an open field, and various reflex and physiological responses. Measurement of locomotor activity is also often included in an FOB. (Modified from Moser, V.C., *Toxicol. Pathol.*, 39, 36, 2011.)

Motor Activity

Motor activity is one component of motor function.[14] Generally described as an unconditioned behavior, motor activity exists at some baseline (operant) level and is a complex behavioral class that includes numerous components such as ambulation, rearing, grooming, and sniffing, all of which can be environmentally influenced. Toxicants may alter motor activity by affecting any or all of its component behaviors. Numerous devices have been designed to measure motor activity. These vary in complexity and in the specific component of motor activity that they measure. One frequently employed device is the figure-8 maze, which consists of a series of interconnected alleys converging on a central open area. Motor activity is detected by photobeams, and an activity count is registered each time a photobeam is interrupted by the animal. In a device such as the open field (Figure 38.4), motor activity is quantified by counting the number of squares entered by the animal within some prescribed period of time. Over the years, such devices have become increasingly automated and generally locate photobeam devices in a manner that permits the detection, in a time course fashion, of different types of motor activity, such as ambulation versus rearing and utilize videotaping and computer-assisted scoring. This is particularly useful as it allows assessment of the time course of activity changes within a session, as reliance on total counts may obscure toxicant-induced differences in the time course of the activity. For example, in an open-field device, one could arrive at the same total number of squares entered in a designated period of time via substantially different patterns of behavior across time. The organism might show an initial period of rapid movement in the open field followed by immobility, or, alternatively, it could exhibit a continuous moderate rate of ambulation. Both patterns could result in the same total

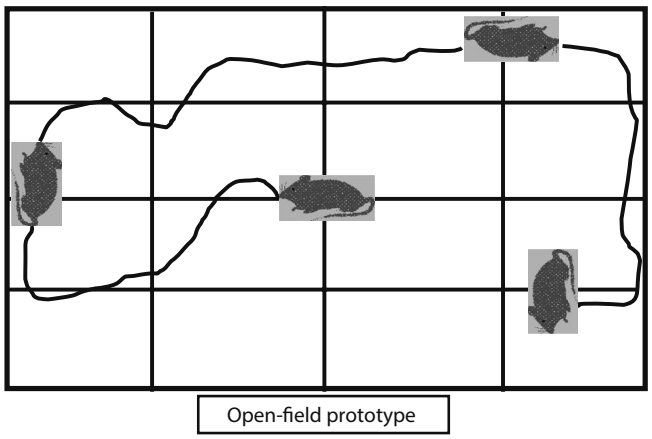

FIGURE 38.4 Open field for the measurement of locomotor activity. In nonautomated procedures, the number of movements per unit time is recorded by an observer. Automated devices typically rely on computerized measurement of photobeam breaks by the movement of the subject to access locomotor activity. Photobeams are typically positioned so as to measure aspects of both horizontal ambulation and rearing in rodents.

number of squares entered, even though the underlying patterns of behavior are quite distinct.

Behavioral Teratology

Although presented as if it were distinct from behavioral toxicity because of its focus on behavioral modifications resulting from toxic exposures during early development, many of the outcome measures of behavioral teratology are the same as those used in behavioral toxicology coupled with the development of physical landmarks and reflexes. Initially, its focus included behavioral changes related to toxicant exposures over the postnatal and possibly into the juvenile and early adult stages of the life cycle. Increasingly, this has extended to later stages of the life cycle based on questions related to the fetal basis of adult disease. Behavioral teratology studies frequently attempt to evaluate multiple behavioral functions, such as motor function and activity and sensory capabilities and learning, in the same experiment. In addition, testing for species-specific behaviors, such as aggression, play, and vocalization, may be included. Table 38.2 lists the criteria specified by the U.S. Environmental Protection Agency (EPA) for its developmental neurotoxicity test guidelines.

What distinguishes behavioral teratology is that testing during the infant, postnatal, and juvenile periods of development sometimes requires modifications of procedures utilized with adults or even the development of new paradigms. In other cases, behavioral paradigms identical to those used in more mature subjects may be employed, albeit with parametric modifications. An example of the former that has been widely used to assess olfactory and motor capabilities early in life is referred to as *homing behavior*, a behavior used by rodent pups to locate the nest should the pup be displaced. In such a test, a rat or mouse pup is placed in the center of a rectangular apparatus in which one side contains clean bedding material and the other side contains bedding from the pup's home cage. The time taken for the pup to orient to or to reach the home cage bedding constitutes the dependent variable of interest. Because this performance depends on memory, olfactory capabilities, and the development of appropriate motor skills, it represents a type of apical evaluation.[15] Other issues related to behavioral teratology, including appropriate fostering procedures to control for toxicant effects on the dam, statistical issues, and summaries of developmental effects of various toxicants, have been reported by others.[16–22]

TABLE 38.2
EPA Developmental Neurotoxicity Criteria

Physical measures	Body weights, sexually dimorphic indices
Brain weights	Regional brain weights on days 4 and 21
Neuropathology	PN days 4, 21; study termination; GFAP
Locomotor activity	N days 13, 17, 21, 45, 60
Reactivity	Auditory startle on PN days 22, 60
Learning and memory	Olfactory discrimination on PN day 21
	Active avoidance (or substitute) on PN day 60

OPERANT BEHAVIOR ASSESSMENT

Scope

The behavioral environment is a dynamic one in which multiple reinforcement processes are operating concurrently to produce, modify, refine, and eliminate aspects of learned operant behavior. The net result is a complex behavioral repertoire that includes a multitude of different behavioral domains and processes, any or all of which may be the target of a toxic agent. Enumerating all such possibilities is beyond the scope of such a chapter and, indeed, constitutes a subject matter having spawned numerous volumes of its own. Consequently, this chapter focuses on seven particular operant behavioral domains that have evolved as primary interest areas in neurotoxicology: motor function, sensory behavior, learning, memory, attention, schedule-controlled behavior, and stimulus and reinforcing properties of toxicants. The techniques and corresponding apparatuses designed to evaluate the entirety of these behavioral domains are numerous and cannot be fully enumerated here. This chapter therefore emphasizes those procedures that are widely used, as well as those that exemplify the range and scope of behavioral technologies.

Apparatus

The basic requirements for an assessment of complex operant behavior must include a defined or specified unit of behavior to serve as the designated response. An environmental consequence or reinforcing event appropriate to the species and experimental parameters is arranged to follow the designated response. For some types of experiments, environmental stimuli of different modalities (e.g., visual, auditory) that can be varied along certain dimensions may be used as discriminative stimuli and conditioned reinforcers. The choice of the operant response, reinforcing consequence, and external or environmental stimuli should be congruent with the physical and behavioral capabilities of the experimental species. Teaching the chimpanzee to vocalize speech sounds proved impossible because of the physical limitations of its vocal apparatus. When the operant response was changed instead to hand signing, experimental studies of the acquisition of verbal behavior proceeded successfully[23] and since have involved computer touch screen responses. Similarly, under certain conditions, visual stimuli may prove inappropriate for rodents, because of the poor visual capabilities of these species, which, instead, depend heavily upon olfactory information in their normal environment.[24]

A broad spectrum of reinforcers has been used in behavioral experiments. The choice depends on the species and the aim of the particular experiment. Reinforcers can include the delivery of food or liquid reinforcers such as water, fruit juice, or saccharin solutions; the delivery of money or tokens (exchangeable for other reinforcers) in the case of humans, the opportunity to engage in wheel running, sexual behavior, or aggressive behavior; the production of heat in a cold environment; the opportunity to self-administer a drug such as cocaine or electrical brain stimulation; and escaping from

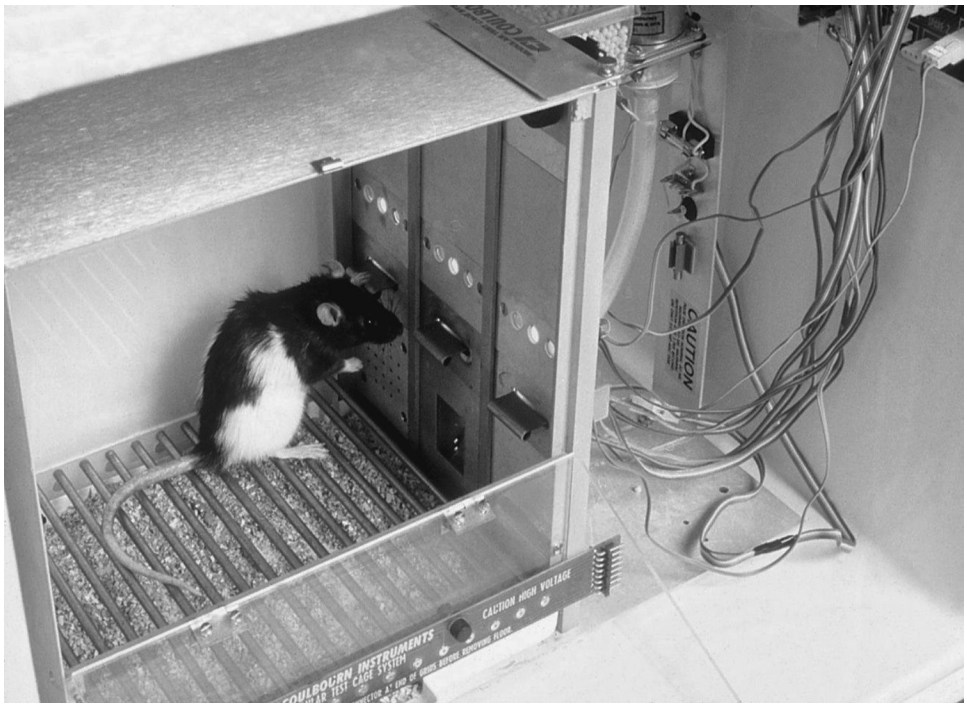

FIGURE 38.5 A prototypical operant chamber (Skinner box) for use with the rat here equipped with three-response levers (left, center, right) and a bank of lights above each lever. Also included are a sonalert for delivering tones, a speaker for the delivery of auditory stimuli (top right), and a houselight for use as a visual stimulus. Food deliveries used as rewards are delivered from a feeder outside of the operant chamber through a plastic tube into a pellet trough, here located below the middle lever. The rat can be seen pressing the left lever.

or avoiding the onset of electric shock. Such a breadth of choice in a standard experimental arrangement allows the experimenter enormous flexibility, such as to compare heat and food reinforcement in metabolically impaired animals.[25]

Many experiments on complex behavior are carried out in operant chambers typified by that illustrated in Figure 38.5. In conventional chambers, response devices typically include levers (rat, mouse, monkey, human), disks (pigeons, monkeys, humans), computer touch screens (monkeys, humans), running wheels (mouse, rat), and cones for snout insertion (mouse, rat, guinea pig). Typically, the execution of the response is operationally defined by the closure of a switch or electrical circuit that can be recorded and acted on by a computer. The operant chamber may contain multiple response devices, usually but not necessarily of the same type. In addition, stimuli of different modalities can be delivered to the chamber by lights, loudspeakers, and such, and the operant chamber may be adapted to utilize different reinforcers. Equally important are the ease and flexibility available to experimentally program behavioral contingencies (rules) in the operant chamber and the precision and resolution with which data can be collected. Computer technology allows responses and other events to be measured and stored sequentially with millisecond resolution. For these reasons, an operant chamber equipped to deliver stimuli of various modalities, containing multiple response devices, and offering a choice of reinforcement delivery systems provides the maximal flexibility and versatility for behavioral studies. It similarly permits a continuum in the pursuit of scientific questions directed by research findings as opposed to research questions that are dictated by the nature of the equipment, particularly in cases where the equipment has been designed to measure only a single specific behavioral function.

Over the years, however, different types of devices, some automated and others not, have been used to measure specific aspects of behavior, some commercially available, others designed to meet the requirements of a particular experiment. This has been the case particularly for the assessment of learning and memory functions, where mazes have been used, including the more traditional mazes such as the T maze and L maze; the radial arm maze, which consists of a central area from which eight or more arms radiate like spokes; and water mazes. The Wisconsin general test apparatus (WGTA) was used to study learning in primates, and modifications have been made to accommodate other experimental species. Nonautomated devices can require a far greater expenditure of personnel time and, ultimately, greater costs to carry out experiments and can also introduce the possibility of experimental bias. Although such devices can certainly provide in some cases a reasonable alternative to an operant chamber, as noted earlier, their generally limited utility for addressing a wide variety of other behavioral questions must also be considered when apparatus decisions are made and where resources are limited.

Shaping an Operant Response

Before implementing a behavioral experiment, an effective reinforcer must be identified, one that can be presented

immediately after the designated response occurs, because delayed reinforcement is less effective. A procedure known as *magazine training* is generally used to ensure the adequacy of the reinforcement contingency in an operant chamber, particularly in experimental studies. Magazine training consists of the presentation, at intermittent intervals, of the reinforcing stimulus, such as a food pellet, independently of behavior. After repeated presentations of food pellets in this manner, a slightly food-deprived organism will reliably approach the feeder and ingest the food whenever the noise generated by the operation of the feeder occurs. A stimulus such as a light is frequently paired with the food delivery and, along with the noise generated by the feeder, comes to serve as a conditioned reinforcer. Such conditioned reinforcers are especially important because they provide the immediate reinforcement for responding given the delay necessarily imposed by the time required for the organism to approach and ingest the food. For some reinforcers, no such training is required. For example, electric shock is used in escape and avoidance procedures in the absence of any prior training of subjects, as is money in human experiments.

Once a reliable reinforcer has been established, shaping of the designated response can proceed. At this point, the reinforcing stimulus is presented only when the organism emits responses that more and more closely resemble the final designated response, a process of reinforcing successive approximations to the desired response. For example, in the case of a lever press response by a rat, reinforcer delivery might first be contingent on touching the lever, then putting two paws on the lever, and eventually applying sufficient force to displace the lever downward and close the associated electric circuit. Reynolds[26] discusses these techniques in greater detail. In some cases, magazine training and shaping procedures can be automated,[27] a major advantage when a large number of experimental subjects must be used, allowing a more high-throughput approach.

Shaping procedures also may be used in other types of apparatus to train the initial response; for example, in the WGTA, the monkey is first shaped to reach out and retrieve food cups on the shelf in front of the enclosures. For other devices, such as mazes and running wheels, little overt shaping may be necessary, as exploring alleys and running usually occur spontaneously at a high enough frequency (high operant level) in rodents to ensure contact with the reinforcement contingency.

Once the organism is emitting the designated response at an adequate rate, it may still be necessary to progress through a series of training conditions before imposing a complex behavioral paradigm of experimental interest, just as a dancer must learn to connect the different steps of a choreographed performance. Likewise, a beginning reader could hardly be expected to tackle the plays of Shakespeare. In a similar way, requiring an organism to emit 100 responses for each food delivery will likely require intervening sessions with smaller response requirements to build response strength and to prevent the behavior from undergoing extinction. It is the shaping process that creates new behaviors and pieces together responses to produce increasingly complex chains of behavior.

An accumulating literature attests to the fact that many of the specific behavioral procedures described here are successfully used with both mice and rats, although differences between the two and between strains of each in operant level behavior may sometimes necessitate alterations in approach. In operant chambers, for example, higher overall activity levels may mean that high levels of lever pressing occur in the absence of any shaping; in this case, additional procedures must be implemented to bring the high rates of behavior under appropriate control of the operant contingencies, such as time-out procedures contingent on excess responding, specific implementation of differential reinforcement for low rates of responding, or the use of response devices such as nose cones rather than levers. Further, many of the behavioral paradigms emphasized in this chapter have been more broadly used across human and nonhuman species with appropriate parametric and response device modifications, an approach that should also facilitate extrapolation in a public health/risk assessment context.

MOTOR FUNCTION

Visible indications of toxic processes in the nervous system often occur as abnormal movements, impaired coordination, slowing of responses, and complaints of weakness. Because any reduction in the capacity for coordinated movement reduces an organism's ability to cope with the demands of its environment, even subtle defects will influence how effectively it functions. Learned motor skills play an especially salient role in human activities. Even apart from the advanced skills of the surgeon or violinist, which are the culmination of years of practice, consider how much we rely on proficiencies in writing and driving as part of our daily activities.

Despite many clear examples, the full scope of contributions to movement disorders by neurotoxic chemicals remains vague. Part of the vagueness arises because the etiology may lie buried many years in the past. For example, understanding whether and how pesticide exposures serve as risk factors for Parkinson's disease,[97] as suggested by experimental models and epidemiological evidence, is hampered by the inability to identify specific past exposures. The emergence of clinical signs may also reflect the diminished ability of compensatory mechanisms during advanced age to overcome the effects of earlier damage—that is, silent damage.[28]

Clear connections between exposure and motoric dysfunction have been established for several metals. Wrist drop afflicted many painters who were occupationally exposed to lead pigments. The cardinal sign of mercury vapor neurotoxicity is excessive tremor. One of the primary signs of methylmercury poisoning is ataxia. Manganese miners display a condition best described as dystonia but with some features of Parkinson's disease. Insecticides are designed as neurotoxicants, and the organophosphorous compounds produce axonopathies that impair both motor and sensory functions. The industrial chemical acrylamide also induces both motor and sensory neuropathies. Certain organic solvents produce central nervous system damage expressed as

motor dysfunction. Many of the chemical classes described in this volume, in fact, even those not classified primarily as neurotoxicants, can induce motor disorders.

The control of posture and movement is anatomically organized in the central nervous system as a collection of diverse motor centers arranged hierarchically from the least integrative, at the level of the spinal cord, through the basal ganglia and cerebellum, to the ultimate level of the cerebral cortex. Weaving through this basic hierarchical structure is a web of enormously complex pathways connecting the various motor centers and involving both afferent and efferent transmission. The total system depends on the functional integrity of many different components, all of which seem to present unique opportunities for the actions of toxic agents. Because they represent a leading clinical problem, the neural basis of movement disorders is the subject of a vast literature.

The basic properties of movement, however, subsume only a few fundamental dimensions described by mass, time, and displacement, expressed in measures such as force, duration, velocity, acceleration, momentum, amplitude, accuracy, and patterning in time. Screening batteries often rely on single global indices, such as spontaneous locomotor activity, which are influenced by many variables independent of motor capacity and do not encompass the full spectrum of motor functions. Some incorporate more specific assays of motor function such as the ability to maintain balance on a rotating rod. Because organisms effect change by producing patterns of muscular contraction, those measures of function likely to prove most sensitive to toxic impairment will typically be based on careful analyses of such patterns and will reflect the integration of multiple systems that yield complex movements.

Learned skills probably offer the most useful baselines for such an assessment because of the flexibility they offer to the experimenter for precise specification of the form of the response. Because of the inherent capacity to compensate for incipient difficulties, however, they are especially challenging to evaluate. For either learned or unlearned motor functions, it seems likely that detection of early subclinical toxic effects will require a detailed quantitative analysis of movement topography.

Response Duration

The time occupied by a particular component of a movement can be studied either as an intrinsic variable, arising indirectly from contingencies applied to another response component such as force, or as an explicit variable specified directly by the experimenter. Especially in the latter instance, the stimuli governing the response are almost wholly proprioceptive, arising from sources such as muscle spindle receptors. Assume a situation in which the required operant response is a lever press and the reinforcement contingency specifies that response durations must exceed *t* seconds to produce reinforcement. If no external stimuli that change systematically with time are presented, the primary stimuli available to the organism with respect to response duration are those arising from muscle and joint receptors. Because such proprioceptors are key elements in the control of movement, measures of response duration could prove useful in evaluating whether these proprioceptors have been damaged by agents such as acrylamide.[29]

Shaping long-duration responses is a fairly straightforward process, as described earlier. Once the designated response, such as lever pressing, has been learned, the additional requirement of maintaining it for a specified duration can be imposed. Early in training, the duration should be short enough that a substantial proportion of responses meet the criterion for reward. The duration, as in all shaping procedures, can then be raised gradually until the final value is attained. Both minimal and maximal durations can be imposed, so a band of durations comes to serve as the criterion. Stevenson and Clayton[30] trained rats to hold down a lever for at least 40 consecutive seconds, after which a white noise signal was sounded. Releasing the bar in the presence of the noise turned it off and triggered a feeder in the operant chamber to deliver a pellet of food.

Relatively few experiments with neurotoxic agents have sought to exploit such possibilities. Cory-Slechta et al.[31] trained rats to respond on a schedule that reinforced only durations above a specified minimum value. After preliminary training, during which the rats were first trained to press a lever for food reinforcement, the experimenters imposed a schedule based on differential reinforcement of response duration. Each lever press that exceeded a specified duration was followed by food pellet delivery. These durations ranged from 0.5 s at the beginning to 6.0 s during later training. Lead treatment reduced durations and also expanded within-group variability.

Several experiments with dogs have relied on response duration as the primary measure of performance. In one, dogs were trained to press a button with their snouts for a food reward.[32] The schedule did not specify response duration directly; instead, it specified that reinforcement would be delivered when 60 s of responding had been accumulated. The dogs adjusted to this contingency by pressing the button between 10 and 20 times for each reinforcement; short responses predominated. Response durations were longer under control conditions than after amphetamine, pentobarbital, or ethanol. In later experiments,[33,34] response duration was made the criterion for a titration schedule, that is, the duration required for reinforcement changed in accordance with the dog's performance. Each session began with a specified minimum duration of 0.25 s. Each time the dog (this time pressing its snout against a panel) ended a response that exceeded the current minimum, the required duration was raised to a higher level. In one variant of the program, the criterion also fell after a series of unsuccessful responses. The dogs learned the progression and, over the course of each session, emitted longer and longer responses. Amphetamine reduced this rate of rise, and alpha-methyl-para-tyrosine (α-MPT), a tyrosine hydroxylase inhibitor, lengthened it, even at doses as small as 3.12 mg/kg (Figure 38.6).

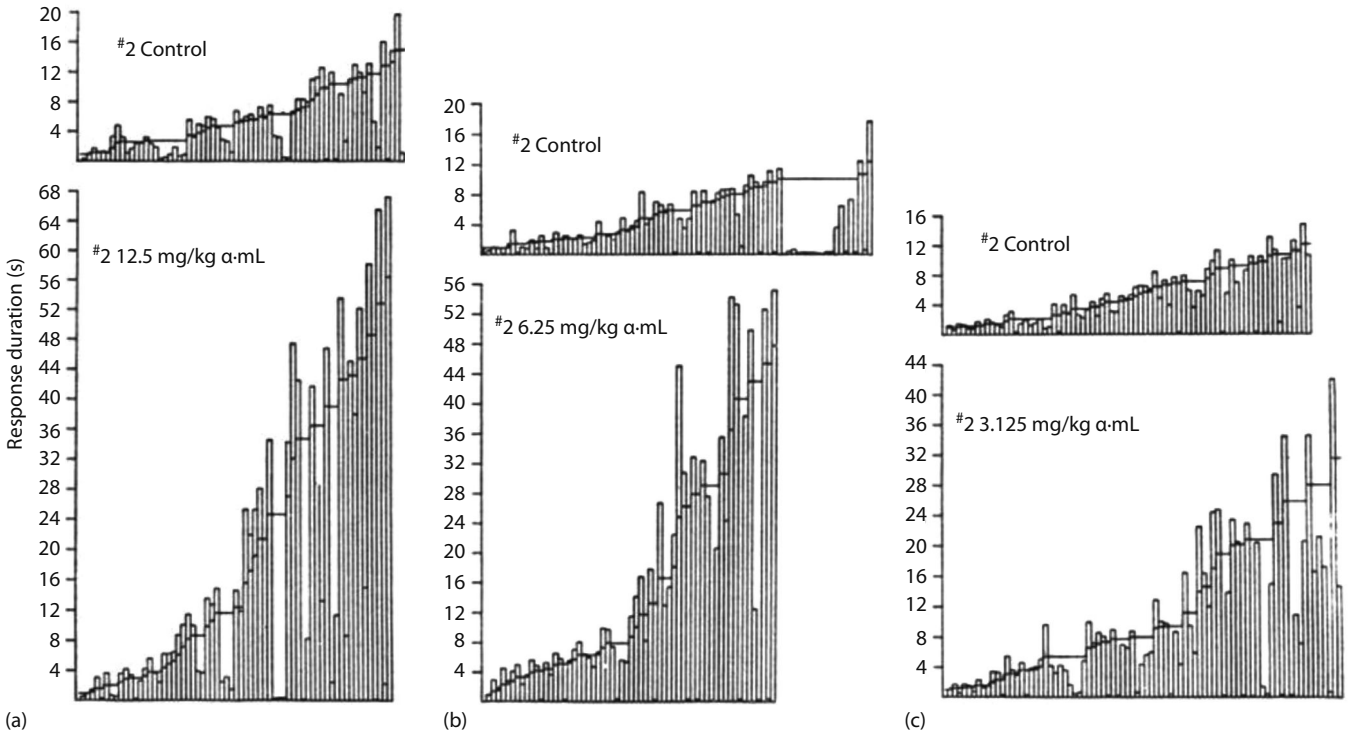

FIGURE 38.6 Complex duration discrimination in Basenji dogs. The dogs were trained to press a panel with their snouts. An experimental session began with a predetermined duration criterion of 0.25 s. Any response duration above the criterion produced delivery of reinforcement (dry dog food) and elevated the criterion by 25% of the difference between the old criterion and the new duration that had exceeded it. This proportion was chosen empirically. If the criterion were raised too quickly, the behavior would be lost, as it often is in shaping procedures that advance so rapidly that they lose contact with the behavior. Each panel shows response durations (seconds) on the *y*-axis in relation to session time on the *x*-axis. Under control conditions, the dogs learned to produce longer and longer durations through the session. The compound α-methyltyrosine, which inhibits the rate-limiting step in the synthesis of catecholamines, enhanced the rate at which successive response durations rose, even at doses many times lower than those used in neuropharmacology experiments. (Adapted from Weiss, B., Amphetamine and the temporal structure of behavior, in Costa, E. and Garratini, S., eds., *International Symposium on Amphetamine and Related Compounds*, Raven Press, New York, 1970, pp. 797–812; Weiss, B., Microproperties of operant behavior as aspects of toxicity, in Bradshaw, C.M., ed., *Recent Developments in the Quantification of Steady-State Operant Behavior*, Elsevier, Amsterdam, the Netherlands, 1981, pp. 249–265.)

Precision

In the absence of clear visual cues, movement precision is also guided largely by proprioceptive information. As an example, Falk and associates[35] trained rats to exert forces within a specified range (15–20 g) for a duration of 1.5 s. The rats responded on a lever that transmitted an electrical signal proportional to the applied force and were reinforced by food pellets for responses meeting the joint force–duration criterion. Training was carried out by approaching the joint criteria of force and duration in small increments and required only 5- to 11-h-long training sessions. With this system, Falk demonstrated that several common central nervous system drugs exerted unique effects on this form of discriminative motor control; for example, the relative amount of time spent within the specified force band varied with the dose of amphetamine and pentobarbital, declining as dose was raised.

A similar device, adapted for monkeys, enabled Preston et al.[36] to study deficits in fine motor control produced by various drugs. One side of a standard metal cage was modified with the addition of a Plexiglas® tube through which the monkey could extend its arm and make contact with a conically shaped response device connected to a force transducer. The monkeys were trained to emit forces between 25 and 40 g for a continuous 3 s to obtain small quantities of water. Various indices of performance demonstrated that the acute effects of methamphetamine, similar to the effects described earlier for rats, impaired performance after repeated high-dose treatment, the pattern of intake adopted by amphetamine abusers.

In another adaptation of this system,[37] monkeys were trained to insert a paw through a slot and to touch a Lucite® plate connected to a strain gauge. The strain gauge output was transmitted to an amplifier, the output of which was coupled to the analog-to-digital converter inputs of a digital computer. The analog-to-digital converter transforms the continuously varying electrical signal from the amplifier into a form that can be processed by the computer. The computer was used to specify upper and lower bounds of the force that defined the response. With this system, the gradual

degradation of fine motor control produced by methylmercury could be traced. The typical pattern before treatment consisted of a precise emission of force within the 25–40 g range specified as the response criterion, maintenance in that range for the required 2 s, and then release. After treatment, the first sign of coordination difficulty was overshoot at the beginning of the response—that is, a transient force above the specified ceiling. This early indication of impaired motor control was followed, after further dosing, by an increasing inability to maintain the force within the prescribed limits. Elsner[38] devised a somewhat similar system to study motor performance in rats, aiming to construct a model reflecting some of the characteristics of attention deficit disorder.

Because rodents remain the most widely used species for routine toxicological testing, additional techniques for the measurement of movement precision in rats and mice, in addition to those mentioned earlier, are especially appealing. All require some investment of time or instrumentation but can yield critical information about the dimensions of motor control. Lesion experiments designed to clarify the functional domain of certain brain areas are a useful source of approaches, and Newland[39] has compiled a table showing which basic tests have shown motor effects of lesions in cerebral cortex, basal ganglia, cerebellum, peripheral nerves, and the neuromuscular junction. In a combination of operant and observational methods, Whishaw et al.[40] examined the impact of motor cortex lesions in rats on their method of grasping food pellets. Before surgery, the rats were reduced to 90% of their initial body weight and trained to eat in a special filming box designed to capture their movements from various perspectives. Video records then were analyzed by a dance notation system adapted to describe animal motor behavior.[41] Such an approach, applied frame by frame, required considerable patience but revealed a spectrum of impairments that were not grossly obvious. A later study[42] used the technique to evaluate motor skills required to reach for food located on a shelf and to manipulate and eat pieces of pasta. Both the pyramidal tract and the red nucleus are also involved in skilled movements, but lesions in these sites suggested a greater role for the pyramidal tract in guiding limb movements.

Another technique designed to evaluate forelimb motor impairment was described by Schrimsher and Reier[43] in an evaluation of cervical spinal cord injury. Rats were trained in a special apparatus to reach into a recessed tray to retrieve a food pellet. Training occurred 2 weeks after body weight had stabilized on a food deprivation schedule. Videotape recordings provided the raw data for analysis. Hypometria turned out to be the primary disability, and it remained permanent in most of the rats. Note that automation of procedures such as the two just discussed, with the potential to yield more accessible and quantitative data, requires little additional training of the animals.[41]

Another situation devised for testing skilled movement in rats is the staircase test,[44] in which rats reach down from a central platform to retrieve food pellets located on each step of two adjacent staircases. The pellets are placed on the staircase and presented bilaterally at seven graded stages of reaching difficulty to provide measures of side bias, maximum forelimb extension, and grasping skill for each paw. Performance is measured by the number of food pellets obtained.

Tremor

Excessive limb tremor accompanies many neurological disorders, is a product of many drug treatments, and can result from exposure to many different classes of toxicants.[45] Tremor is associated with exposure to metals such as mercury and manganese; to insecticides such as chlordecone, dieldrin, and the organophosphates; and to solvents such as carbon disulfide. In a survey, Anger[46] noted tremor as a response to 177 chemicals or chemical classes. Newland[47] describes some of the characteristics of abnormal tremor associated with lesions or chemical exposures. Because of its pervasiveness as a marker of nervous system dysfunction, many techniques have been developed to measure tremor. The availability of inexpensive digital computers and appropriate programs makes the task of recording and analyzing tremor much simpler now than in the past. Because abnormal tremor is a marker for many different neurological syndromes and can be induced by damage at many sites in the nervous system, experimenters have to be wary of ascribing too much specificity to it.

Tremor is one of the cardinal signs of excessive mercury vapor exposure. Wood et al.[48] studied several women exposed to vapor in a factory workroom devoted to pipette calibration. When first seen clinically, the women exhibited visible tremor. After a prolonged absence from the factory, the pathological tremor faded. To follow the course of recovery, and especially to relate it to diminished blood mercury levels, a system was devised to quantify tremor using a strain gauge to which was attached a Lucite slot in which the patient placed her finger. She was instructed to maintain a force within a range designated by lights to mark the upper (40 g) and lower (10 g) bounds. The output of the strain gauge was transmitted to the analog-to-digital converter of a digital computer for processing to express the continuously varying electrical signal corresponding to the output of the strain gauge in the form of tremor frequency. Because physiological tremor is composed of many different frequencies, the analysis is designed to yield their relative contributions to the total signal. The algorithm by which this analysis is performed is called a *fast Fourier transform*.

Fast Fourier transforms demonstrated two features of the tremor induced by mercury vapor exposure (Figure 38.7). First, as expected, the total amount of tremor was greater than any seen with normal subjects. Second, and unexpected, the distribution of the relative amounts attributable to different frequencies showed multiple modes, indicating a very complex signal whose component could not be seen clinically. Eventually, the amplitude of the tremor returned to normal levels, and at the same time, the multiple modes collapsed into one dominant peak. Later, Langolf et al.[49] used a similar technique to monitor workers in a chloralkali

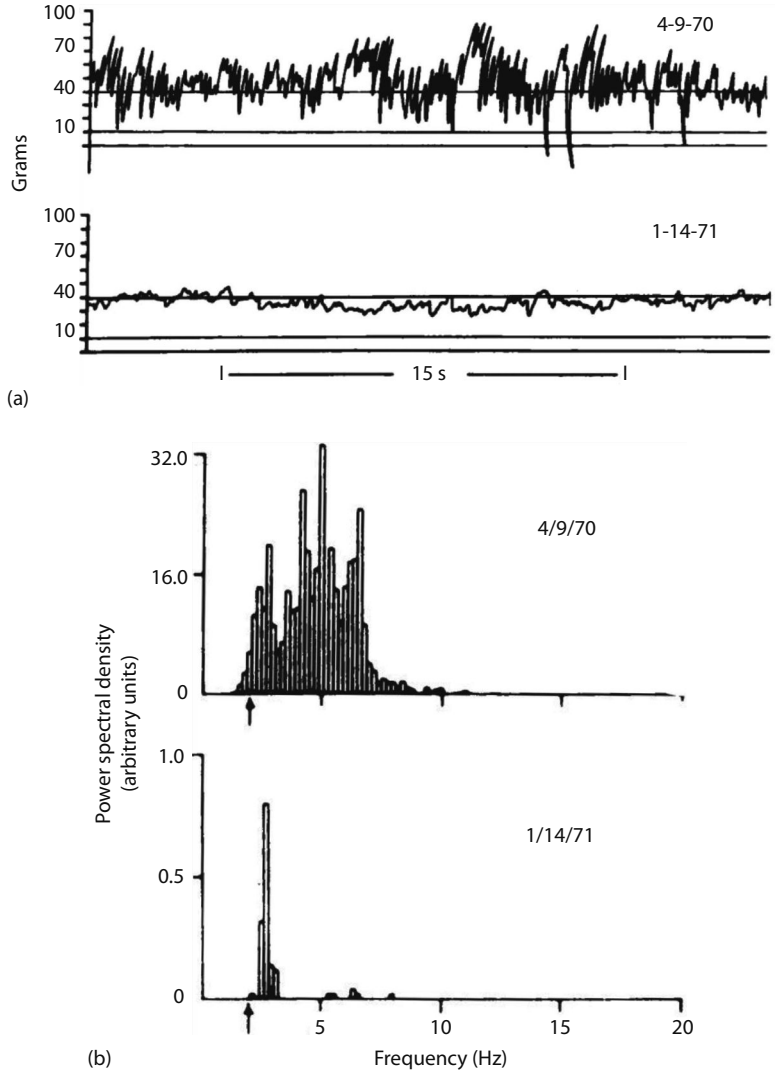

FIGURE 38.7 (a) Tremor tracings from a female worker chronically exposed to mercury vapor in a factory area devoted to pipette calibration. The worker rested a forefinger in a Lucite® slot attached to a strain gauge and was requested to maintain a force between 10 and 40 g, as signaled by lights. Strain-gauge output was amplified and transmitted to a digital computer for analysis. The upper tracing was recorded shortly after the worker entered the hospital for treatment. The lower tracing was recorded 9 months later, with no intervening workplace exposure and a marked decrease of mercury blood levels. (b) Power spectral density plots of tremor corresponding to racings shown in (a). These plots show the amount of total power (variance) contributed by each component frequency in the tremor spectrum and were calculated by fast Fourier analysis. They emphasize the feature of tremor, such as multiple modes, that cannot be evaluated by ordinary clinical examination. (From Wood, R.W. et al., *Arch. Environ. Health*, 26, 249, 1973. With permission.)

processing plant who were exposed to mercury from the massive electrodes used in such plants. They found that as urine mercury rose, the distribution of tremor frequencies began to show multiple modes. With removal from exposure and a parallel decline in urinary concentrations of mercury, the secondary modes declined.

Several later studies have confirmed the usefulness of this method, called *power spectral analysis*, for monitoring workers exposed to mercury. Chapman et al.[50] compared battery workers exposed to mercury with controls by instructing them to maintain a steady force on a displacement transducer. All of the battery workers were judged asymptomatic after interviews and clinical examinations; however, differences in power spectra were able to separate exposed from control workers. One distinguishing feature consisted of peak frequencies in the spectrum. The exposed workers generally showed a displacement toward the higher frequencies. Chapman et al.[50] noted similar findings in workers exposed to carbon disulfide in the grain industry and concluded that frequency differences more effectively indicate neurotoxicity than amplitude differences. The investigators noted that such subtle changes in tremor characteristics are not apparent in clinical examinations and are seen at relatively low exposure levels.

As the preceding examples illustrate, tremor measures may be sensitive to disturbances of motor function that are not clinically apparent; however, as Beuter and de Geoffroy[51] emphasize, the data yielded by tremor measurement techniques depend on the apparatus, the procedure, and the data analysis.

Studies of manganese-exposed workers by Roels et al.[52] tried to assess tremor by using a device that required the subject to hold a stylus in a hole without touching the sides. Small-diameter holes offer a greater challenge than large-diameter holes. Each contact is recorded as the completion of an electrical circuit. Workers with higher urinary values of manganese produced a greater incidence of contacts. A much more extensive test battery was applied to another population of workers exposed to manganese[53]; these data also indicate subclinical deficits in such populations. Both of these studies suggest that the methods used by Newland et al.[54] to study manganese neurotoxicity in monkeys might be directed to human assessment.

Strength

Complaints of weakness appear after exposure to acetylcholinesterase inhibitors, manganese, and other agents. In fact, weakness is one of the most frequent subjective indices of neurotoxicity. Although simple procedures for assessing strength in rodents, such as forcing them to pull against a spring (grip strength), have been devised, complex learned performance offers more direct answers to the kinds of questions likely to reflect human complaints or observations, such as the relationship between blood pressure and effort and greater sensitivity to subclinical effects.[55] With an apparatus similar to a rowing machine, requiring simultaneous applications of force by the legs as well as the arms (Figure 38.8), Newland and Weiss[56] found that low doses of *d*-amphetamine and L-dopa reduced rates when the behavior was maintained by multiple fixed-ratio (FR)/fixed-interval (FI) schedules. Domperidone, a peripheral dopamine-blocking agent, counteracted the effects of L-dopa, suggesting that at least some of these effects arose peripherally. These techniques are readily adaptable to questions about complaints about weakness and fatigue arising from other agents and, because they are based on apparatus developed for human physical conditioning, could play a role in human toxic assessments as well.

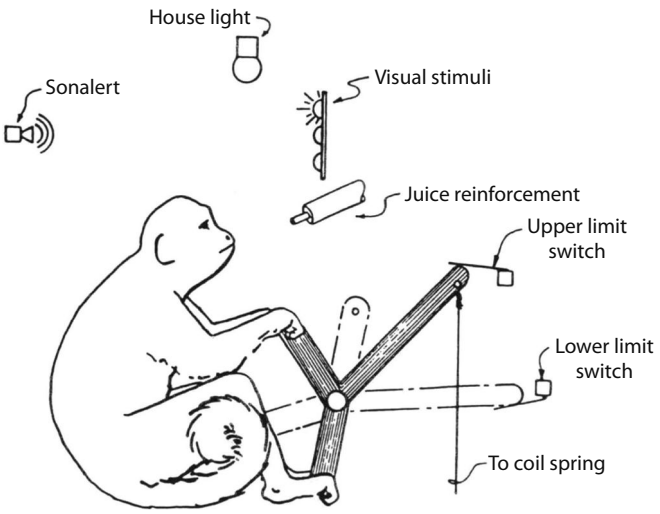

FIGURE 38.8 Apparatus for testing strength and endurance. As in rowing, the monkey pulled the lever with its arms while simultaneously thrusting with its legs. A complete response was defined in three steps: (1) beginning from the home position by closing the lower limit switch, (2) closing the upper limit switch, and (3) opening the lower limit switch. To meet these criteria, the monkey had to move the response device through an arc length of 10 cm against a 40 N (4 kg) circular spring. A brief tone followed each complete response. The visual stimulus panel indicated the schedule component in effect. (From Newland, M.C. and Weiss, B., *Pharmacol. Biochem. Behav.*, 36, 381, 1990. With permission.)

Lead at high doses, such as observed in painters exposed to lead pigments, produces neuropathies such as the syndrome of wrist drop due to radial nerve damage. Newland et al.[57] showed that prenatal lead exposure, at levels (21–760 μg/dL) insufficient to induce overt motor dysfunction, can cause deficiencies in strength. Squirrel monkey subjects, at 3–7 years of age, were trained to pull a T-shaped bar against a 1 kg spring through a distance of 1 cm. On FR schedules, which tend to evoke high rates of responding, the lead-exposed monkeys showed a higher incidence of incomplete responses than control monkeys. The authors concluded that in utero lead exposure at these levels produces subtle impairments in motor function detectable years after birth.

Studies such as those just described indicate how corresponding questions in toxicology may be approached. In its advanced stages, manganese intoxication results in signs sometimes interpreted as Parkinsonism but, as argued by Barbeau,[58] more closely corresponds to dystonia. In its earlier stages, feelings of weakness and excessive fatigue predominate. As with many questions of neurotoxicity, tracking its progression provides the key to identification of underlying neural substrates and appropriate measures on which to base quantitative risk estimates. The exercise device described earlier[56] offers such a means to track progression.[59] After training, monkeys were followed during a sequence of manganese or vehicle treatments. Performance measures included number of missed or incomplete responses, response durations, and interresponse times (IRTs). Figure 38.9, depicting a history of more than 400 days, shows the effect on one index: number of misses or failures to pull through the complete required arc. After the first manganese treatment (10 mg/kg i.v.), the frequency of misses on the FR component jumped sharply. Although this index occasionally drifted downward, it never returned to a pretreatment baseline. Additional manganese treatments produced further variability. Overt signs, such as tremor and dystonias, appeared late in the course of the treatment. Even with the cumulative dosing regimen adopted for this experiment, total exposure amounted to a small fraction of the doses reported by Suzuki et al.[60] to induce overt signs of manganese toxicity.

Gait, Balance, and Coordination

Some measures used to evaluate gait and balance have been described, such as the quantification of Parkinson's disease outcomes in clinical studies and their analogs in the animal laboratory—that is, studies of changes in the walking patterns of rats treated with organophosphate compounds[61] or the kind of kinematic analysis undertaken by Cohen and

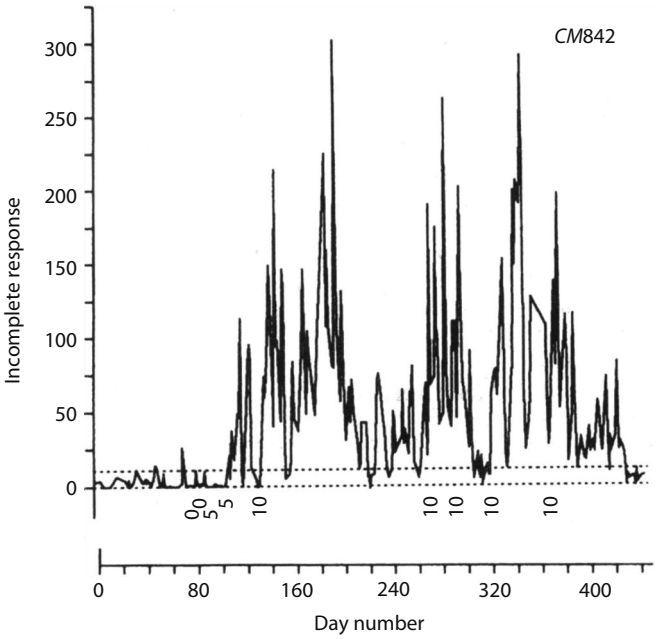

FIGURE 38.9 Performance of a Cebus monkey on a device shown in Figure 38.8 during a course of manganese treatments. Incomplete responses designate those defaulting on the criteria, typically because of failure to operate the upper limit switch. The performance charted was maintained by the FR 20 component of the FR FI multiple schedule of reinforcement. Manganese chloride was administered intravenously in doses of 5 or 10 mg/kg at times indicated on the baseline; saline infusions are represented by zeros. Dotted lines show the 5th and 95th percentiles calculated from baseline sessions. (From Newland, M.C. and Weiss, B., *Toxicol. Appl. Pharmacol.*, 113, 87, 1992. With permission.)

Gans[62] to describe the patterns of rat locomotion in running wheels. Kulig and Lammers[63] reviewed a wide range of techniques currently in use to investigate motor dysfunction. These techniques and approaches have now become increasingly refined and automated through videotaping and computer-assisted scoring and considerations for use described.[64]

A procedure frequently used for assessment of coordination, endurance, and balance is the rotarod. A mouse or rat is placed upon a rotating cylinder, the speed and rate of acceleration of which can be manipulated; the time the animal stays upon the device is measured. Albeit simple and shown to be affected by various neurotoxicants, this procedure can also be associated with significant behavioral problems, including animals who simply learn to engage in behaviors incompatible with staying on the device and which can confound the interpretation of any shortened time staying on the device.[65]

Wolthuis and Vanwersch[61] designed an ingenious procedure for recording and analyzing coordination deficits in rats. A traditional running wheel was modified with the addition of flanges spaced at 30° intervals around the interior surface. The wheel was driven at specified rates by a motor. The ability of rats to step from flange to flange as the wheel rotated was recorded on a videotape, and the analysis was performed by automated measures of the placement of the hind feet, which had been coded by color so appropriate software could be used to measure positions in the video image. With this system, the experimenters could demonstrate that organophosphate treatment produced coordination deficits not readily detectable by common early screening techniques such as the rotarod and hind limb foot splay.

Running wheels also served Cohen and Gans[62] in studies aimed at providing a detailed analysis of rat locomotion, given that the motor activity of rats is frequently monitored to determine the effects of physiological and behavioral variables. Adult male rats were trained to run in an activity wheel. Photoflood lights provided the S^D for running, and mild electrical stimuli delivered through an electrode provided a source of aversive reinforcement to encourage running. Electrodes implanted in forelimb muscles allowed the investigators to monitor muscle activity at the same time movement was being filmed. In an analysis of this functional morphology, running could be shown to represent a *complex and highly adaptable grouping of motor sequences*. The rat's forelimb was shown to act as a steering, propulsive, and supportive device with complicated temporal relationships defining their joint actions. Studies such as these underscore the fact that simple locomotor activity, which has been adopted widely as a component of screening batteries for neurotoxicology, or assays such as hind limb foot splay offer a misleading, perhaps even deceptive, simplicity and sometimes a lack of sensitivity with respect to motor function deficits. Moreover, these earlier studies have led to increasingly automated and commercially available means for acquiring such data.

One of the most easily recognized adverse effects of ethanol is incoordination. Newland and Weiss[66] arranged a situation to try to quantify some of the less overt consequences of ethanol consumption (Figure 38.10). They trained squirrel monkeys to grip a rod attached to the hub of a rotary transformer whose output voltage was proportional to angular displacement. The monkeys received juice reinforcement for maintaining the rod within 15° of horizontal for 8 s. The output voltage was sampled for 5.12 s, and tremor was measured using power spectral analysis.[47] Ethanol greatly reduced the amplitude of tremor, even at doses of 0.25 g/kg. It also tended to flatten the spectral distributions.

Simple tests of motor function almost always are included in screening batteries for humans. One of the most common is finger-tapping rate. Subjects or patients are asked to tap a key or button as rapidly as possible during a test period of several seconds. Both the preferred and alternate hands are usually tested. Chaffin and Miller[67] included both finger and toe tapping in a test battery applied to chloralkali workers exposed to metallic mercury and were able to show that performance correlated with exposure. In a more complex version of the tapping task, the subjects tapped between two copper plates with a stylus connected to a timer so that the intervals between taps could be recorded.[68] In addition, variations in difficulty were introduced by changing the size of or distance between the plates. With a similar technique, Sanes et al.[69] found slower rates in patients with Parkinson's disease than in controls.

Tests developed originally for assessing manual dexterity in workers also have been adopted for human behavioral

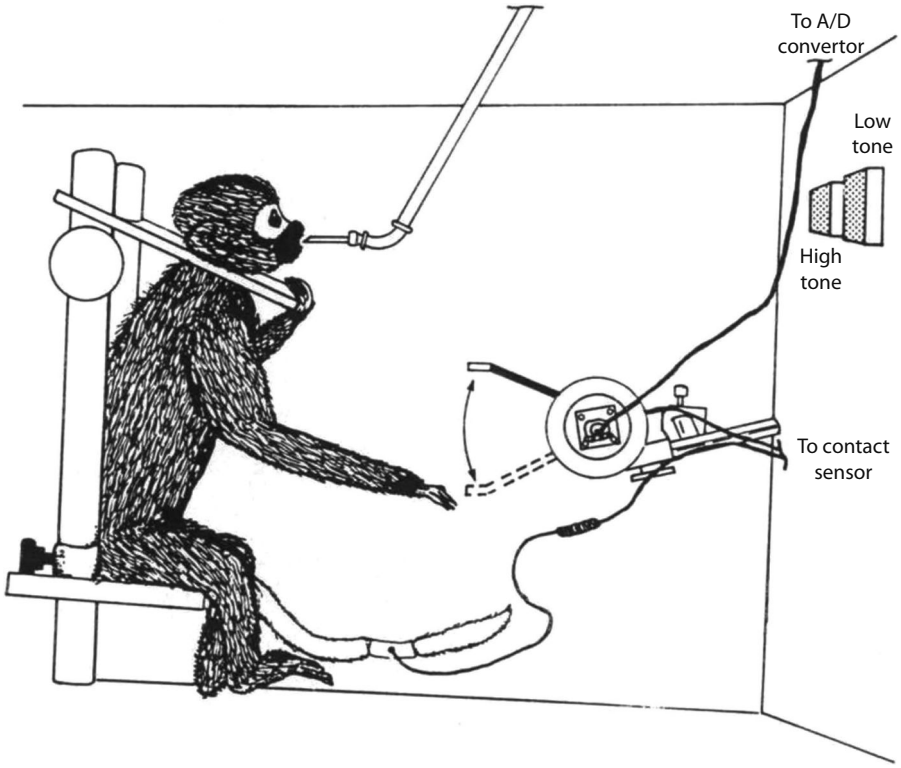

FIGURE 38.10 Depiction of a squirrel monkey (*Saimiri sciurea*) responding on a tremor assessment apparatus. The bar was insulated everywhere except the handle to limit response topography. A contact sensor circuit between the bar and the monkey's tail closed when the bar was gripped. The position of the bar provided a continuous electrical signal that was transformed into a digital code by the analog–digital converter input of the computer that controlled the experimental contingencies. When the bar was held in range, a tone sounded. If held for a sufficient duration, a high-frequency tone burst sounded; on a random ration 2 schedule of reinforcement, a pulse of fruit juice was delivered to the monkey. (From Newland, M.C. and Weiss, B., *J. Stud. Alcohol*, 52, 492, 1991. With permission.)

toxicology studies. The Santa Ana test requires the subject to remove pegs from holes in a board, turn them 180°, and reinsert them. The number rotated correctly within a specified time is taken as the score. It is similar to a test known as the *grooved pegboard*. Hanninen[70] first used the test in a study of workers exposed to carbon disulfide and then included it in the screening battery developed at the Finnish National Institute of Occupational Health. The Purdue pegboard, also developed to assess manual dexterity in factory workers, requires subjects to place pins in a series of holes, but they can then be asked to place collars or washers on the pins. Baker et al.[71] included it in a test battery and noted that it is one of the tests deemed acceptable for occupational studies by a World Health Organization (WHO)–National Institute for Occupational Safety and Health (NIOSH) expert committee.

SENSORY FUNCTION

Almost all behavioral processes depend ultimately on an organism receiving information from its environment. Many behavioral deficits can be traced to disturbances in the way this information is received or processed. Moreover, sensory systems seem to be special targets of certain agents. Acrylamide and methylmercury toxicity are characterized during their earliest stages by loss of sensitivity to touch. Both agents also damage the visual system, although by different mechanisms. A large number of agents, in fact, impair visual function, sometimes so subtly that human victims may be unaware of the deficit. Individuals with defective color vision or areas of scotoma often are detected only on clinical examination. Hearing is degraded by toluene, lead, methylmercury, salicylates, certain antibiotics and diuretics, and noise. Cadmium exposure and chronic solvent exposure have been reported to impair the sense of smell. Behavioral assessments of sensory function can be especially useful because they provide an integrated evaluation of the entire system, starting at the receptors and progressing through the intermediate to the final processing stages of the central nervous system. Furthermore, it may prove misleading to rely mainly on histopathology as the primary index of sensory system toxicity. The intactness of the visual system, for example, cannot be judged simply by examining the retina with a fundoscope or by the gross histological appearance of the visual pathways. The ability to discriminate colors requires a verbal response in humans and some form of motor response in trained animals.

Psychophysics

Psychophysics is a branch of psychology that studies the relationships between sensory stimuli and behavior.[72] One of its

central themes is the concept of a sensory threshold, or the limits of sensitivity for a particular sensory modality, defined as the minimum energy necessary to produce a sensation. Even with the trained observers who typically served as subjects in such experiments in early years, variation from trial to trial in sensitivity was apparent, leading to the development of procedures to cope with these fluctuations and to obtain precise estimates of thresholds. These methods have been refined and expanded since their introduction and have broad applicability across species.

A crucial ingredient of sensory testing is precise specification of the stimulus and its properties. Such descriptions are often lacking in much of behavioral toxicology, as in the reaction time literature discussed later in this chapter. Light and sound stimuli are used without any apparent reports of their parameters, such as brightness and loudness, despite evidence, extending to the beginning of the century, that reaction time latencies are related directly to stimulus amplitude. Many of the devices marketed for sensory testing are also deficient; rather than providing direct measures of stimulus qualities such as vibratory stimulus amplitude, they simply use a dial setting on a potentiometer, say, for specification.

The behavioral measurement of sensory function in laboratory animals also embraces a long history and was one of the earliest topics addressed by experimental psychologists and physiologists, including Pavlov. The development of operant behavior technology contributed major advances to the precision with which sensory function could be determined and to a marked elevation in the status of animal psychophysics. As Stebbins[73] noted, "Training and testing procedures based on the principles of operant conditioning have shown that animals can report on their sensory capabilities in as precise and reliable a fashion as humans." The basic technique in animal psychophysics is to reinforce responses to specified physical characteristics of the stimulus or stimuli. These stimuli serve no eliciting role. They exert control over behavior because they are associated with a particular history of reinforcement. This feature is of special relevance for extrapolation to humans, as many measurement techniques are applied to animals and humans with equal facility. All psychophysical experiments whether conducted with animals or humans are based on a discriminative response by the subject, lever presses and verbal responses happen to be functionally equivalent, a threshold is a statistical concept, it does not imply an absolute physical limit, and its value depends on the methods used to estimate it.

Psychophysical Methods

In most instances, the aim of psychophysical testing is to derive a threshold. During the extensive history of psychophysics, many procedures were developed for the collection of data (Tables 38.3 and 38.4). Most of these have been effectively translated into procedures that can be used with animals. As noted earlier, thresholds are not absolute values but statistical functions of the techniques used to derive them. For example, if the subject is called on to designate the presence or absence of a stimulus, the threshold is typically defined as the stimulus value at which it is detected on 50% of the trials. If the subject is required to discriminate a variable stimulus from a standard, the threshold is typically defined as the magnitude of the variable stimulus that can be differentiated from the standard on 75% of the presentations.

TABLE 38.3
Basic Psychophysical Methods

Method of limits	Series of stimulus intensities ascend and descend from above and below thresholds, respectively.
Method of constant stimuli	Equally spaced stimulus values are presented in random sequence.
Method of adjustment	Stimulus intensity is varied by the observer to exceed and dip below detection limits.
Adaptive methods	Stimulus intensity rises with failure of detection and falls with correct detection.

TABLE 38.4
Response Requirements for Psychophysical Assays

Forced-choice procedures	Subject must choose among alternatives.
Yes–no procedures	Subject responds if stimulus is detected and refrains if it is not.
Rating procedures	Subject reports likelihood of presence or absence using rating scale.

The traditional *method of constant stimuli* presents the observer with between five and nine different stimulus values, each tested repeatedly in a random sequence. The proportion of *yes* responses (indicating that the observer detected a stimulus event) to each stimulus intensity is calculated, a function drawn, and the threshold usually taken as the intensity yielding a 50% incidence of such responses. The correspondence with dose–response functions and their analysis is obvious. For animals and nonverbal humans, such as the mentally retarded, the *yes* response is converted into an action such as a lever press. Even for capable adults, actions such as key presses or computer touch screen responses are now preferred because they are compatible with computer-automated stimulus presentation and response recording. A modification of these traditional procedures known as a *forced-choice paradigm* requires the subject to choose between two stimuli presented either consecutively or simultaneously and so has the advantage of less ambiguity about whether or not a stimulus has been presented, thus controlling for attempts at guessing. As noted earlier, the threshold in this situation is calculated as the stimulus magnitude corresponding to 75% correct detections, because 50% incidence corresponds to chance. In the *method of limits*, the observer is presented with a series of stimulus intensities that begin either well above or well below the presumed threshold. The limits of detection are approached in steps of either diminishing or increasing stimulus values. With alternating descending and

ascending series of stimulus values, the transition intensities between *yes* and *no* responses in both directions become the value from which a threshold value is computed. An ascending series, for example, begins with a stimulus intensity well below the limits of detection. After each trial on which the observer fails to report detection, the intensity of the next stimulus is raised by a prescribed amount. Once the observer reports detection, the series is ended and the intensity recorded. A descending series is conducted in the same way, except that it begins with a stimulus intensity well above the detection limit. Thresholds are typically calculated as the average value or midpoint at which a transition between detection success and failure occurs.

In one variation of the method of limits, called variously the *up-and-down*, *staircase*, or *titration* method, stimulus intensity is modulated in accordance with the responses of the subject, so the threshold can be tracked continuously. A correct detection lowers the next stimulus amplitude, and an incorrect response raises it. This is the most efficient system for calculating thresholds and was a clever variation introduced into audiometry by von Békesy.[74] Because titration procedures are designed so the amplitude of the stimulus is governed by the behavior of the subject, they proved especially suitable for studies of analgesia,[75] providing a means for tracing the impact over time of drugs such as morphine and aspirin on thresholds of sensitivity to shock delivery. Alternative techniques would have required suprathreshold aversive stimuli to be applied to the animals, with inevitable behavioral disruption and unnecessary pain.

The theory of signal detection,[76] first proposed in the 1950s, conceives of the situation as one in which the task of the observer is to distinguish or detect the presence of a signal or stimulus against a background of random activity or noise. Techniques derived from the theory permit the experimenter to take into account variables such as guessing habits or biases on the part of the observer that lead to false detections. Unlike classical psychophysics, these techniques can make explicit the consequences of correct detections and false reports, such as in the form of monetary gains and losses.

In all psychophysical experiments, precise specification of stimulus attributes is essential,[77,78] a facet that may require complex equipment and procedures and thus partly explains why psychophysics remains a relatively neglected component of toxicology. Animal psychophysics introduces further complications, deriving mostly from the extended training sometimes required to extract precise and reliable data. Such investments in time and apparatus are worthwhile when the results are coupled closely to problems of human toxic exposure. Circumstances in which animal and human data require reconciliation are likely to generate questions that mandate the application of complex procedures for sensory assessment. For this reason, as in the other sections, the more advanced techniques are featured. Numerous descriptions of simpler approaches, such as reflex measures,[79] have already appeared in the toxicology literature, whereas the more complex procedures and the contexts in which they confer special advantages are less abundant.

Vision

Vision is the sensory system that has received the most attention from toxicology, where it is represented by at least two books[80,81] and chapters in textbooks.[82] Toxic reactions can take place at many different sites in the visual system. Grant[80] has compiled a list of over 2800 agents that can induce visual system toxicity. Corrosive chemicals can damage the cornea, certain drugs can induce cataract formation in the iris, many different chemicals can damage the retina, and even that part of the cerebral cortex subserving vision can be a toxic target. Vision is unique in the way that information is transmitted along the system. The representation of the visual field on the retina, which contains the light receptors and constitutes what has been labeled a retinotopic map, preserves an analogous spatial distribution in the neuronal pathways ascending from the periphery to the final cortical map. Although ancillary influences may act on these pathways and the distribution of information in the cortex involves secondary projections, parallel and topographic segregation in the primary projection areas is preserved with remarkable fidelity. This feature of the visual system makes testing simple and complicated at the same time.

Color sensitivity, for example, is localized to the center of the visual field (the fovea), in receptor elements called *cones*, which are coded chemically to respond to light of different wavelengths. Deficits in color discrimination arise when the function of these receptors is impaired. Humans, however, are often unaware of such deficits because they learn to compensate by relying on brightness or other contextual cues, which is why specialized tests are used to detect color blindness. An illustration of how easily color discrimination deficits may be masked was provided by the experience with the monoamine oxidase inhibitor pheniprazine, which had been prescribed to treat depression. The treated patients developed red–green color blindness, a toxic effect of which neither the patients nor their physicians were aware. Only later, when normal visual function could be recovered in a relatively small fraction of the patients, did clinical investigators learn of this problem. Once they did, it was possible to demonstrate a corresponding effect in pigeons, whose color discriminative capacity is close to that of humans.

Hanson et al.[83] trained pigeons to peck a translucent disk on which different colors could be projected. The birds were reinforced with food for pecking the disk when it was illuminated by green and orange stimuli but not by blue, yellow, and red stimuli. Discriminative responding quickly appeared; few responses were made in the presence of the negative stimuli. After about a month of daily administration of pheniprazine, however, discriminative capacity was lost, with pigeons responding equally to all stimuli. (The experimenters took care to match brightness so it could not be used as a cue.) When treatment with pheniprazine ceased, discriminative ability gradually recovered. This fairly simple discrimination procedure could have been used to preclude a serious, irreversible effect in humans had it been implemented.

Color vision deficits are recognized as one outcome of occupational exposure to organic solvents. Earlier, Raitta

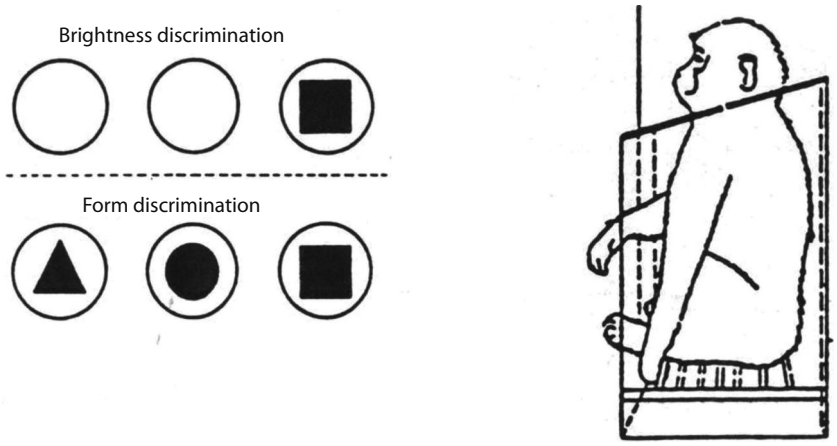

FIGURE 38.11 Testing geometric form discrimination in monkeys exposed to methylmercury. The monkey faced a panel of three disks. When examined for the ability to distinguish shape, the monkey was required to press the disk on which the square was projected. The position of the square varied randomly from trial to trial. When examined for its ability to perform simple brightness discrimination, the monkey was required simply to indicate which disk contained the square. (On the figure, the light and dark areas are reversed for ease of presentation.) Correct responses were reinforced by pulses of fruit juice. (Adapted from Evans, H.L., Early methylmercury signs revealed in visual tests, in Hutchinson, T.C., ed., *Proceedings of the International Conference on Heavy Metals in the Environment*, Vol. 3, University of Toronto Institute of Environmental Studies, Toronto, Ontario, Canada, 1978, pp. 241–256; Evans, H.L. et al., *Fed. Proc.*, 34, 1858, 1975.)

et al.[84] had noted such deficits among workers in the viscose rayon industry, where they are exposed to carbon disulfide, by using the Farnsworth–Munsell 100 hue panel, which requires subjects to arrange a series of 85 reference caps representing incremental changes in hue. Color vision function is based on the ability of the subject to place the color caps in order of hue. Modifications of the 100 hue test include the Farnsworth–Munsell dichotomous D-15 color test, requiring the subject to arrange 15 numbered disks with different hues, and the Lanthony D-15d desaturated hue panel claimed to be more sensitive. Geller and Hudnell[85] have noted the high incidence of errors sometimes made by control subjects and suggested revised test protocols to improve its diagnostic value.

Numerous studies, based on such techniques, have now been published in support of the claim that workplace organic solvent exposure impairs color vision. They implicate styrene, toluene, tetrachlorethylene, xylene, methyl ethyl ketone, and others.[85–87] A common finding in these studies is blue–yellow confusion, but red–green confusion seems to be a more specific marker for solvents.

Color vision deficits due to solvents are likely due to retinal damage or dysfunction. Toxicants that damage visual pathways at upstream sites in the central nervous system create other kinds of deficits that depend on the site of damage. Methylmercury is a potent central nervous system poison that in primates, including humans, tends to be most lethal to nerve cells buried deep in the folds of the cortex. The peripheral projections of the visual fields lie within the medial portions of the occipital cortex along the calcarine fissure, which is a principal site of damage induced by methylmercury. Humans who have undergone serious exposure show constriction of the visual fields, sometimes progressing to severe tunnel vision and, occasionally, blindness. Korogi et al.[88] found, by magnetic resonance imaging, considerable atrophy in several brain regions, including the calcarine fissure, of Japanese victims of methylmercury poisoning. Evans et al.[89,90] traced this progression in monkeys and related it to exposure and tissue levels of methylmercury (Figure 38.11). Monkeys were first accustomed to perching in a primate test chair. During testing, they faced a panel containing three Lucite disks illuminated from behind by three geometric forms—a square, a circle, and a triangle. Pressing any one of the disks closed a circuit and allowed the experimenter to record the source of the response and to arrange certain consequences as a result. In this experiment, the monkeys were reinforced with a small amount of fruit juice for pressing the key with the square.

The reasons for the choice of a geometric form discrimination to trace the progression of methylmercury toxicity illustrate how animal psychophysics is applied to toxicological questions. If methylmercury preferentially damages cortical cells receiving projections from the peripheral areas of the visual fields, then visual discriminations at low luminances should be differentially impaired. The periphery is represented in the retina by visual elements called *rods*, which are sensitive to low light levels. The cones in the center of the field, which are responsible for color vision and for fine acuity, function at high light levels. To detect a differential effect in the central and peripheral visual fields, the forms were illuminated on different occasions with a range of luminance values, the lowest of which made the forms visible only after the monkey had remained in the dark for at least 10 min so the rods had become adapted. Monkeys treated chronically with methylmercury began to show deficits in visual function revealed earliest by diminished accuracy on the form discrimination at the lowest luminances. Only much later did damage progress far enough in the cortex to produce deficits in discrimination at the higher luminances (Figure 38.12).

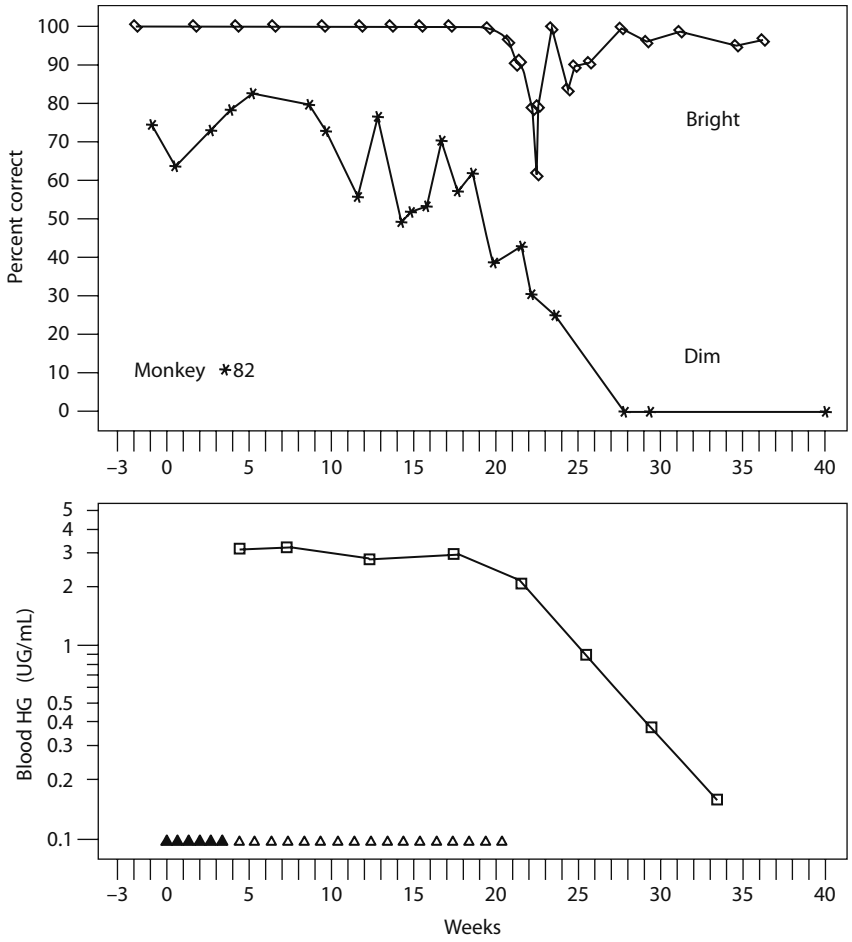

FIGURE 38.12 Form accuracy (top plot) of a monkey (*Macaca speciosa*) treated with methylmercury (see Figure 38.11). The methylmercury was administered in doses of 0.5 mg/kg on occasions marked by small triangles on the tower graph. Resulting blood levels are traced by the connected squares (bottom plot). Performance under dim (scotopic) target luminances deteriorated before performance under bright (photopic) luminances and eventually reached zero, where it remained even after treatment ceased. Note the sharp fall in photopic accuracy beginning at about week 20. At that time, methylmercury administration was discontinued and was followed by recovery, but performances at scotopic luminances continued to deteriorate, suggesting that sensitive assays might help identify toxicity at a stage during at which damage is still reversible. (Adapted from Evans, H.L., Early methylmercury signs revealed in visual tests, in Hutchinson, T.C., ed., *Proceedings of the International Conference on Heavy Metals in the Environment*, Vol. 3, University of Toronto Institute of Environmental Studies, Toronto, Ontario, Canada, 1978, pp. 241–256; Evans, H.L. et al., *Fed. Proc.*, 34, 1858, 1975.)

The primary health concerns aroused by methylmercury arise from its effects on brain development. Because visual deficits are such a conspicuous feature of adult poisoning, Rice and Gilbert[91] characterized visual function in two groups of monkeys (*Macaca fascicularis*) exposed developmentally to methylmercury. One group was dosed from birth onward with 50 μg/kg/day. A second group was exposed in utero by dosing the mother with 10, 25, or 50 μg/kg/day and was then exposed postnatally until 4.0–4.5 years of age with the same dose the mother had received. The authors based their assessment on what is now conceived by vision scientists to be the principles on which the visual system functions. They view the visual system as basically a frequency analyzer, responding to variations in both time and space. Variations in time are exemplified by flickering light sources. Spatial variations are illustrated by almost all natural scenes containing different textures. Low spatial frequencies are represented by objects with relatively broad features, such as faces. High spatial frequencies correspond to fine details, such as the print on the page. Figure 38.13 shows the kinds of variations in a visual display defined by spatial frequency. Spatial and temporal visual function was tested in both groups.

For spatial testing, subjects viewed a display composed of gratings or alternating light and dark bars. These bars were not sharply defined stripes but varied sinusoidally so the highest (lightest) luminance in the modulated signal represented the peak and the lowest (darkest) luminance the trough. In correspondence with temporal stimuli, the variables comprise spatial frequency (bar width), mean luminance, and contrast between peak and trough. High spatial frequencies are represented by narrow bars, which require intact visual acuity, and low frequencies by wide bars.

To access temporal acuity, the subject is asked to indicate whether a target, such as an oscilloscope screen, is flickering

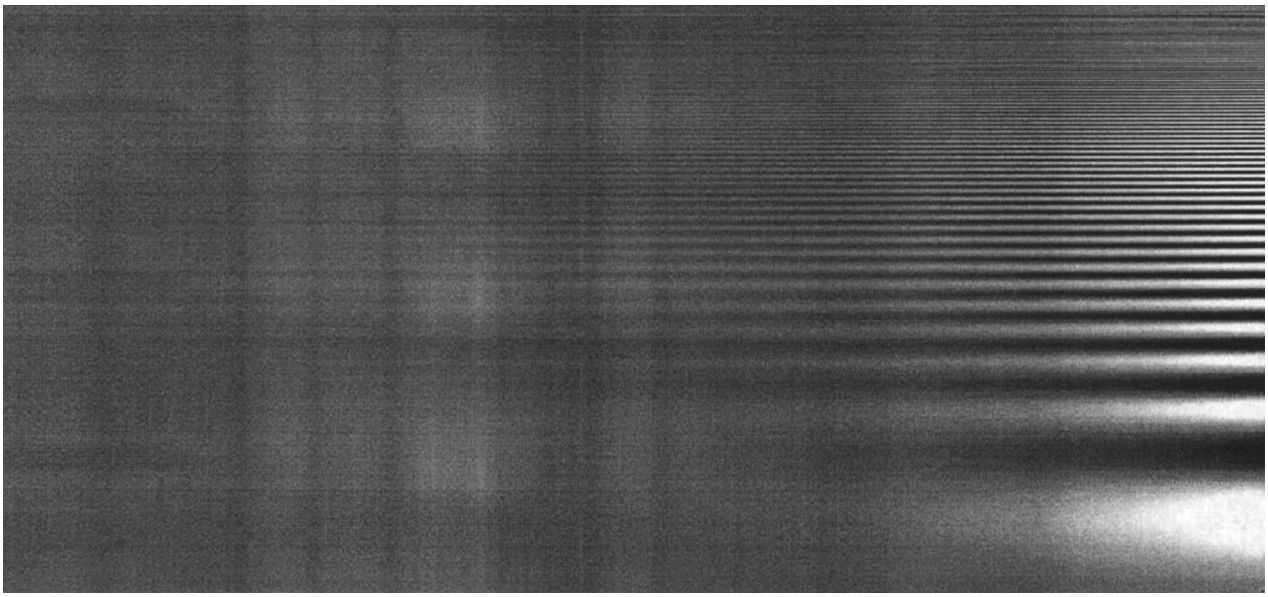

FIGURE 38.13 Spatial contrast model. Spatial frequency decreases from top to bottom, and contrast decreases from right to left. A conventional contrast sensitivity plot would be based on the display of a grating with a specified visual angle and would indicate the contrast level at which the grating appears uniform.

or steady. The ability to do so is measured by raising the frequency of flicker to a rate at which it no longer appears to vary. The current approach varies in both depth of modulation, or the difference in luminance between the brightest and dimmest extremes of the light source, and the mean luminance around which these values fluctuate sinusoidally.[92] Some neuropsychological test batteries[93] continue to rely on the old method, designed to provide a measure called *critical flicker frequency* (CFF), which typically flashes a bright light source on and off at different rates. The CFF is defined as that frequency at which the subject reports a shift from a flickering to a steady stimulus.

Earlier, Rice and Gilbert[94] had reported spatial contrast sensitivity deficits in the group dosed only postnatally. Monkeys exposed both in utero and postnatally exhibited deficits in both high- and low-luminance spatial sensitivities. They also exhibited deficits in low-frequency, high-luminance temporal discriminations (flickering light), while low-luminance temporal vision was superior to that of control monkeys. Those monkeys exposed from birth displayed superior low-luminance temporal vision and no impairment of high-luminance temporal vision. Constriction of the visual fields was not detected. The authors suggest that the pattern of visual deficits produced by methylmercury exposure during development differs from that seen in the adult and speculate that the developing visual system may be able to remodel in response to early damage by a neurotoxic agent.

Similar approaches indicate that the visual system is subject to damage from the axonopathic agent acrylamide. Until the report by Merigan et al.,[95] it had been assumed that acrylamide neurotoxicity, expressed predominantly as a central–peripheral distal axonopathy, was largely reversible. Moreover, none of the previous publications, including those reporting cases of human poisonings, had mentioned visual deficits. Merigan et al.,[95–97] in a series of experiments with acrylamide, trained monkeys to choose between two targets presented on two oscilloscopes. The monkeys sat in a special test stand positioned before a response panel that supported two push buttons and a spout for juice delivery. Before a test trial began, both screens were illuminated evenly. At the onset of a trial, a tone sounded and the test stimulus appeared on one of the oscilloscopes. For acuity testing, the test stimulus was a vertical grating. For tests of temporal resolution, it was a flickering screen. If the monkey pressed the push button corresponding to the position of the test screen (left or right), it received a juice reward. Each response terminated the trial. An adjusting or titration procedure governed the stimulus parameters. Correct responses made the gratings finer on the next trial during acuity testing and raised flicker rate during flicker fusion testing. Incorrect responses drove the stimulus values in the other direction. The positions of the variable and steady targets shifted randomly from trial to trial and stimulus position, and characteristics were controlled by a digital computer.

Treatment with acrylamide continued until the appearance of overt toxic signs, then it was stopped and recovery monitored. All measures of function recovered except for visual acuity, defined as the ability to resolve gratings at the highest contrast (Figure 38.14), which evidenced only partial recovery. A subsequent series of histological studies[98–100] demonstrated that the source of this deficit was the destruction of a class of cells in the retina that project to a particular site in the lateral geniculate nucleus of the midbrain, which in turn projects to the visual areas of the cerebral cortex. The degree of persisting functional impairment noted in some of these studies, however, might go unnoticed by many, if not most, people, just like color blindness.

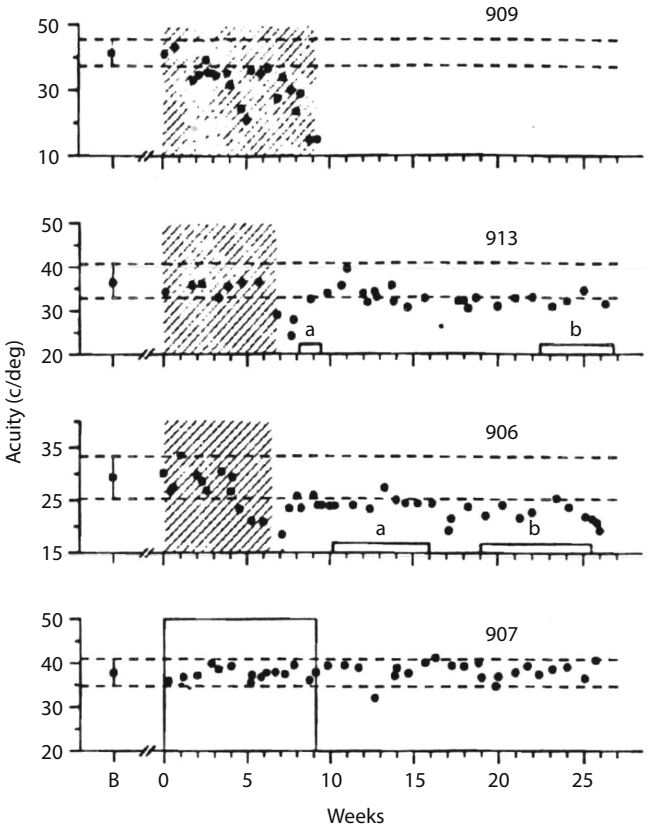

FIGURE 38.14 Visual acuity plots of four monkeys (*M. nemestrina*) dosed with acrylamide. The monkeys faced two high-resolution oscilloscopes during testing. One displayed a grating and the other a uniform field, according to a randomly chosen sequence. For expressing acuity, grating contrast, which describes the differences between the brightest and dimmest areas of the vertical grating pattern, remained constant at 0.55. Spatial frequency, based on the width of light and dark bars of the grating and expressed as cycles per degree of visual angle, was varied in steps according to an up–down procedure to obtain a threshold for the monkey's ability to distinguish a grating from a uniform display. Hatched areas show the period of acrylamide treatment. Acrylamide (10 mg/kg) was administered five times weekly until ataxia appeared. Monkey 907 received only sham treatment. The bottom panel shows the mean of eight baseline sessions (±2 SD), which was extended across the duration of the experiment (dashed tines). The intervals marked *a* and *b* for monkeys 913 and 906 designate collection of contrast sensitivity measures. Monkey 909 was sacrificed at the end of dosing for neuropathology. Monkeys 913 and 906, despite some recovery, failed to reach predosing acuity. (From Merigan, W.H. et al., *Invest. Ophthalmol. Vis. Sci.*, 26, 309, 1985. With permission.)

Merigan et al.[101] and Eskin et al.[102] adopted a similar approach to study the visual toxicity of carbon disulfide. Monkeys were exposed in inhalation chambers to 256 ppm for 6 h daily, 5 times each week, for 7 weeks. The visual acuity thresholds of the two exposed monkeys indicated severe functional losses after about 5 weeks. Further testing revealed a seven- to tenfold loss of acuity from which only one of the exposed monkeys partially recovered. Flicker fusion thresholds, however, showed much smaller, reversible effects. Retinal examinations by fundus photography and fluorescein angiography showed no evidence of the kind of damage to the vasculature, such as microaneurysms, reported in exposed workers,[103] nor was there any other clinical indication of damage. These results, like those of the acrylamide studies, argue that advanced psychophysical testing methods are the most dependable sources of information about the neurotoxic subclinical potential of agents acting on sensory systems. Color vision deficits, as noted earlier, have been observed among workers exposed to carbon disulfide[84] and among those exposed to solvents such as toluene.[104] These deficits were not accompanied by cogent evidence of ocular pathology. Further, Merigan[105] later showed impaired color discriminations in monkeys exposed to acrylamide and confirmed that the class of retinal ganglion cells damaged by acrylamide carried color information. For these experiments, contrast sensitivity measures were based on red–green and yellow–blue contrasts as the grating components.

Hearing

The most frequent cause of hearing loss after aging is exposure to excessive noise. Several classes of drugs also impair hearing. Some (e.g., salicylates) produce transient effects such as tinnitus. Others, such as the aminoglycoside antibiotics (streptomycin and kanamycin), damage the hair cells in the cochlea, where the mechanical movements of sound are transformed into nerve impulses. Loop diuretics and quinine, especially with prolonged administration, can cause similar damage. It is now standard practice for such classes of drugs to be tested for auditory system pathology, but behavioral testing may detect impairment at a stage when the cessation of treatment leads to recovery.

Stebbins and his coworkers[106] have produced an extensive body of data on ototoxicity from which two important conclusions have emerged. First, they demonstrated that hearing can be assessed by many different behavioral methods and in many different species. Second, they established correlations between histopathology and the results of behavioral testing that yield valuable information about how the auditory system works. In this work, a trial begins by pressing a key or contact-sensitive plate, a response that activates a light. At a variable time after light onset, the acoustic stimulus, usually a pure tone, is presented. If the subject responds during the tone, the reinforcer is delivered immediately. Premature releases are followed by termination of the trial and a delay of 6–10 s before the next trial. Trials on which no tone is presented (*catch* trials) are interspersed among the tone trials to estimate the subject's tendency to guess. Training is continued until guessing is reduced to a low, stable rate. Both the method of constant stimuli and the up–down or titration method have been used in these studies.

Measurements of relative loudness, which involve stimulus intensities well above threshold, are made with a modification of these methods. The animals are trained by differential reinforcement, to respond quickly, say within 500 ms.

This is basically a reaction time situation, and, as noted earlier, because greater stimulus intensities produce shorter response latencies, loudness, which is a subjective variable, can be measured in animals. For example, response latency in monkeys falls from about 900 ms at a sound pressure level of 10 dB to about 200 ms at a level of 90 dB. With these techniques, in species as diverse as macaque monkeys, cats, guinea pigs, and chinchillas, these investigators have been able to demonstrate the effects of aminoglycoside antibiotics on the progression of hearing loss and both the temporary and permanent consequences of exposure to noise. The earliest effects are seen at the high frequencies, a typical finding. Mattson et al.[107] notes that such specificity makes it unlikely that ototoxicity would be detected with the usual elementary screening techniques because their stimulus dimensions tend not to be precisely described.

With continued treatment, losses extend to lower and lower frequencies. Such a progression has its morphological counterpart in the hair cells attached to the basilar membrane, which stretches along the winding spiral structure of the cochlea. If the cochlea is examined at an intermediate stage of hearing loss, when high- but not low-frequency discrimination is impaired, it shows damage to hair cells in the lower half of the cochlea, where high frequencies are represented, but no damage to the upper half of the cochlea, where the basilar membrane responds maximally to low frequencies. Given such correlations as a guide, it has become possible to largely confirm the assertion that frequency is coded according to location along the basilar membrane from the base to the apex of the cochlea.

Lead and methylmercury have both been documented as ototoxicants. Schwartz and Otto[108,109] reported that thresholds to a 2 kHz tone rose almost linearly with blood lead levels in subjects 14–19 years of age. This function is based on conventional audiograms from more than 4000 individuals surveyed in the second National Health and Nutrition Examination Survey (NHANES II). In another analysis, based on a subset of Hispanic subjects between 6 and 19 years of age, an increase in blood lead level from 6 to 18 μg/dL was associated with a 2 dB loss of hearing at all frequencies tested. Although a difference of such magnitude is seemingly of little consequence to an individual, it is significant to a population because of the great magnification it undergoes at the extremes of the distribution.[37]

Complaints of hearing difficulties were voiced by individuals from Minamata, Japan, who were heavily exposed to methylmercury, but intensive testing beyond conventional audiometry has not been carried out. Rice and Gilbert[110] surveyed auditory function in *M. fascicularis* monkeys exposed to methylmercury from birth to 7 years of age. The testing was conducted at 14 years of age, after the monkeys were trained on an up–down detection procedure, as described earlier. Exposed monkeys generally exhibited deficits at frequencies above 10 kHz. The age at which these deficits began to appear is not known because auditory testing did not begin until long after exposure had ended. Another group of monkeys was exposed from gestation to 4 years of age.[111] As in the postnatal group, both mothers and offspring received daily doses of 0, 10, 25, or 50 μg/kg. Pure tone detection thresholds were determined at 11 and 19 years of age by the up–down psychophysical procedure. The degree of deterioration of hearing thresholds between 11 and 19 years of age in the treated monkeys greatly exceeded that observed in the controls and supports speculations that accelerated impairment of function during aging is one possible consequence of developmental exposure to neurotoxicants.

Other instrumental techniques have also been applied to questions of sensory function. A series of studies by Pryor and associates, for example, using conditioned avoidance responding, established that hearing loss could be induced in rats by chronic exposure to common organic solvents such as toluene.[112] Rats were trained to jump and grasp a pole upon presentation of a tone to avoid an aversive electric shock delivered through a grid floor in the chamber.

Most of the testing methods described earlier command extensive resources and time for their execution and limit the number of subjects that can be evaluated, and as such, they are not suitable for inclusion in a preliminary screening battery designed to identify potential hazards. Their advantage lies in their ability to respond to questions arising at later stages of evaluation. Even so, there is constant speculation about how these methods might be replaced by less expensive and lengthy approaches. One attractive alternative is the reflex modulation paradigm. It is based on the ability of a low-intensity stimulus, delivered before a stimulus intense enough to elicit a startle response, to modify the amplitude of that response.[113] The startle stimulus is typically a loud sound, and the response is measured in rats by confining them to a platform connected to a force or acceleration transducer. If a prepulse, such as a soft sound, appears at an appropriate interval, such as 80 ms before the startle stimulus, the amplitude of the startle stimulus will be reduced. If the prepulse stimuli consist of pure tones of various frequencies and energies, it is possible to chart what in essence is a conventional audiogram because the amount of reduction is directly related to prepulse amplitude.

Applications of the auditory startle response technique have established the potency of solvents as ototoxicants; for example, rats exposed to trichloroethylene at 4000 ppm for 6 h a day for 5 days showed elevated thresholds at 8 and 16 kHz, perhaps due to a loss of spinal ganglion cells.[114] Startle also can be inhibited by cutaneous and visual stimuli and by brief gaps in a continuous auditory stimulus. The reflex modification technique, nevertheless, is limited to relatively simple questions. It would be unsuitable for plotting contrast sensitivity functions, such as those reported by Merigan and his coworkers in studies of acrylamide and carbon disulfide, or for evaluating disorders of color sensitivity. The latter demand an extremely subtle form of stimulus discrimination that could be achieved only by highly trained and motivated subjects. What seems most reasonable is to include reflex modulation in preliminary screening batteries and to reserve the complex psychophysical methods for advanced questions.

Somesthesis

The skin contains a heterogeneous population of receptors whose central representation is equally diverse. Mechanoreceptors are specialized to respond to deformations of the skin. Some respond on the basis of depth of displacement, some on the basis of velocity, and others on the basis of acceleration. Still others are activated by the movement of hairs. Additional receptors are responsive to temperature. Free nerve endings are thought to subserve pain. Pathways transmitting information from the skin travel to the central nervous system through the peripheral nerves and then, except for the head region, penetrate the spinal cord through the dorsal roots. The head region is supplied by the 12 cranial nerves. At the level of the cerebral cortex, the skin surface is represented in accordance with the density of receptors in various areas, so the cortical areas devoted to the face and hands greatly exceed those devoted to the back, buttocks, and other low-density areas. A corresponding nonspecific system, which is less defined and which includes the reticular formation of the midbrain, makes a wider range of connections as it ascends and is thought to serve an arousal function.

Most disturbances of somesthesis are attributed to impaired function of the peripheral nerves (i.e., peripheral neuropathy), although one prototypical agent, acrylamide, apparently exerts its earliest peripheral effects on the acceleration receptors known as Pacinian corpuscles. Damage anywhere along the pathways from receptor to cortex may produce such disturbances, but methylmercury is the only poison clearly documented to be involved at the cortical level. A wide array of chemical classes, as listed in Table 38.5, seems to produce their somatosensory effects primarily through peripheral nerve damage, although some act as well at subcortical and cortical levels. This list includes metals, solvents, organophosphates, and other chemicals. A more complete list would include drugs from many different therapeutic classes.

In assessing the scope of such a dysfunction, histology will be of limited utility when the basic question is the risk to the health of a specific population such as workers. Moreover, the typical clinical neurological examination is generally inadequate because its aim is to uncover frank disease. The clinician may prick the skin with a pin, draw a wisp of cotton across the skin, or ask the patient to judge whether a tuning fork is vibrating. Even the most controlled of these stimuli, the tuning fork, is a crude device held in a hand that itself has an oscillation of much greater amplitude than is discriminable at the skin. The threshold for vibration detection at a frequency of, for example, 150 Hz lies close to 1 μm and requires precise instrumentation, calibrated to 0.1 μm, to determine. The simple vibration devices marketed by several producers and often used in clinical studies are too gross to yield useful data; moreover, they tend to be used with improper psychophysical procedures.[77,78] A major flaw is their lack of direct specification of stimulus characteristics, particularly amplitude. Some of the commercial units express stimulus output as volts, which refers to the setting on a dial; others use unspecified units that are equally arbitrary.

Despite the requirements for precision, however, vibration sensitivity testing has particular utility as a method for assessing sensory neuropathy. One is its underlying structural and functional diversity. It is thought to be mediated by at least two sets of large-diameter myelinated fibers originating from two kinds of receptors: the Pacinian corpuscles, presumed to be maximally responsive to stimulus frequencies from about 100 to 400 Hz, and Meissner corpuscles, maximally responsive to frequencies of about 50 Hz and below. As in the visual and auditory systems, then, vibration sensitivity can be evaluated by determining detection thresholds across a broad range of frequencies.

Monkeys and humans possess about the same degree of sensitivity to vibration and can be tested in essentially the same way. Maurissen and Weiss[115] tested monkeys perched in a primate chair, with one hand restrained by a Plasticine mold fitted individually for each monkey. An electromagnetic vibrator drove a rod in contact with the middle finger and was positioned to indent the skin at a constant depth in the absence of vibration. It is essential to use a vibrator powerful enough to maintain its calibrated amplitude when opposing the mechanical resistance of the skin. (One flaw of some commercial instruments advertised for vibration testing is their sensitivity to pressure, which renders them unsuitable for precise measurement of displacement.) The monkey's other hand was free to respond on a key. The frequency and amplitude of stimuli were determined by function generators whose outputs, in turn, were governed by parameters entered into a computer program that controlled the sequence of experimental events and that stored the results.

One experiment with this system sought to trace the onset and development of sensory neuropathy induced by the axonopathic agent acrylamide[116] in *Macaca nemestrina* monkeys. During training, they learned to press and release the key to obtain juice, succeeded by a phase in which they learned to wait for the onset of vibration delivered to the other hand before releasing the key. In the final protocol, a tone signaled the beginning of a trial. The monkey then held down the key. After a foreperiod of variable length, a vibratory stimulus was presented. If the key were released during this period, juice delivery followed. On catch trials, inserted to discourage and compensate for random guessing and on which no vibration occurred, the monkey earned juice

TABLE 38.5
Chemicals Impairing Vibration Sensitivity

Acrylamide	Triorthocresyl Phosphate
N-Hexane	Lead
Carbon disulfide	Cyanide
Arsenic	Chlorobiphenyl
Methylmercury	Methyl bromide
Thallium	Methamidophos
Methyl-*n*-butyl ketone	

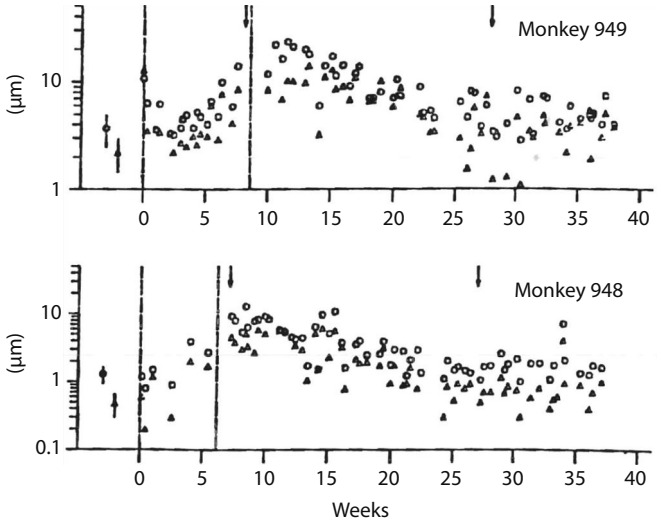

FIGURE 38.15 Vibration sensitivity of two monkeys (*M. nemestrina*) assessed by determining thresholds to stimulation by a vibrating rod applied to a finger. The rod was driven by an electromagnetic vibrator controlled by a voltage determined by the computer program that managed the experiment. The monkey's paw was maintained in a stable position by a Plasticine cast. The subjects were trained to press a telegraph key with the unrestrained hand when a vibratory stimulus was detected and to respond only at the end of the trial period when a stimulus was not detected. Correct responses were reinforced by the delivery of fruit juice. Baseline thresholds (±2 SD) are plotted in the area preceding acrylamide dosing at week 0 for vibration frequencies of 40 Hz (circles) and 150 Hz (triangles). Dashed vertical lines indicate the beginning and end of acrylamide treatment (10 mg/kg, five times weekly). Thresholds, given as vibration amplitude in grams on the ordinate, rose slowly for several weeks during treatment, then slowly declined. (From Maurissen, J.P. et al., *Toxicol. Appl. Pharmacol.*, 71, 266, 1983. With permission.)

delivery by holding down the key during the tone and releasing it only after the tone ceased. Correct responses lowered the amplitude of the stimulus on the next trial and incorrect responses raised it, as in the vision experiments described previously.

Figure 38.15 shows the results of this experiment. After stable baselines were established, monkeys were given 10 mg/kg acrylamide monomer in apple juice on weekdays during the treatment period. In addition to testing two vibration frequencies, 40 and 150 Hz, the monkeys were also tested, in a separate but identical apparatus, for electrical sensitivity. In this apparatus, the rod in contact with the monkey's finger was an electrode through which a 60 Hz electrical stimulus was delivered. As during vibration testing, correct detections lowered the amplitude of the stimulus on the next trial, and incorrect responses raised it. Gross motor deficits were assessed with an apparatus that allowed an observer to record the length of time required to retrieve marshmallow from a mesh grid. Body weights and the occurrence of standard clinical signs also were recorded.

As the figure shows, vibration thresholds rose soon after dosing began. Once dosing ended, they fell slowly after several weeks. Electrical sensitivity remained stable, presumably because it is subserved by small, unmyelinated fibers that are not as sensitive to acrylamide as the large, myelinated nerve fibers that carry information from the Pacinian corpuscles and other receptors that respond to vibration. A second course of acrylamide treatment, following the return to baseline sensitivity, duplicated the results obtained during the first course.[116] What is especially notable about both sets of data is the absolute sensitivity to vibration. Note the baseline thresholds, that lie in the range of a few micrometers. As noted earlier, measuring such thresholds, which is required if early intervention is sought, requires instrumentation that can be calibrated to within tenths of micrometers.

Rice and Gilbert[117] used this method to determine the effects of methylmercury and lead on vibration sensitivity. Methylmercury-exposed monkeys consisted of the same group described previously in discussions of visual and auditory functions. Lead-exposed monkeys had been treated throughout life. Paresthesias comprise the earliest indication of methylmercury neurotoxicity in adults, and peripheral neuropathies are a recognized consequence of lead exposure. Of five monkeys dosed with methylmercury from birth to 7 years of age, four exhibited reduced sensitivity to vibration when tested at 18 years of age. Two monkeys dosed prenatally through 4 years of age exhibited reduced sensitivity at 15 years of age, while two monkeys treated with higher doses showed little impairment. Lifetime lead exposure produced ambiguous but suggestive indications of impairment.

Quantitative sensory assessments are becoming more routine in clinical neurology because of the recognition that the traditional clinical methods are insensitive,[118] and vibration sensitivity is one of the more common indices. Quantitative measures are especially useful for monitoring patients treated with drugs, such as cancer chemotherapeutic agents, that induce peripheral neuropathies, so treatment can be interrupted before permanent damage is inflicted. Despite the availability of instrumentation capable of the precise control of amplitude and frequency, however, considerable misunderstanding remains about the appropriate procedures, including confusion as to the difference between psychophysical methods for the presentation of stimuli and the type of responses with which they are used. Maurissen[78] describes some of the typical errors made by investigators.

Smell

Olfaction is a primitive sense in the context of evolution. Its representation in the brain takes a different course than the senses already described because the olfactory pathways bypass the thalamus and travel directly to the piriform cortex. Recognition of odorants by the olfactory receptors comprises the first stage in odor discrimination, a process that is beginning to yield to a molecular understanding of odorant recognition. Only then do they link to subcortical structures in the limbic system, which includes the amygdala and hypothalamus. Because the limbic system is associated with behaviors such as those involved in reproduction, olfaction

carries a crucial responsibility in species as well as in individual adaptation to the environment. Even in humans, the sense of smell, although not critical in meeting most environmental challenges, is nevertheless the source of both pleasant and unpleasant stimuli that contribute to the quality of life. Moreover, some chemicals announce the approach of dangerous ambient levels by stimulating olfactory receptors, so diminished smell sensitivity might pose a danger.

Disorders of olfaction recently entered the argument about whether or not chronic, low-level exposure to volatile organic solvents inflicts neurotoxic consequences. Schwartz et al.[119] examined workers in the paint industry who had been exposed to a variety of solvents, all in settings well enough regulated that exposure concentrations did not exceed the threshold limit values (TLVs®). The workers were tested by asking them to identify standardized odor patches produced by the Monell Center at the University of Pennsylvania. Workers exhibited a lowered discriminative capacity. Such a test does not afford precise control over parameters such as concentration; it is a fairly blunt instrument designed for screening large groups. Precise control is achievable in both animal and human studies, although it requires specialized instrumentation, as shown by recent attempts to provide adequate precision for clinical use.[120] In animal studies, operant behavior, based on the use of odor as a S^D has proven successful.[121] In the presence of one odor, responses are reinforced with food delivery; in the presence of another odor or a neutral stimulus, responses are not reinforced. A simple discrimination procedure described by Enwere et al.[122] allows generation of actual concentration-effect curves for odor and determination of a threshold, based on techniques that are similar to those used in a later section (stimulus properties of chemicals). Differences in responding serve to index the ability of the subject to distinguish the specific odors at varying concentrations. A special reason for not neglecting olfaction is its exquisite elaboration in rodents, the most common laboratory species. Odor discrimination learning occurs rapidly in rats[123] and also seems sensitive to toxic intervention.

LEARNING

Learning refers to the process of behavioral adaptation to changes in environmental contingencies—that is, behavior in transition. As Laties[124] pointed out, "There can be as many ways of studying learning as there are ways of confronting organisms with changed reinforcement contingencies and then watching them adapt to the new contingencies." The procedures and apparatus devoted to the study of learning are so diverse and numerous that an adequate description of their domain is beyond the scope of this chapter. As previously noted, this chapter focuses on approaches that have been most frequently utilized, as well as those most promising for illuminating behavioral and neurobiological mechanisms of toxicity. It also highlights some of the important methodological issues involved in interpretation of changes in learning. Research reviews have described in greater detail the effects of specific toxicants on learning, as well as other procedural issue.[125–128]

Simple Mazes

The evaluation of learning processes has frequently been based on procedures involving a response choice from which measures including accuracy and latency can be derived. Before the advent of more sophisticated technologies, the assessment of learning often relied on the use of simple mazes. For example, in a T maze, so named because of its shape, the subject is reinforced for choosing (entering) the arm of the maze designated as the correct side, whereas entering the wrong arm results in no reinforcer delivery (extinction). Such procedures can be modified to some extent by the inclusion of external discriminative stimuli to signal which arm was the appropriate choice; for example, the black arm is the S^+ and the white arm the S^-. Typically, learning under such conditions is determined by the number of errors (entries into the wrong arm), the number of trials to some specified accuracy criterion, and the latency (time) from leaving the base of the T to enter the appropriate arm of the maze on each trial.

Such procedures not only have proven useful as indices of learning impairments, but they also entail potential disadvantages and caveats with respect to interpretation of outcome. Because such devices are not easily automated, they are personnel intensive; for example, after each trial, the animal must be replaced in the start box by the experimenter for initiation of the next trial. The necessity of handling animals and experimenter intrusion into the recording of data introduce the possibility of experimenter bias, unless procedures are carried out by personnel blind to all experimental conditions. Additionally, demands on personnel time by nonautomated versions of mazes markedly increase the operational costs of such procedures and may limit the number of experiments that can be undertaken. Objections to purchasing fully automated equipment such as operant chambers, based on the expense of instrumentation, often neglect to account for personnel costs incurred by procedures relying on human intervention. While such approaches have been facilitated by videotaping, computer scoring can still be time-consuming.

Moreover, simple mazes represent relatively simple learning tasks, thus decreasing sensitivity to drug or toxicant effects. The relative rapidity with which learning occurs also renders such baselines ineffectual for assessing learning deficits following exposures to toxicants that have a delayed onset of action or for detecting chronic effects or for tracking reversibility of any observed learning impairments. Finally, and perhaps most importantly, these dependent variables may be influenced by changes in other behavioral processes, limiting the ability to define a learning impairment. For example, the time to traverse the maze can be affected by changes in motor function. Increases in time to reach the correct arm via, for example, motor dysfunction can also delay time to reward itself a variable known to retard learning. Olfactory cues left by experimental animals can be shown under some conditions to influence the performance of animals tested

later. Such difficulties in controlling variables known to influence behavior may result in replication failures both within and across laboratories. Interpretation of a change in behavior as a true learning impairment, therefore, imposes the need to eliminate these potential confounds.

Radial Arm Maze

The radial arm maze is a more complex learning task (Figure 38.16) that can be partially automated. It consists of a central area from which, typically, eight arms radiate like spokes, and a single food pellet is available in each arm. The subject then has access to eight reinforcer deliveries, one in each of the eight arms radiating from the central compartment. The accuracy, efficiency, and speed with which the organism learns to retrieve all eight food deliveries constitute the data of interest, with maximal efficiency requiring only eight arm entries to collect all eight food pellets. The radial arm maze has been shown to be sensitive to the effects of a variety of toxic agents and prenatal insults. For example, Walsh et al.[129] reported an impairment of reacquisition of radial arm maze performance in rats that had been treated with trimethyltin (TMT), a toxicant that damages the hippocampus, a brain structure known to play a prominent role in learning and memory functions. Because it presents a more complex problem to the animal, the radial arm maze has advantages relative to the simpler mazes described earlier, with respect to sensitivity to detecting toxicant effects. Modification of the standard procedures used with the radial arm maze can make it amenable to evaluation of repeated learning (RL) and thus of long-term effects, delayed onset effects, and reversibility. Peele and Baron,[130] for example, accomplished this by baiting only four of the eight arms; the particular four arms that were baited changed during each successive experimental test session. Nevertheless, in parallel with the simpler maze procedures is the possibility that changes in the baseline may reflect not changes in learning processes per se but rather the effects of the toxicant on motor function, sensory capabilities, etc., that must be accounted for in the interpretation of behavioral changes.

Water Mazes

The water maze is a learning paradigm based on negative reinforcement procedures. A rodent is placed in a large pool of water that has been made opaque; escape from the water, that is, the reinforcer, is possible only by finding a platform submerged just under the surface so as to be invisible. Dependent variables include the number of trials to learn where the platform is hidden and the latency to find the hidden platform on each trial. The water maze has been employed widely in behavioral toxicology, in neuroscience,

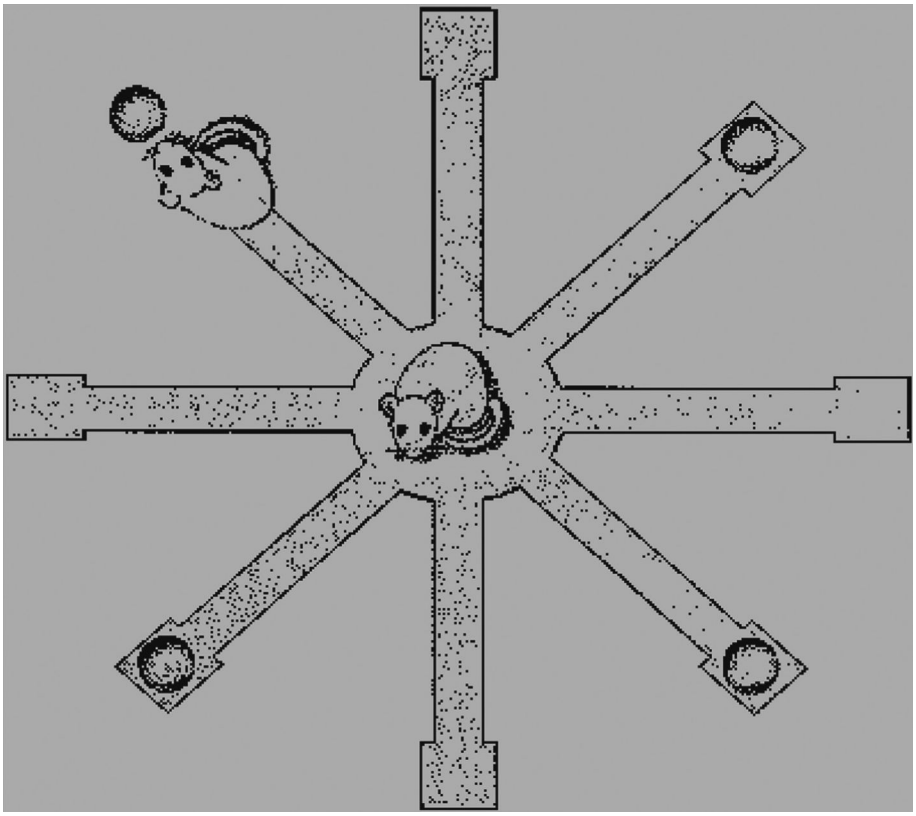

FIGURE 38.16 A prototypical radial arm maze for use with a rodent. The subject is typically placed in the center arena from which some number of arms radiate. Food reward is available under different configurations in various arms. Indices of performance include latencies to obtain rewards, number of arms visited to obtain rewards, and errors (e.g., returning to unbaited arms or arms in which the reward had already been obtained).

and in aging studies because of its ostensible simplicity and the lack of a training requirement or any food deprivation procedures. Performance on the maze has been reported to be influenced by a variety of manipulations, including lesions, toxicants, drugs, and aging. The procedure cannot be fully automated, rendering it a personnel-intensive approach to learning. Moreover, in the configuration described, it represents a relatively simple learning assay and thus may be of limited sensitivity and utility for studies of delayed onset of effect, reversibility, chronic exposure, etc. This simplicity can be addressed to some extent by movement of the platform to new locations requiring learning of a new spatial route for escape.

Like the maze procedures described earlier, it is important to note that the ability to define a selective effect on learning may be confounded by concurrent changes in other behavioral functions. These include impaired motor function, where, for example, a weaker animal will have to exert more effort to swim, a highly effortful response. Body temperature is also a determinant of performance, as has been demonstrated in aging animals. Changes of as little as 1°C in the water temperature may significantly alter performance, making the response more stressful and effortful.[131] Sensory alterations could impair the ability to utilize environmental stimuli to guide the response, and studies have shown that olfactory cues[132] in the water from prior subjects can influence the performance of animals tested subsequently. Such possibilities require the use of additional probes or experiments (e.g., cued platform, swim speed, endurance) for evaluation of a true learning alteration. Furthermore, in some studies, floaters have been identified who must be eliminated from the experiment, thus enhancing the possibility of experimental bias. Although frequently employed, the use of motor activity to rule out motoric deficits as contributing to latency differences in a water maze is unlikely to be adequate, given that swimming represents a far more effortful response than simple ambulation. Additionally, the task has been shown in particular to produce a significant neuroendocrine stress response, including increases in corticosterone levels and altered neurotransmitter levels, effects that may interact with the independent variable.[133,134]

Discrimination Paradigms

Discrimination procedures in operant chambers represent another approach to the evaluation of learning. Technically, the discrimination paradigm reinforces the designated response in the presence of one stimulus (S^+) but not in the presence of another (S^-). As an example, a child's asking for a cookie after eating dinner (S^+) is likely to pay off, whereas asking before dinner (S^-) is not. Given such training, responding becomes confined to those periods during which the S^+ is present and has a low probability of occurrence during S^- presentations. In an experimental situation, a lever press response may be reinforced only when a red light is on but never when the stimulus light is green. The dependent variable of primary interest is the proportion of the total responses occurring on the correct (S^+) lever (accuracy) and the number of sessions or session time until some specified accuracy criterion has been achieved. Discrimination paradigms are of two types: simultaneous or successive. In a simultaneous discrimination, both stimuli are presented at the same time; if only a single response device is available, responding on this device is reinforced during S^+ presentations but not during S^- presentations in a successive discrimination.

Discrimination paradigms carried out in operant chambers as shown in Figure 38.5 offer the distinct advantage of being conducted as free operant rather than discrete trial procedures such as must be used in the maze techniques described earlier. In trial procedures, the time between each trial or opportunity to respond (i.e., the intertrial interval) is determined by the experimenter, and a trial ends with a designated response. The necessity for an intertrial interval using mazes is imposed by the requirement of removing the animal from the reinforcement delivery site back to the start box. In free operant procedures, a response by the organism initiates a trial and no intertrial interval is necessarily imposed between responses. The advantage of this approach is that the subject's rate of responding can be used as a potential index of motivational and motor effects of a treatment, which may contribute to any presumed effect on discrimination learning (see also later discussion). Another advantage of the free operant procedure is that initiation of the trial by the subject rather than by the experimenter ensures the subject's attention to the relevant environmental stimuli and improves accuracy.[135]

A relatively simple operant discrimination procedure is exemplified by the study of Hastings et al.[136] examining the impact of neonatal lead exposure of rats in which a simultaneous visual discrimination task using lights as stimuli proved sensitive to lead exposure. In this discrete trial procedure, a trial was initiated by the insertion of two levers into the operant chamber. The light above one of these levers was illuminated, signaling it as the correct lever (S^+), whereas the other light was not illuminated (S^-). The lever associated with S^+ varied randomly from trial to trial. Criterion performance, defined as 90% correct responses during a daily test session, was reached significantly more slowly by lead-exposed rats.

An example of the kinds of complex problems that may be designed to confront the organism is a conditional discrimination procedure shown by Rice[137] to be impaired in monkeys following lead exposure. One of three disks (the sample) was illuminated with one of three colors on this delayed matching-to-sample paradigm. A press on this sample disk darkened it. After a delay period, which was constant under some conditions and variable under others, all three disks were illuminated, each with one of three colors. If the monkey pressed the disk with the color that had been presented on the sample disk (i.e., if it matched the previously presented sample), a fruit juice reward was delivered. The discrimination is described as conditional because the correct response for any trial is conditional on the sample stimulus for that trial. In this experiment, differences between control and lead-treated monkeys were not observed in the initial acquisition of the

behavior, but appeared when delays were imposed between the conditional stimulus and matching stimuli, with shorter delay values in treated monkeys impairing performance to a greater extent than in controls. In this matching-to-sample paradigm, the response to the sample (conditional) stimulus itself is called an *observing response*; requiring a response to this sample stimulus ensures that the organism is attending to the relevant stimuli when a trial or experimental sequence begins and improves the accuracy of performance.[135] A variant of the matching-to-sample procedure, the oddity paradigm, requires the organism to choose the stimulus that does not match the previously presented sample.

Typically, a specified criterion defines learning in discrimination procedures, such as 8 correct responses in a block of 10 trials. In a discrete trial experiment with both a control group and a treated group, the group mean total number of trials to reach the specific criterion can be compared. Using the free operant procedure, the proportion of the total responses occurring during the S^+ presentation (i.e., the correct responses) may be contrasted between the two groups. Alternatively, an organism can be used as its own control, in which case the accuracy of performance or the rate of learning can be compared before and after treatment. This approach has been made possible by the development of procedures that allow the repeated measurement of learning (discussed in the following).

Repeated Learning

As previously pointed out, one major limitation of many of the simpler procedures described is that, once the correct response has been learned, performance rather than learning is being measured. To pursue issues such as time course of a toxicant's effects on learning, chronic toxicity, or reversibility of toxicant effects, such procedures offer limited utility. Some, however, can be modified to allow repeated measurements of learning. In a discrimination reversal task, for example, acquisition of the original discrimination can be followed by a reversal of the S^+ and the S^-, that is, the stimulus–reward contingency is reversed until the learning criterion is met for the new discrimination problem. Multiple reversals of this sort can be carried out and behavioral adaptation evaluated by measuring the number of trials to criterion on each reversal. After a number of such reversals, however, subjects may acquire the concept of *reversal* and effectively learn the discrimination in a single trial.[138] Bushnell and Bowman[139] found that monkeys exposed to lead showed an increase in the number of trials to criterion and in the number of errors on the first reversal of discrimination problem, but no effect on six subsequent reversals. Likewise, water maze procedures can be modified to permit repeated measurement of learning by moving the location of the submerged platform after the subject has successfully learned its initial location.

An automated paradigm designed specifically to provide a measure of RL was developed for human subjects by Boren.[140,141] This procedure requires the subject to learn a new response sequence or response chain during each experimental session and has since been widely used to study the effects of drugs on learning. In an experiment with pigeons,[142] three stimulus keys in a chamber were all illuminated simultaneously by one of four colors. The pigeon's task was to peck the correct key in the presence of each color—for example, if the key was yellow, peck the left key; if green, peck right; if red, peck center; and if white, peck right. In such a case, if the sequence of light presentations was yellow, green, red, and white, then the correct sequence of responses would be left, right, center, and right. The association between color and key position, however, changed with each successive experimental session, and the subject was required to learn a new four-response sequence. Each correctly completed sequence was followed by food delivery. With training, the number of errors (incorrect responses at any point in the sequence) per session stabilizes, yielding a steady baseline from which drug or toxicant effects on learning can be assessed repeatedly. Thompson and Moerschbaecher[143] have studied the effects of several drugs on the baseline in nonhuman primates.

Paule and McMillan[144] used a variant of this procedure to track the time course of trimethlytin (TMT) effects on learning in rats. In their incremental repeated acquisition procedure, the sequence length was incremented during the course of a session from one- to five-member sequences. By separating certain types of error classes in their analyses, they were better able to understand the particular behavioral processes affected by TMT. Their observations indicated a differential time course for TMT effects on various classes of errors and showed that acquisition of early responses in the sequence was disrupted to a greater degree than the later stages of the sequences, suggesting an effect on learning, while the recall necessary for the longer sequences remained intact.

Another variant of the repeated acquisition procedure devised for rodents by Cory-Slechta and colleagues[145–147] extended the explanatory power of this procedure. Through microanalyses of response patterns and patterns of errors, it could be shown that different drugs could achieve what appeared to be a similar effect (namely, decreases in overall accuracy) through very different patterns of errors. For example, the noncompetitive glutamatergic antagonist MK-801 decreased overall accuracy on the repeated learning (RL) paradigm by increasing the frequency of preservative (repetitive) errors, while scopolamine, a cholinergic muscarinic antagonist, increased the frequency of errors composed of incorrectly skipping forward or backward through the sequence.

Before classifying the effects of a toxicant as one on learning, however, the critical issue of specificity raised earlier must be addressed. Changes in accuracy on learning paradigms may well arise from nonspecific behavioral deficits such as changes in motor activity or function, sensory capacity, motivation, or other unrelated factors, as noted previously. Studies have shown that aging-related impairments in performance by rats in a water maze can be attributed to hypothermia induced by the temperature of the water and inefficient body temperature regulation.[131] Odor trials can likewise affect performance in the water maze.[132] The use

of odor trials as a confounder of learning deficits in conventional mazes is a well-documented phenomenon. Diminished motivation could increase latency in a maze, increasing the delay to reward, which, by itself, would slow the rate of learning, as noted earlier. For the maze procedures described earlier, the potential influence of changes in motor, sensory, and motivational functions and other influences such as temperature-related effects must be ruled out using an independent procedure—that is, by carrying out additional experiments or probes.

One approach that has been widely adopted for assessing the contribution of such nonspecific effects to learning with the repeated acquisition paradigm is to alternate the RL component with a performance (P) component during each experimental session (technically, a multiple schedule of reinforcement; discussed below). The learning or acquisition component, as described previously, requires the organism to learn a new response sequence during each successive experimental session. In contrast, the response sequence reinforced during the P component remains *constant* across sessions, so the organism is simply performing an already learned or rote response sequence. By alternating the RL and P components during each experimental session, such as after every tenth reinforcer delivery, the experimenter can separate, during the same session for each individual animal, drug- or toxicant-induced changes in learning from nonspecific changes in performance or motivation. This is based on the premise that if a compound selectively affects learning, then decreases in accuracy will only be manifest during the learning (acquisition) component of the schedule, as no learning is required in the P component. Because intact motor, sensory, and motivational processes are required in both components, impairments of these functions would result in decreases in accuracy in both the RL and P components.

This approach demonstrated selective effects of lead on learning as illustrated in an extreme case in Figure 38.17,[146] which compares the behavior of a typical control rat to that of a rat exposed to 250 ppm lead acetate from weaning. The particular session depicted began with a performance (P1) component, which was followed sequentially by the learning component (RL1), a return to the P component (P2), and a final presentation of the learning component (RL2), with these components alternating after 25 reinforcer deliveries. Reinforcement followed each completion of a correct sequence. Both rats had received extensive training on the schedule. The control rat (top record) evidenced high levels of accuracy in the first P component, generating a high rate of reinforcement delivery and a relatively low rate of errors, as expected. After the onset of the learning component (RL1), indicated to the subject by a change in illumination in the operant chamber, the rat was required to learn a new three-response sequence. As anticipated, the error rate was initially high, producing a lower rate of reinforcement delivery. By the end of the learning component, however, the rate of errors had begun to decline, and the rat was earning more food deliveries as it gradually began to learn the correct sequence. The acquisition process was even more pronounced during the second presentation of the learning component (RL2), as the rat continued to learn the correct sequence for this particular session. The bottom record of Figure 38.17 shows the rather dramatic impact of lead exposure that was selective for the learning component of the schedule. As can be seen during this particular session, the lead-exposed rat earned virtually no food deliveries during the learning component (RL1 and RL2), emitting hundreds of incorrect responses over the course of both presentations of the learning component, even while exhibiting high-accuracy levels during both presentations of the P component, rapidly earning all 25 available reinforcers. By undertaking a microanalysis of the various patterns of responses and classes of errors, it was determined that the detrimental effect of lead on learning occurred via an increase in response perseveration—that is, repetitive responding on the same lever or repetitive iteration of the same sequence of responses during the learning components.

Any learning assay also must consider the values of the experimental parameters and nature of the problem selected, because such factors influence the sensitivity of the task to disruption by chemical agents. For example, Winneke et al.[148] investigated the effects of lead exposure on the acquisition by rats of both a form and a size discrimination. The form discrimination required rats to distinguish between vertical and horizontal stripes. On the average, only eight training sessions were required by rats to reach criterion accuracy, and the procedure did not differentiate the lead-exposed from the control groups. In contrast, size discrimination, in which a small circle had to be distinguished from a larger circle, proved to be a much more difficult problem, requiring more than 20 training sessions; it revealed a substantial impairment due to lead. A similar effect was reported by Carlson et al.[149] in lead-exposed sheep. Such data demonstrate the important role of task complexity and difficulty in modulating sensitivity to disruption by chemical agents.

Often, the degree of difficulty of the task can be equated with the degree of stimulus control over the performance—that is, the strength of the stimuli controlling the response; for example, Laties et al.[150] required rats to press a fixed number of times on one of two levers, then respond once on the alternate lever for reinforcement. Completion of an insufficient number of responses on the first lever before switching to the alternate lever resets the response requirement. During some parts of the session, a light and tone signaled that the response criterion on the first lever had been met; in other parts of the session, no external stimulus signaled the completion of the response requirement. As would be expected, accuracy of performance was superior during the signaled components of the session, and much higher doses of *d*-amphetamine[150] or toluene[151] were required to disrupt the signaled (S^D) than the unsignaled performance (unlabeled, no S^D). A similar example is exemplified by the data presented in Figure 38.17. Responding on the P component of the multiple RL and P schedule represents a far less difficult discrimination and maintains higher overall levels of accuracy. These findings indicate that behavior strongly controlled by environmental stimuli, such as is the case during the signaled component of

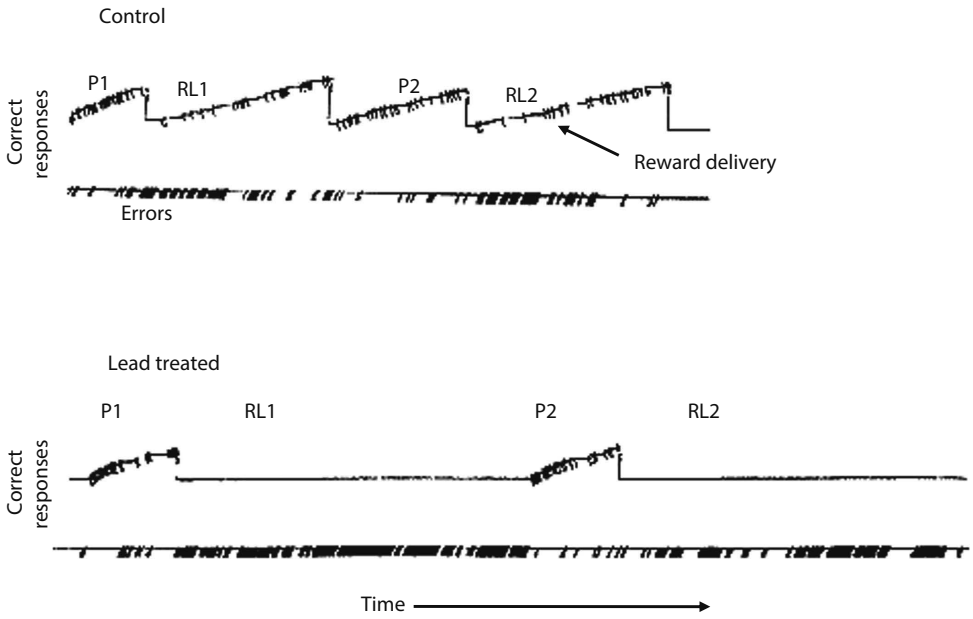

FIGURE 38.17 Cumulative records of performance on a multiple schedule of repeated learning (RL) and performance (P) in a rat exposed chronically to distilled water (top record) versus 250 ppm lead acetate drinking solutions over the course of a behavioral session from left to right. The multiple schedule involved an RL component, which required learning a new three-response sequence during each successive experimental session. These alternated with a P component in which the three-response sequence remained constant across sessions. The top tracing of each record shows the correct responses, which cumulate vertically. Each pip on the correct response curve depicts the delivery of a reinforcer for correctly completing the sequence of three responses required for reward. The pen resets to the baseline with each transition between the performance and RL components. The bottom tracing for each subject shows errors that occurred during the components. The lead-treated rat earned virtually no food deliveries (i.e., completed no correct sequences) during the RL components of the session despite normal performance under conditions where no learning was required (P component). This was not due to a lack of responding, as the rat emitted hundreds of errors during this period. (Adapted from Cory-Slechta, D.A., unpublished data; Cory-Slechta, D.A. et al., *Behav. Brain Res*., 102, 181, 1999.)

Laties et al.,[150] is less easily disrupted than behavior under weaker stimulus control. Thus, the degree of stimulus control over behavior can markedly influence its susceptibility to the effects of drugs of toxicants.

MEMORY

Memory is a term that has been used both to describe and to attempt to account for the behavior of remembering or the influence on behavior of previous events. Experiences are said to be stored in memory, in encoded forms, such as neural engrams or, more recently, as electrical fields, and later recalled from storage by some type of retrieval system. Many theorists have adopted the vocabulary of computer technology to describe memory processes, despite the lack of evidence of operational correspondence. In a behavioral analysis, memory may be best understood and experimentally approached in terms of stimulus control. Remembering is really another way to assert that the probability of certain learned responses is increased. Heise[127] referred to the fact that, while learning is "manifested behaviorally by acquisition, an enduring change in behavior, … memory may be defined as the preservation of the learned behavior over time." Experimentally, memory is indicated by the accuracy of a response after a delay (retention interval) between the occasion for learning and the test for recall. A distinction is typically drawn between short-term memory, occurring over relatively short delay periods, and long-term or virtually permanent memory. Obviously, the temporal parameters of these two subclasses are species dependent.

Novel Object Recognition

A technique that was actually described many years ago, but now increasingly used in neurotoxicology with rodents, is novel object recognition. This paradigm rests on the fact that exposure to a familiar object and a novel object will evoke greater approach and contact with the novel object, and as such is premised on the assumption that the preference for the novel object indicates a memory for the already familiar object. Procedurally, the rodent is initially exposed to two objects in an apparatus for some period of time, after which it is removed. After some time delay, dependent upon experimental conditions, the subject is placed back in the apparatus where one of the familiar objects has been replaced with a new object and the amount of time and/or contacts with each object, the familiar and novel, recorded.[152]

While its simplicity and easy implementation favors it's use, there are several issues that need to be considered in data interpretation. For example, frequently, only data from the second session, that is, that including the familiar and novel object, are presented, precluding the ability to ascertain

whether behavioral differences were also present in the initial session, when both objects are effectively novel, that could have influenced performance in session two (testing of the novel object), for example, side bias or hyperactivity. Other issues related to data interpretation, including that of novelty preference, and involvement of working memory in the paradigm have been described.[153]

Avoidance Behavior

As noted earlier, the various techniques devised to assess memory typically involve the measurement of response accuracy after various delays. An effect of a drug or chemical on memory is suggested by an increased impairment of accuracy with increasing delay values (retention intervals). Avoidance of an aversive stimulus, such as electric shock, is one frequently used measure of both learning and memory. Several different variants of these procedures have been employed; some are discrete trial procedures, and others are free operant procedures. The frequently used passive avoidance paradigm (also called fear conditioning) assesses the tendency of the subject to avoid the site of previous shock delivery. A mouse that avoids returning to a normally preferred dark chamber where it previously has been shocked is said to be exhibiting a memory of that event. Active avoidance paradigms require a specified response to be emitted to postpone an impending shock onset. In some cases, the apparatus used is a two-compartment chamber in which the subject switches compartments at the appearance of a stimulus that signals impending shock (Figure 38.18).

Although useful as a screen for potential memory dysfunction, it is important to note that there are also interpretation issues associated with such approaches, especially the passive avoidance paradigm. As with learning, a drug or toxicant can alter performance in memory paradigms through indirect changes in nonmnemonic functions. For example, motivation to avoid shock can be produced by differences in shock sensitivity prior to the training phase in passive avoidance paradigms. If the toxicant decreases shock sensitivity per se, then treated animals will be less motivated to subsequently avoid the shocked compartment during the memory testing phase, as shock level is an important determinant of subsequent memory. Similarly, in active avoidance paradigms, toxicant-induced decreases in shock sensitivity would diminish response rates to avoid impending shocks. The drug or toxicant may also modify activity levels (e.g., induce hypo- or hyperactivity), thus increasing the probability of the subject returning to the shocked compartment independently of remembering in the passive avoidance paradigm. Furthermore, state-dependent learning may alter subsequent retention for passive avoidance training if the animal is trained during drug or toxicant exposure but retested under nonexposed conditions. In other words, the response may have an altered probability of recurrence during the retest simply because the environmental stimulus conditions (nondrugged) differ from stimulus conditions during training (drugged) and thus may be independent of memory. Such possibilities must then be sorted out in additional experiments that probe these confounding interpretations. Other problems that can be encountered with these simple avoidance paradigms include the fact that variability of response among animals tends to be high, often necessitating large groups of subjects. Additionally, some rats fail to learn such procedures and may be discarded as slow learners, biasing the sample population for unknown reasons.

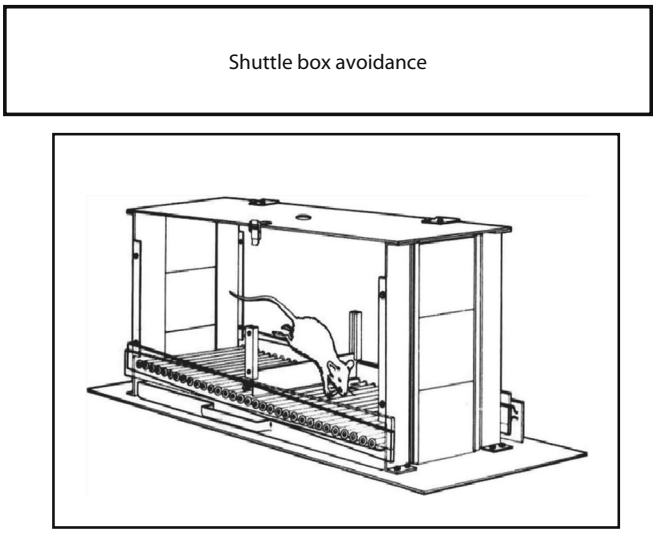

FIGURE 38.18 Shuttle box avoidance apparatus. In this active avoidance paradigm, the rat can avoid impending shock or escape an ongoing shock by shuttling to the other side of the chamber, thus terminating an aversive stimulus.

Delayed Alternation

Delayed alternation requires a subject to alternate responses on each of two response devices such as levers or nose cones for reinforcement (Figure 38.19),[154] that is, a response on device A followed by a response on device B produces a reinforcer, a return to device A, another reinforcer, and so on. After initial training, a delay requirement is interposed between the alternating responses, so only alternations separated by at least the required delay interval are reinforced, rendering this a memory paradigm. Generally, the delay value varies randomly from trial to trial across a series of values designed to include accuracy levels ranging from chance to virtually 100% during an experimental session, allowing the assessment of accuracy by delay (retention interval) information from each experimental session. A response occurring before the end of the delay period typically resets the delay requirement, with the particular delay values specified being dependent on the experimental species. Importantly, on some trials, there is no delay. This no-delay condition is critical for evaluating the contribution of non-memory-related changes in behavior to any presumed memory impairment. Since no memory is required in the absence of a delay, accuracy values should not decline in response to treatment in the no-delay condition. Typically, one also expects to see a greater decline in accuracy with increasing delay values in drug- or

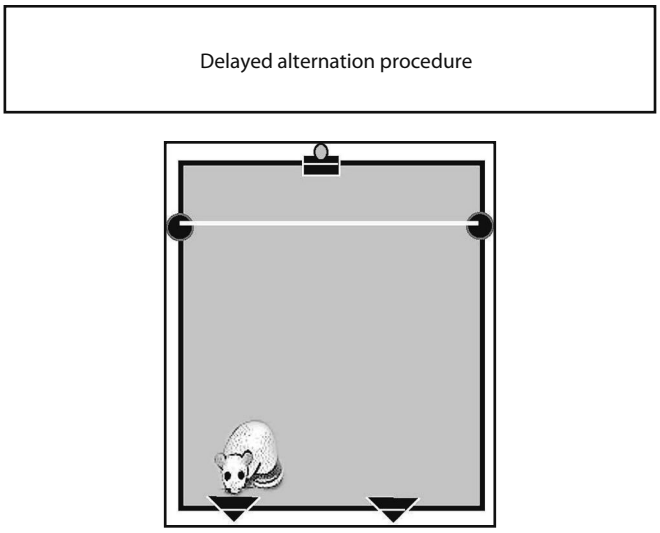

FIGURE 38.19 Schematic of the delayed alternation procedure. This paradigm requires the subject to alternate responses between two response manipulanda for reward (in this case, to nose poke first on the right manipulanda and then on the left manipulanda for reinforcement). The next reinforcement delivery then requires a switch back to the right manipulanda. For the assessment of memory function, a delay between these response opportunities is imposed, requiring the subject to recall on which manipulanda the previous response occurred and thus alternate accordingly. This delay value typically varies from trial to trial during an experimental session so a complete function of accuracy by delay length (retention interval) can be generated for each experimental session.

toxicant-treated groups. Using this procedure, Bushnell[155] reported that TMT could produce deficits in memory processes in rats, with TMT accelerating the decline in the delay interval function relative to control but not affecting accuracy on the zero-second delay trials. These data are consistent with the notion of a selective impairment of remembering.

Delayed Matching to Sample

The matching-to-sample procedure described earlier becomes a memory paradigm when delay intervals are imposed between the presentation of the sample stimulus and the later presentation of the matching stimuli options. As with delayed response procedures, a range of delay values is presented in a semirandom manner throughout the course of an experimental session, allowing the collection of accuracy by delay interval information in every session. Taylor and Evans[156] reported impairments in delayed matching-to-sample performance in pigeons at an exposure concentration of toluene that maintains self-administration behavior (discussed later) in the primate.[157] This impairment was not interpreted as an effect on memory, however, because accuracy was significantly impaired even in the no-delay condition.

As in the case for learning, then, changes in performance on a memory task may have little to do with memory but may instead be indirectly influenced by other nonspecific behavioral effects such as changes in motivation, arousal, sensation, and perception. For example, a toxicant that increased the rate of responding might produce premature responding to the comparison stimuli in a matching-to-sample task and thereby increase error rates. Alternatively, decreased rates of responding might increase the latency to make the response in a choice situation and thus increase the functional delay interval. One efficient way to assess the contribution of nonspecific effects in free operant memory procedures is to examine changes in the rate of responding as well as changes in accuracy in the presence of a no-delay condition. Nonspecific effects on levels of arousal or motivation may be reflected in altered response rates. The no-delay condition permits drug or toxicant effects not related to memory to be determined, as no remembering is required at the zero-second delay. A further condition supporting the interpretation of a true memory impairment is that the magnitude of the decrease in accuracy increases with increasing delay value relative to controls[127] and that similar effects of the toxic agent can be demonstrated in other memory paradigms using other stimulus and response conditions.

SCHEDULE-CONTROLLED BEHAVIOR

Every experimental procedure using operant behavior is based on the principle that behavior is generated, altered, refined, and eliminated by its consequences. Seldom, however, is each and every instance of a specific behavior in the natural environment followed by reinforcement. Continuous or invariant reinforcement is infrequent; instead, intermittent reinforcement is the rule. Paychecks are typically distributed on a weekly, semiweekly, or even monthly basis. Not every visit to the mailbox will be rewarded by the arrival of the letter we are awaiting. Often, a string or chain of responses may be emitted before there is a reward, much as the pianist finishes playing an entire piece of music before the audience applauds. Similarly, the child may correctly put together several puzzle pieces or even the entire puzzle before the parent praises the child. In the wild, predation and foraging behavior are certainly maintained under conditions of intermittent reinforcement. Besides the economy achieved by intermittent presentation of reinforcement, behavior maintained under conditions of intermittent reinforcement is actually considerably more robust than that maintained by continuous reinforcement.[158] For example, a response that has been reinforced on every occurrence declines much more rapidly during an extinction procedure (withholding of reinforcement) than does behavior that has been intermittently reinforced. Put another way, continuously reinforced behavior is much less resistant to extinction than that maintained by intermittent payoff. Many parents have learned, to their distress, how occasional reinforcement of a temper tantrum (failure to ignore it) may subsequently increase the magnitude and persistence of the behavior.[159] The intermittent reinforcement occurring during gambling can sustain high and persistent rates of behavior.

In the human environment, reinforcement schedules—that is, the nature of the rules by which reinforcement is allocated—may be complex. The laboratory offers the

experimenter direct control over these contingencies and thereby allows a more precise analysis of the ways in which the scheduling of reinforcements controls various aspects of responding, including its temporal distribution, force, rate, and resistance to extinction. The study of reinforcement schedules is a discipline in itself.[160–162] Of most relevance to toxicology, an extensive body of literature has accrued describing the effects of a wide variety of central nervous system drugs on schedule-controlled behavior.[8,9,163–168] This permits comparison of toxicant effects on schedule-controlled responding to those of central nervous system compounds whose mechanisms of action are generally well understood. The effects of numerous toxicants on schedule-controlled behavior have since begun to be enumerated.[169–174]

Schedules of reinforcement are especially important to behavioral toxicology, as reinforcement schedules govern the rate and pattern of behavioral responding involved in different behavioral processes.[170] The rate of learning, for example, may well be influenced by the reinforcement schedule according to which the reinforcer is presented. The response may be only slowly acquired if initially reinforced only infrequently. Whether the response is learned at all may depend on the strength of competing responses, the magnitude of reinforcement, and the concurrent availability of competing schedules of reinforcement. In addition, changes in schedule-controlled behavior may actually underlie behavioral changes that are attributed to impairments of other behavioral processes. Decreases in rates of responding produced by a toxicant may be incorrectly interpreted as a decline in the rate of learning in a discrimination procedure. Schedules of reinforcement can also be used as an index of learning processes per se—that is, whether treated subjects learn the behavioral patterns characteristic of the particular schedule under study and whether changes in parametric values of the schedule produce corresponding changes in schedule-controlled performance at that same rate in treated and control subjects. To truly understand the behavioral effects of a toxicant, then, requires an understanding of its impact on schedule-controlled behavior, as behavior occurs at given rates and in particular pattern over time.

Simple Schedules

Although a virtually limitless number of ways to schedule reinforcement delivery are possible, simple schedules are based on either number of responses or time. Four simple schedules have been defined: the fixed interval (FI) and the variable interval (VI), both of which are time-based reinforcement schedules, and the fixed ratio (FR) and the variable ratio (VR), both of which are response-based schedules. Characteristic patterns of performance eventually emerge on each of these schedules, known as steady-state performance.[175] The temporal pattern of responding as well as drug effects on the performance are frequently very similar across species,[165] a feature providing confidence in cross species extrapolation and the continuity of behavioral processes across species. Another advantage of schedule-controlled behavior is that the pattern of performance is often remarkably stable over prolonged periods, a decided advantage for tracking the progression of toxicity and for assessing reversibility.

Typically, after the designated response has been shaped, the schedule of interest is then imposed directly, although it may reach its final parametric value only after a series of gradual changes from the initial parametric values. For example, in studying FR performance in a rat, the experimenter will most likely carry out several sessions at lower FR values before imposing a final value as high as 100. This prevents *ratio strain* (irregular pauses occurring between responses on an FR schedule[160] associated with high FR values) and prevents extinction of the response.

Interval schedules stipulate that a certain amount of time must elapse following a previous response before the next occurrence of the response will be reinforced. On an FI schedule, the time period is constant and typically is measured from the previously reinforced response. For example, an FI 5-min schedule reinforces the first response occurring at least 5 min after the preceding reinforced response; responses during the 5 min interval have no programmed consequence. The FI schedule typically generates a characteristic scalloped pattern of performance, as shown in Figure 38.20, which consists of a pause after reinforcement delivery (designated as the postreinforcement pause time [PRP]) followed by a progressive increase in the rate of responding (running rate) as the time for the next food delivery approaches. In the human environment, studying for a scheduled exam has features resembling performance maintained by an FI schedule, that is, normally, little or no studying occurs early in the semester, but as the time for the midterm approaches, the rate of studying begins to escalate and is highest right before the exam. The species generality of the scalloped pattern of FI performance is amply demonstrated in Figure 38.21 across species, type of response, and type of reinforcer. Human FI performance shows a similar scallop under many, although not all, conditions.[176,177] Sensitivity of the FI schedule to a variety of toxicants has been demonstrated.[171]

The typical, though grossest, measure of schedule-controlled behavior is absolute or overall response rate, which is simply the total number of responses divided by total session time. Often, a full understanding of chemical modification requires a microanalysis to dissect the behavior into more elementary components. Like the separation of various classes of errors in a learning or memory experiment, schedule-controlled performance can be differentiated into component parts, which permits a more precise understanding of the manner in which the schedule controls performance. Such an analysis can suggest the possible behavioral processes that are altered by a chemical and point to directions for further analyses and manipulation.

FI performance provides an example. The schedule has been the focus of much experimental study in part because it exemplifies temporal control over behavior. As previously described, FI performance is characterized by a pause after reinforcement delivery (Figures 38.20 and 38.21). The length

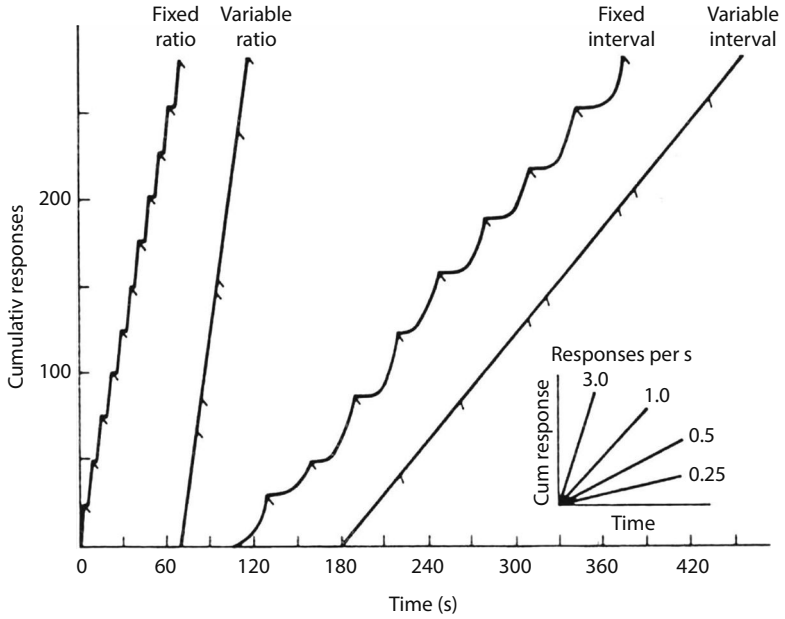

FIGURE 38.20 Characteristic behavior on each of the four simple schedules of reinforcement: the FR, VR, FI, and VI. In these records, responses cumulate vertically, and the slope of the line indicates the rate of responding; examples of slope-rate comparisons are shown in the inset. Each pip on the response curve shows where a reinforcer was delivered according to schedule contingencies. The FR schedule generates a very high rate of responding, with short pauses (indicated by horizontal periods on the curve) following each reinforcer delivery. The VR schedule generates an even higher rate of responding with little or no pausing after reinforcement, as the very next response may produce reward. The FI schedule produces a scalloped pattern of responding characterized by pauses after reinforcement delivery followed by a gradually increasing rate of responding as the time of the next available reinforcer delivery approaches. The VI schedule generates a very stable moderate rate of responding with almost no pausing, as the time to the next available reinforcement opportunity is unpredictable. (From Seiden, L.S. and Dykstra, L.A., *Psychopharmacology: A Biochemical and Behavioral Approach*, Van Nostrand Reinhold, New York, 1977. With permission.)

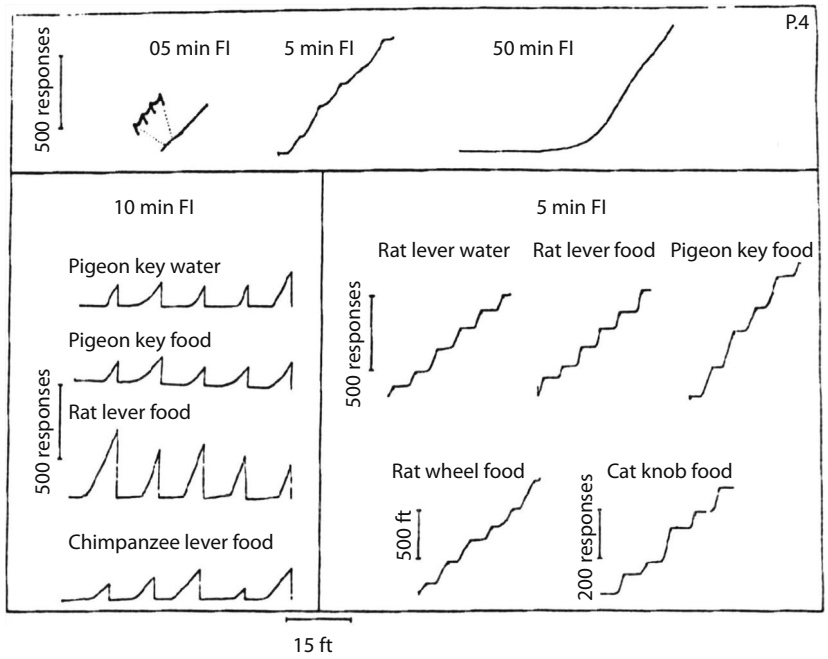

FIGURE 38.21 Species generality of characteristic FI schedule-controlled behavior. Responses cumulate vertically in these records, and time is represented horizontally. The top panel shows the behavior of an individual pigeon pecking a key for food at three different FI values; the general scalloped pattern persists despite the 100-fold change in time value. Performance on an FI 5 min schedule is shown in the bottom right panel. As can be seen, FI performance is remarkably similar for different species (rat, pigeon, cat) with different responses (lever press, key peck, wheel running, and knob pushing) and different reinforcers (water, food). The bottom left panel shows performance on an FI 10 min schedule of reinforcement; in this case, the pen resets to the baseline after each reinforcer delivery. Again, comparable FI performance occurs across species, response, and reinforcers. (From Kelleher, R. et al., *Ergebnisse der Physiol.*, 60, 1, 1968. With permission.)

of this postreinforcement pause (PRP) time (time from food delivery to the first response in the next interval) depends on the length of the FI[178]: the longer the interval, the longer the pause. The PRP is followed by a long period of shorter pauses, interspersed with short bursts of responding. As the interval progresses, the pauses cease and are replaced by alternating periods of moderate and high response rates until reinforcement delivery.[179] The rate of responding during an interval, after the PRP is subtracted out, is referred to as the *running rate*. With the aid of a computer, the actual times between each successive response, the interresponse times (IRTs), can be collected, a frequency distribution of various length IRTs generated, and sequential patterns of IRTs reconstructed. The characteristic scalloped pattern of performance is usually described by one of two measures. The index of curvature[180] specifies the extent to which the scallop deviates from a straight line (a constant rate of responding throughout the interval), and the quarter-life[181] is defined as that proportion of the interval required for the first 25% of the total responses in the interval. It is these microanalyses that may permit detection of a toxicant effect and understanding of its behavioral mechanism of action. In the case of low-level lead exposure in rats, for example, the proportion of short IRTs (0.5 s or less) is consistently increased by lead exposure, even while increases in overall response rate may be less impressive.[182] A similar effect has been reported in chronically exposed monkeys.[183]

The type of effect produced by lead exposure on FI schedule-controlled behavior might be viewed as a loss of discriminative control by the schedule.[171,184] The behavioral mechanism might be explained as a failure of the FI schedule to exert temporal control over behavior or as a failure to learn to discriminate interval length. This hypothesis earned further support from two additional experiments. In one study,[31] rats were required to hold down the lever for 3 s for each food delivery. Control rat median response durations were between 2.5 and 3 s, while some lead-exposed rats exhibited a much higher proportion of response durations too short to produce reinforcement, even after a tone stimulus was subsequently added to signal that the required duration had been met. In another experiment,[185] reinforcement depended on separation of responses by at least 30 s (a schedule known as DRL or differential reinforcement of low rate). Lead-treated monkeys acquired the performance more slowly than controls, as indicated by a higher frequency of nonreinforced responses during the initial sessions. These findings with lead are consistent with other hypotheses regarding its behavioral mechanisms of action.

In contrast to lead, certain pesticide classes alter both response rate and the temporal patterning of FI behavior, suggesting actions through different behavioral and neurobiological mechanisms.[171] Both organochlorine pesticides[186] and formamidine compounds[187] have been shown to decrease rates of responding on the FI schedule and to disrupt the normal temporal pattern of responding. An example is provided in the cumulative records shown in Figure 38.22.

When relevant information about a toxic agent is scarce, the FI schedule offers several distinct advantages as an early

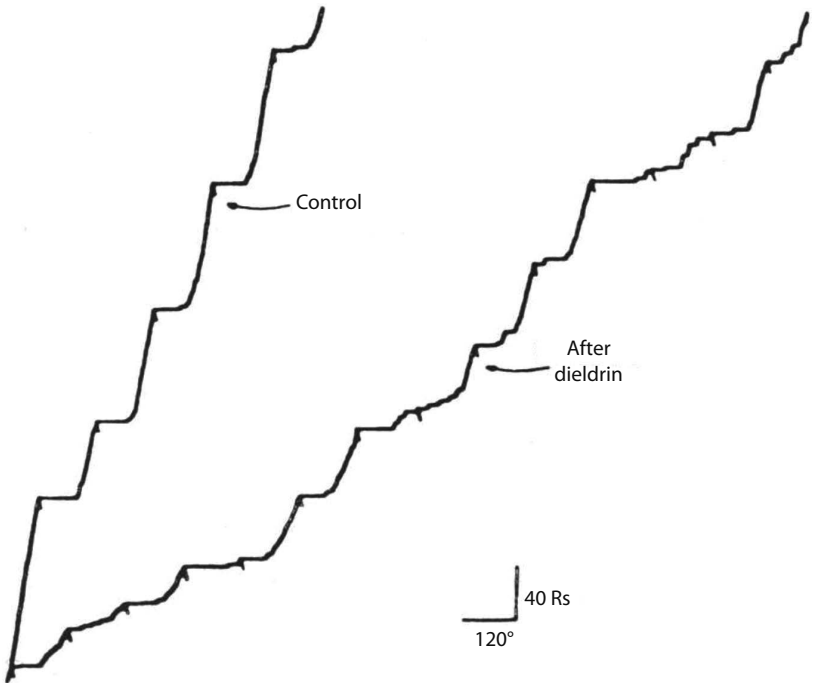

FIGURE 38.22 Cumulative records of the performance of a Japanese quail on an FI, 2 min schedule of reinforcement. Responses cumulate vertically, and time is represented horizontally. Each downward deflection of the pen (pip) indicates food delivery. Performance under control conditions, shown in the left panel, is the typical scalloped pattern characteristic of FI behavior (see Figures 38.20 and 38.21). After 5.0 mg/kg dieldrin treatment (right cumulative record), the response rate is decreased, as indicated by the shallower slope of the curve, and the typical pause after reinforcement delivery is disrupted. (From Burt, G.A., Use of behavioral techniques in the assessment of environmental contaminants, in Weiss, B. and Laties, V.G., eds., *Behavioral Toxicology*, Plenum Press, New York, pp. 241–263, 1975. With permission.)

test for behavioral toxicity. Because the schedule reinforces only the first response after the end of the interval, and because responses during the interval have no programmed consequence, response rates during the interval itself can vary quite broadly before they are sufficient to alter the frequency of reinforcement. This feature may explain in part why FI performance is often more sensitive to drugs than ratio-based schedules of reinforcement[188–190] on which a decrease in the rate of responding necessarily decreases reinforcement frequency (discussed later).

The patterns of behavior by the three other simple reinforcement schedules are shown in Figure 38.20. On a VI schedule, the intervals between reinforcement availability are determined on the basis of time, with the specific value varying from interval to interval and the mean of those values indicated by the schedule parameter value. For example, on a VI 30 s schedule, the specified interval between reinforcement opportunities will vary from one reinforcement delivery to the next, but the average of all the values will be 30 s. As indicated by Figure 38.20, the VI schedule generates a moderate but steady rate of responding, with little pausing evident after the reinforcement, consistent with the lack of predictability of reinforcement availability. Reinforcement may be available immediately after the previous reinforcer delivery or may be delayed. Reynolds[26] cites as an example a busy signal on the telephone, with the caller continuing to make the response that is reinforced (by a ringing sound) on a VI schedule because of the variable length of telephone conversations. The steady persistent pattern of responding on the VI schedule would suggest its utility as a baseline for detecting toxic effects; however, little information in this regard is currently available, even though alterations in VI performance certainly are produced by central nervous system agents.[191]

Ratio schedules require a specified number of responses for reinforcement. On the FR schedule, the requirement remains constant; for example, each completion of 100 responses produces food delivery on an FR 100 schedule. The piecework system used early in U.S. history serves as a classic example; salespeople working exclusively on a commission basis is another. The FR schedule typically generates a pattern of performance in which there is a characteristic pause after reward delivery, the length of which is related to ratio size, followed by a very rapid rate of responding until the ratio requirement is completed. The high, constant response rate on the FR schedule is the result of the relation between rate of reinforcement and rate of responding; the faster the ratio is completed, the sooner reinforcement delivery occurs. As a result, short IRTs tend to be differentially reinforced, amplifying the relationship. As mentioned earlier, the rate of responding on the FI schedule can vary widely before affecting the frequency of reinforcement delivery; however, decreases in response rate on the FR schedule necessarily decrease the rate of reinforcement. It is precisely this difference in the contingencies controlling the two performances that generates the stark contrasts in FI and FR schedule-controlled performance.

Like all schedules, the FR schedule can be analyzed into its component parts, which generally include measurement of the length of the postreinforcement pause and the running rate, as well as an examination of the IRT distribution. Such a microanalysis reveals that the effect of chronic lead exposure on FR performance is to increase the median IRT[169] and thus to decrease response rates. A variety of CNS agents have been shown to alter FR performance,[188,191,192] as have various toxicants, including metals and pesticides.[171] Gentry and Middaugh[193] used simple FR schedules to examine the long-term consequences of prenatal ethanol exposure. In that study, offspring were tested under an FR 1 schedule for 10 sessions, followed by an FR 20 for 9 sessions, and finally an FR 100 for 4 sessions. The increase in response rate across sessions and ratio values was significantly depressed in the groups exposed to ethanol prenatally.

On a VR schedule, the response requirement varies from reinforcement to reinforcement, with the mean of those values designated by the schedule parameter value. On a VR 50, for example, the average response requirement will be 50, but the value will vary from one ratio to the next. One commonly cited example of a VR schedule in the human environment is gambling. The slot machine may pay off on the average once every 100 plays, but the number of plays between payoffs varies unpredictably. Another example includes the sale of real estate. As can be seen with gamblers, the VR schedule generates very high and consistent rates of responding with little or no pausing (Figure 38.20). Both the VR and the FR schedules generate very high rates of responding, but they differ in the characteristic pause after reinforcement, seen only on the FR. This difference in response pattern between the two schedules derives from the fairly constant amount of time required to complete the fixed number of responses on the FR schedule. One hypothesis is that reinforcement delivery on the FR then becomes a stimulus associated with a subsequent period of nonreinforcement or extinction, decreasing response probability, as it represents the earliest part of such a temporal interval. In contrast, reinforcement opportunity on the VR schedule is unpredictable. A reinforcement may follow after any number of responses since the preceding food delivery; thus, reinforcement delivery itself does not become a stimulus that indicates absence of reinforcement availability. The VR schedule is comparable to the VI schedule in that both maintain fairly constant rates of responding with little or no pausing after reinforcement. The VR, however, generally maintains the higher rates of responding of the two because the faster the ratio is completed, the sooner reinforcement delivery occurs. On the VI schedule, higher rates of responding cannot accelerate reinforcement availability. Sensitivity of the VR schedule to toxic agents remains relatively unexplored.

The differences in performance on these four simple schedules of reinforcement thus reflect the very different contingencies of reinforcement. Comparing the effects of a chemical agent on the various schedules can provide a better understanding of the mechanisms by which drugs or toxicants modify behavior or the behavioral mechanisms by which

changes occur. For example, the suppression of response rate on all schedules might suggest a nonspecific effect of a treatment on antecedent factors, such as the motivational level of the subject (i.e., an alteration in functional deprivation conditions). An effect specific to a schedule would implicate the unique contingency of that schedule and its underlying neurobiological substrates for further study.

Several lines of evidence[165,194] indicate that the type of consequence is less important in determining the behavioral effect of a chemical than is the schedule according to which such consequences are presented. Kelleher and Morse[189] found that both amphetamine and chlorpromazine had similar effects on behavior maintained on a given schedule (a multiple FI–FR, described later), regardless of whether it was maintained by food reinforcement or escape from electric shock. Weiss and Laties[195] studied the effects of amphetamine, chlorpromazine, and pentobarbital on the behavioral regulation of temperature. Shaved rats, placed in a cold compartment, could warm themselves by responding on a lever that turned on a heat lamp for a short period of time. Amphetamine increased the frequency of responding even while elevating skin temperature above normal. Despite accelerating heat loss, chlorpromazine decreased the rate of turning on the heat lamp.

Another variable of importance in determining drug or toxicant effect is the baseline rate of responding.[191] Many compounds, including stimulants, barbiturates, minor tranquilizers, and opiates, have been found to increase the length of short IRTs and to decrease the length of long IRTs on certain reinforcement schedules. In other words, many agents increase low rates of responding and decrease higher rates of responding, thus yielding what are called rate-dependent effects. For amphetamine, such effects have been noted in several species and across a wide variety of reinforcement schedules.[196] Thus, on an FI schedule, low rates of responding early in the interval may be increased, while the higher rates of responding occurring just prior to reinforcement may be decreased, leading to a loss of the scalloped pattern of responding characteristic of FI performance. One notable exception to the rate-dependency phenomenon is responding suppressed by punishment, which may be even further decreased by amphetamine.[197] Thus, the baseline rate of responding may be a determinant of toxicant effects.

The designated response to be reinforced can take many forms, depending on the species and the experimenter's goals. For pigeons, pecks on a disk serve as the operant. Tepper and Weiss[198] found that the exposure of rats to concentrations of ozone as low as 0.12 ppm disrupted operant wheel running reinforced under an FI 10 min schedule. Also, the simple lever press can be elaborated into a more complex requirement; for example, the response may consist of holding down the lever for a specified minimum duration, a performance disrupted by chronic lead exposure.[31] A force requirement can also be specified, or the animal may be required to emit a series of responses with different topographies, such as a wheel run followed by a lever press. Another kind of complex response with an extensive literature in behavioral pharmacology specifies that only lever presses separated by a specified interval of time will be reinforced (i.e., the DRL schedule, which makes the pause part of the operant response).

Temporally based schedules of reinforcement also have been used to study processes analogous to retention (memory). Consider the FI schedule of reinforcement. Food delivery is programmed to follow the first response occurring after a specified interval has elapsed since the preceding food delivery. Responses emitted before the end of the interval carry no penalty. The DRL schedule previously described is another type of interval schedule but does prescribe a penalty for early responding in stipulating a minimum interval (and sometimes a maximum) between successive responses. Neither schedule conventionally provides external stimuli, such as lights or tones, to indicate the passage of time. Both schedules generate distinct temporal patterns of responding. FI schedules foster patterns in which little or no responding occurs at the beginning of the interval, which has become a S^D for the absence of reinforcement availability, but as the time of the next food delivery approaches, the rate of responding increases. Subjects experienced on the DRL schedule learn to separate successive responses by enough time to earn a high percentage of reinforcements. Increases in response rate on a DRL schedule, such as found by Colotla et al.[199] to be produced by solvent exposure, then decrease the rate of reinforcement.

One criticism leveled against the use of temporally defined schedules of reinforcement in a memory context is the inability to provide a within-session manipulation of the temporal intervals to be discriminated. Although multiple FI–FI schedules (discussed later) represent an alternative approach,[160] more direct psychophysical techniques for estimation of time intervals are available.[200] In some duration estimation procedures, for example, a stimulus of specified length is presented and the subject makes a choice response to indicate whether the stimulus was short or long. Using a two-key procedure, Stubbs and Thomas[201] presented tone stimuli to pigeons that varied in discrete intervals from 1 to 10 s. Tones 5 s or less were defined as short and were reinforced after a response on a red key, whereas responding on a green key was rewarded after the presentation of long tones. Amphetamine increased the proportion of long tones that were discriminated as short. Using a variant of this procedure, Daniel and Evans[202] reported that acrylamide caused a significant decrement in duration discrimination accuracy, which recovered only gradually.

Complex Schedules

A major advantage of schedule-controlled performance is the flexibility of schedule combinations, so more than one baseline can be studied concurrently in the same subject. This approach compounds the amount of information obtained in an experiment. In a multiple-schedule format, the most common combination, component schedules alternate during the

course of an experimental session. FI and FR schedules have been frequently used as the component schedules because of the marked differences in the contingencies of reinforcement on these two schedules (described earlier). On a multiple FI–FR, the two schedules may alternate either on the basis of time (e.g., every 15 min) or after a certain number of reinforcers have been delivered (e.g., switch to the other schedule after every 10 reinforcer deliveries). Different external stimuli are used to indicate to the organism which schedule component is in effect at a given time; for example, for pigeons, a red light might be illuminated throughout each FI component and a green light used to signal the FR component. After some training, each light serves as a S^D controlling the performance typical of the reinforcement schedule with which it is associated.

The multiple FI–FR schedule of reinforcement has been used by several investigators to study a range of toxicants, such as mercury vapor[203] and methyl *n*-butyl ketone.[204] In those studies, differential sensitivity of the two schedules was not demonstrated, indicating that these toxicants, at the doses used, produced a generalized impairment of performance. In contrast, Levine[205] demonstrated a greater sensitivity of FI than that of FR performance in pigeons exposed to carbon disulfide, a difference similar to that reported for many drugs. A decline in FI rate occurred after a single 8 h exposure (day 1), whereas the concurrent FR performance remained intact. A 2 day, 8 h exposure to carbon disulfide was required to disrupt FR responding. Similarly, Leander and MacPhail[187] studied the effects of the pesticide chlordimeform on a multiple FI 1 min–FR 30 schedule of reinforcement (Figure 38.23) and noted reductions in FI performance at doses lower than those required to disrupt FR performance. Wenger et al.[206] tracked the consequences of TMT exposure in C57B/6N mice responding on a multiple FR 30–FI 10 min schedule of reinforcement. Rates of responding on the FI schedule had increased substantially within 3 h of the administration of TMT, whereas FR performance was as yet unchanged. Markedly divergent effects of TMT on these two schedules were observed 5–9 days after injection, with substantial rate increases on the FI and decreases on the FR. Such divergent drug or toxicant effects reflect the different reinforcement contingencies of

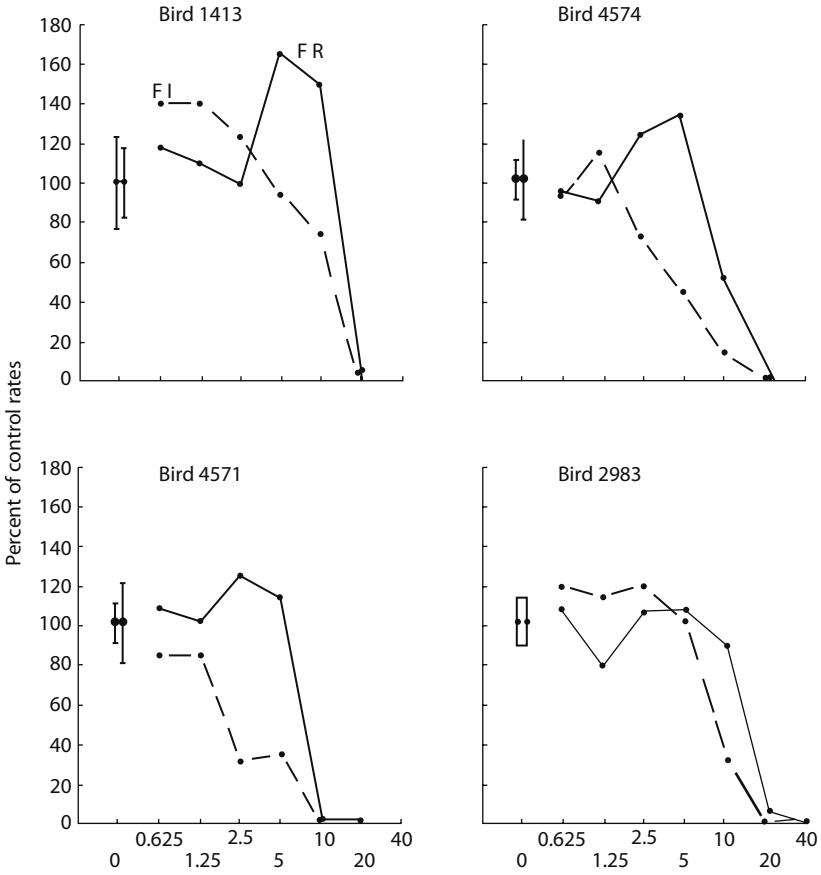

FIGURE 38.23 Effects of acute administration of chlordimeform on a multiple FI, FR schedule of food reinforcement in pigeons. Changes in response rates (total responses divided by session time) are plotted in relation to increasing dose of chlordimeform separately for each component and for each of four subjects. Points and brackets above 0 indicate the mean ± SE under control conditions. As is evident, overall response rates (ordinate) decreased with increasing dose of chlordimeform (abscissa). FI performance was generally more sensitive, however, as it declined at lower doses than those required to disrupt FR performance. (From Leander, J.D. and Macphail, R.C., *Neurotoxicol. Teratol.*, 2, 315, 1980. With permission.)

the two-component schedules and the very different behavioral performances they produce. These examples illustrate how the schedule of reinforcement itself may be a powerful determinant of the effect of a chemical agent.

A mixed schedule of reinforcement operates identically to a multiple schedule, but without external stimuli to indicate which schedule component is in effect.[26,160] The only stimuli available to the subject, then, come from its own behavior in relation to the reinforcement schedule contingencies; thus, comparisons of drug or toxicant effects on multiple versus mixed schedules permit an assessment of the role of S^D control over behavior. In that context, Leander and McMillan[207] compared the effects of chlorpromazine on the performance of pigeons under a multiple FR 30–FI 5 min schedule and a mixed FR 30–FI 5 min schedule of reinforcement. The contingencies of reinforcement were identical on the multiple and the mixed schedules; they differed only in the extent of stimulus control. On the multiple schedule, blue and red key lights were illuminated during the FR and FI components, respectively, while a white key light was illuminated during both components of the mixed schedule. A dose of 3.0 mg/kg chlorpromazine decreased FR response rates on the mixed schedule, whereas a dose of 100 mg/kg was required to produce an equivalent decrease in FR response rates on the multiple schedule. These results again demonstrate the modulation of the effects of a chemical agent by environmental conditions—in this case, the degree and nature of the stimulus control over the performance. A toxicological example is provided by work using a fixed consecutive number (FCN) schedule of reinforcement.[208] On this baseline, pigeons were required to peck eight or nine times on one key and then one time on a second key for reinforcement. Methylmercury exposure shortened the run of responses on the first key below the required level. When an external stimulus was added to signal the completion of the eight to nine required responses, however, the effects of methylmercury were eliminated. A return of the effects of methylmercury on the FCN baseline reemerged when the external stimulus was again removed, a further example illustrating how strong external stimulus control can overcome a toxicant-induced discriminative deficit.

A chained schedule of reinforcement also has different stimuli associated with each component of the schedule, but it requires the completion of the entire sequence of schedule components for reinforcement delivery. For example, on a chained FI 5 min–FR 30 schedule, an external stimulus first signals the FI component and the subject is then required to complete the FI with a response after 5 min has elapsed. This event produces a change in the external stimulus, which acts both as a conditioned reinforcer (S^r) for the FI performance that preceded it and as a S^D for the FR schedule component that will follow. After completing the FR requirement, the reinforcer is delivered, and the chain starts again. A tandem schedule of reinforcement is equivalent to the chained schedule, but no external stimuli indicate which component is in effect.

Wood et al.[151] compared performances on FR 8–FR 1 chained and tandem schedules to determine whether the behavioral effects of toluene were modulated by stimulus control. Both schedules required the completion of the FR 8 on one lever, followed by a single response (FR 1) on the other lever for food delivery. During the chained schedule, the light above the first lever served as the S^D for the FR 8 component. Completion of the FR 8 activated a tone and illuminated the light above the second lever, signaling the subject that a response on the alternate lever would now result in reinforcement delivery. The first of these two stimuli, then, served as the S^D for FR 8 performance, while the second acted as both a conditioned reinforcer for FR 8 performance and the S^D for the FR 1 component, with completion of the latter followed by food delivery. On the tandem schedule, only the house light was illuminated, and it remained illuminated during both the FR 8 and the FR 1 components. Clear differential effects of toluene were observed under these conditions, with performance on the chained schedule, which was under stronger external stimulus control, disrupted far less than the unsignaled tandem performance.

In the complexity of the natural environment, we are routinely faced with many contingent relationships simultaneously, sometimes requiring choices among them. Concurrent schedules, an experimental simulation of such circumstances, facilitate a study of choice by making it possible to select among concurrently operative reinforcement schedules. In an experimental chamber, each such choice would be associated with a separate response device.[209] The choice of component schedules used will, of course, depend on the experimental question of interest. In addition to securing data about preferences for the available reinforcement options, concurrent schedules permit analyses of the behavior of switching between schedule options, a category of responding under the control of the contingencies governing choice. As might be expected, under some conditions,[210] schedule-controlled performance maintained by concurrent schedules differs from the performance observed under conventional schedule conditions.

Despite the wealth of information about complex behavior such as choice yielded by concurrent schedules, few studies of chemical effects on such baselines have as yet been conducted. Newland et al.[57,211,212] used concurrent schedules of reinforcement to examine the effects of in utero exposure to lead, methylmercury, and elemental mercury vapor in squirrel monkeys. Random-interval (RI) schedules were programmed on two response levers, with the reinforcement density always being richer on one of the levers. RI schedules are similar to VI schedules, as the intervals between available reinforcement deliveries are truly random and the RI schedule parameter represents the average of these values. Under steady-state conditions, the behavior of the control monkeys was indeed sensitive to the differences between the two levers in reinforcement density, as indicated by the relative distribution of responses across the levers, that is, monkeys allocated proportionately more time to the response lever associated with the greater reinforcement density. In contrast, the behavior of monkeys exposed to methylmercury and elemental mercury and with blood lead values in excess

of 40 μg/dL was more biased and less sensitive to the differences in reinforcement rates. Further, as exemplified by the results with mercury vapor, when the relative reinforcement densities of the two levers were switched, as shown in Figure 38.24, control monkeys gradually shifted to the new, richer lever, whereas the offspring of females exposed to mercury vapor changed more slowly, or not at all, or even in the wrong direction. These effects led the authors to suggest that one behavioral mechanism by which these exposures altered behavior was by causing insensitivity to alterations in reinforcement contingencies.

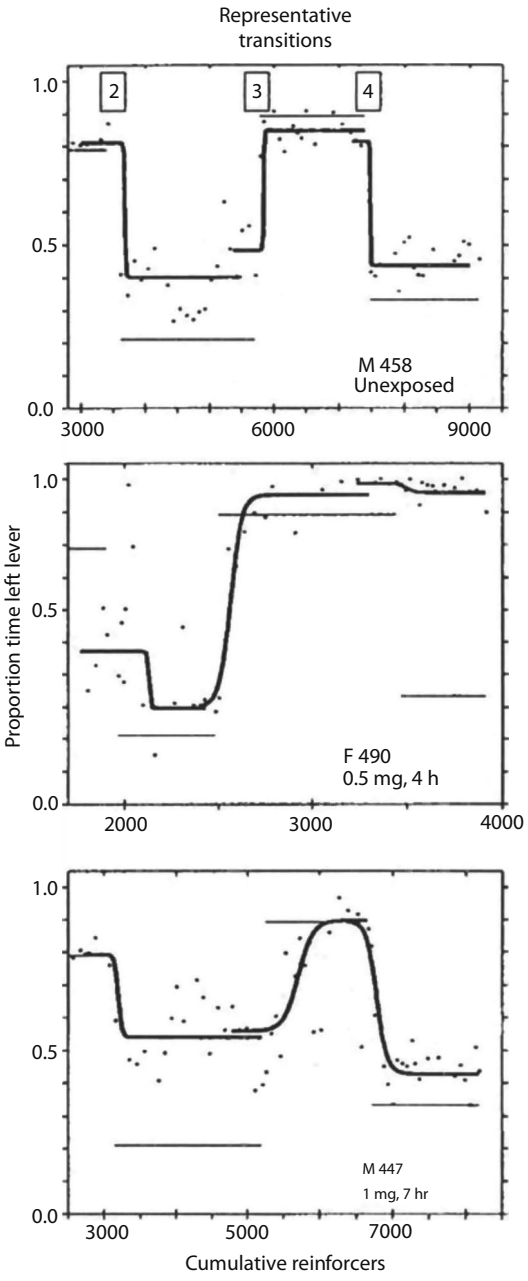

FIGURE 38.24 Plot of transition performance in one control monkey (M 458) and two monkeys whose mothers were exposed to mercury vapor during gestation. Proportion of time on the left lever should track the proportion of reinforcements (shown by the unconnected horizontal lines) on that lever. The response allocations (proportion of time on the left lever) of the control monkey shifted rapidly when the proportion of reinforcers programmed for the left lever was changed. The two exposed monkeys proved less sensitive to the change in contingencies. The heavy black line shows the fit of a logistic equation to the plotted points. (From Newland, M.C. et al., *Toxicol. Appl. Pharmacol.*, 139, 374, 1996. With permission.)

STIMULUS PROPERTIES OF CHEMICALS

Chemical agents or drugs themselves may function as unconditioned stimuli, as discriminative stimuli, and as reinforcing stimuli.[213] These roles correspond to those of conventional exteroceptive stimuli in controlling behavior. As reinforcing stimuli, they may pose the problem of dependence and the allied problem of abuse. Chemicals can function as discriminative stimuli in drug discrimination paradigms to provide information about the central nervous system properties of the agent and its neurochemical mediation. Chemicals have also been shown to have the capacity to act as positive reinforcing stimuli in a drug abuse context, as well as negative reinforcing stimuli that provoke avoidance behavior.

Chemicals as Discriminative Stimuli

The capacity of a chemical to act as a S^D and the neurochemical mediation of its central nervous system effects can be evaluated by a simple discrimination procedure referred to as the *drug discrimination paradigm*. Before an experimental training session, an organism is injected with, or exposed to, a specified dose of an agent (drug, toxicant) or an appropriate vehicle. Subsequently, responding on one of two levers is reinforced if the vehicle was administered prior to the session, but on the alternate lever if drug or toxicant administration preceded the session. The order of administering the chemical or drug versus vehicle across sessions is random, so the organism is not trained to respond to the pattern of chemical and vehicle administration. Typically, responding on the appropriate lever is reinforced according to an FR schedule, such as FR 10 or FR 20. Optimal training parameters of this procedure have been reported.[214] Accuracy of the discrimination within a session is defined arbitrarily (usually about 80%) and measured from the allocation of responses during the first FR completed. This restriction of the accuracy determination to response allocation during the first FR of the session is necessary because the first food delivery itself signals to the subject on which lever responding will be reinforced during the session. The establishment of a discrimination between the drug or chemical and the vehicle is arbitrarily defined, such as 8 of 10 consecutive experimental sessions in which the within-session accuracy criterion was met. The number of sessions required to establish such a discrimination depends on many factors, including parametric aspects of the procedure and training dose of the agent.

Once a discrimination is established—that is, once the subject has learned to accurately report whether it received

a drug or vehicle injection by appropriately responding on the lever consequent to its administration—the dose of the agent administered is varied during special very short–duration test sessions (generalization tests) designed to prevent any training to these other doses of the chemical. This procedure generates a dose–effect function relating the proportion of responding on the drug or chemical lever to the dose of the chemical; the ED_{50} value can then be extrapolated from this function. The procedure can be used to relate toxicant-induced changes in neurotransmitter function to changes at more molecular levels of analysis and to determine the neurochemical basis of behavioral toxicity.[171] For example, Cory-Slechta and colleagues[27,171] used this procedure to evaluate changes in dopaminergic and glutamatergic functions in relation to lead exposures. Rats were trained to discriminate either a D_2 dopamine agonist from saline or a D_1 agonist from saline using standard drug discrimination procedures. When the dose–effect curves depicting the proportion of drug lever responding to various doses of these agonists were determined, the ED_{50} values for postweaning (see Figure 38.25) and postnatally lead-exposed rats were significantly shifted to the left of those of control, consistent with dopaminergic supersensitivity. A previous study also has shown a lead-induced subsensitivity to a *d*-amphetamine drug stimulus.[215]

Chemicals as Positive Reinforcers

Operant techniques for experimentally evaluating the efficacy of drugs as reinforcers were developed in the 1960s for both rat[216] and monkey.[217] Animals are often equipped with intravenous catheters attached to infusion pumps through which specified amounts of the drug are administered when response requirements are met by the organism. The development of such procedures established the capability for an experimental analysis of drug dependence, and the resulting literature demonstrated unequivocally the correspondence between most of the substances self-administered experimentally and those abused by humans. Consequently, these techniques are commonly relied on today to evaluate the abuse liability of newly synthesized compounds. In some cases, animals are first trained to self-administer cocaine. The test compound is then substituted for cocaine, and its efficacy in maintaining responding is determined. Abuse potential is not, however, limited to drugs. Volatile materials and aerosols frequently encountered in occupational settings and also commercially available are likewise subject to abuse by inhalation. Using such procedures in squirrel monkeys, Wood demonstrated self-administration of toluene and nitrous oxide,[157,218] two substances that are also abused by humans. Other toxic compounds reported to engender

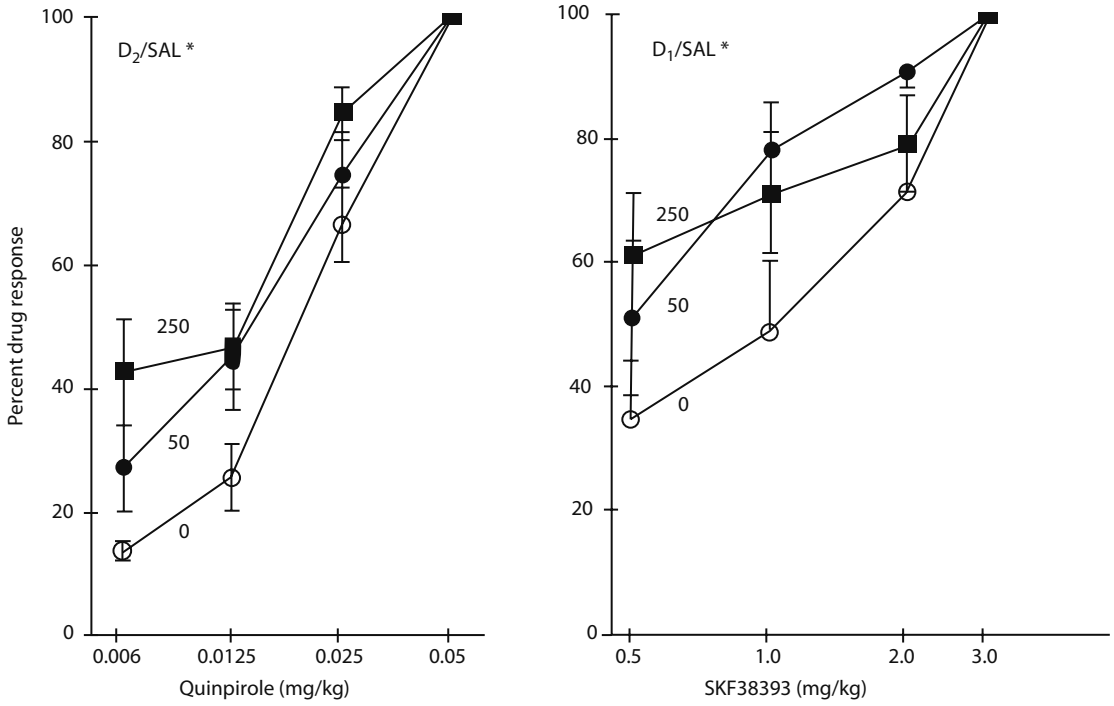

FIGURE 38.25 Generalization dose–effect curves for control (0 ppm; open circles) and 50 (filled circles) and 250 ppm (filled squares) lead-exposed groups trained to discriminate a dopamine D_2 receptor agonist from saline (left panel) or a dopamine D_1 receptor agonist from saline (right panel) using a drug discrimination paradigm that reinforced responding on one lever if the session was preceded by saline administration and on an alternate lever if dopamine agonist administration preceded the session. Test sessions involved administration of lower doses of the agonist. Each data point shows a group mean ± SE based on $n = 10$. The ability to discriminate drug from saline declines with decreasing dose of the drug as expected. These dose–effect curves were shifted to the left following lead treatment, indicating supersensitivity to the dopamine agonists. (From Cory-Slechta, D.A. and Widzowski, D.V., *Brain Res.*, 553, 65, 1991. With permission.)

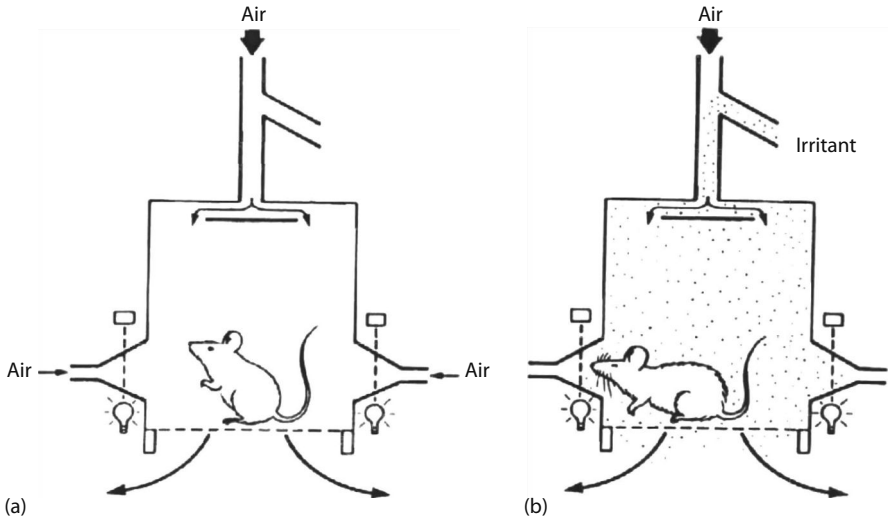

FIGURE 38.26 Schematic drawing of an exposure chamber used to study the irritant properties of compounds. Panel (a) shows the situation before irritant delivery; panel (b), during irritant delivery. The chamber atmosphere was introduced at the top, and a baffle ensured even mixing. The mouse stood on a perforated stainless steel platform though which the atmosphere exhausted. The irritant was added to the dilution air immediately above the chamber. Delivery of the irritant could be terminated by a nose poke, as shown in panel (b), which interrupted a light beam and was then recorded. Only one of the sensors shown was the active sensor for terminating irritant deliveries; the other served to measure the specificity of any behavioral changes. When the irritant exposure was terminated, either by a response or the end of the trial, a stream of clean humidified air was delivered through each cone to minimize the delay of irritant termination after a response had occurred. (From Wood, R.W., *Toxicol. Appl. Pharmacol.*, 50, 157, 1979. With permission.)

self-administration in humans include *n*-hexane, gasoline, vinyl chloride, and other organic solvents and volatile agents.

Chemicals as Negative Reinforcers

Drugs or chemicals may also function as negative reinforcers and maintain escape or avoidance behavior. As shown in Figure 38.26, for example, mice will respond to terminate the flow of ammonia through a chamber.[219] Response latency and incidence are directly related to the concentration of ammonia. In other studies, similar aversive properties of ozone,[220] acetic acid,[20] and formaldehyde[221] have been demonstrated. Also, Tepper and Weiss[198] found that rats working under an FR 20 schedule of reinforcement made fewer responses to release a brake on their running wheels, an avoidance of exercise that increased ozone flow into the lungs. Thus, such techniques can be used to assess both the pleasurable and aversive/irritating properties of a chemical, properties previously considered subjective and consequently barred to experimental evaluation.

BEHAVIORAL TESTING IN INVERTEBRATE ORGANISMS

Increasingly, invertebrate organisms are being added to the arsenal of neurotoxicity testing given the opportunities they afford for more accelerated study and incorporation of genetic variants, among other features. With this has come the development of increasing means of carrying out behavioral testing. While the types of complex cognitive functions described previously cannot be fully encompassed in these species, there are nevertheless mechanisms devised for simple measures, particularly those related to motor function and cognition and in some cases including both respondent and operant conditioning approaches. Multiple reviews have recently been published summarizing such approaches for zebra fish[222–224] and for *Drosophila*.[225–229]

HUMAN BEHAVIORAL TESTING

With the recognition that toxic exposures in the workplace might lead to functional disturbances too subtle to be detected by ordinary clinical or neurological evaluation, a growing body of research based on clinical psychological testing methods began to develop in the early 1970s. Gamberale et al. [230, p. 359] cogently described the rationale for behavioral testing:

> The growing interest in the measurement of performance is most probably due to the sensitivity shown by these methods in unveiling changes in the human organism that otherwise would not be detected. By now, the evidence that these changes are some of the earliest indicators of the occurrence of health effects has become unequivocal. As a consequence, the measurement of performance has come to be regarded by many as a device of major importance for monitoring hazards to health and safety in the work environment.

At the same time, awareness grew that equivalent problems in the community, such as the adverse consequences of excessive lead exposure in children, also required a greater reliance on quantitative behavioral methods rather than clinical examinations.

A salient example of an occupational issue is the claim, advanced most consistently by Scandinavian investigators,

of a syndrome, characterized by behavioral and neurological abnormalities, associated with chronic exposure to organic solvents.[231,232] Because the diagnosis of organic solvent syndrome or toxic encephalopathy makes a worker in those countries eligible for a disability award, official recognition of such a syndrome carries considerable economic implications. A similar point with respect to environmental issues is embodied in the discussion of what constitutes excessive lead exposure in children. If lowered scores on intelligence tests can be detected at blood lead concentrations of 10 or even <10 μg/dL, then sources of environmental lead must be much more stringently controlled.

Testing Approaches

Neuropsychological test methods, confined previously to clinic or laboratory settings, have been increasingly adapted to serve in both field and population studies. Although in principle both clinical neuropsychology and experimental psychology flow from common tenets, there are marked differences in history and tradition. Laboratory animal testing is based on the traditions of experimental psychology and the technology refined by behavioral pharmacology. Much of human psychological testing is rooted in questions of clinical diagnosis. Many of the tests that were most widely applied in occupational settings were designed to originally classify subjects or patients into various diagnostic categories or to characterize areas of deficit. The assessment of brain damage was the major focus of the specialty of neuropsychology.

Both standard clinical neuropsychological methods and those developed in experimental psychology have now come to be used in occupational and environmental studies. Advanced tests of sensory and motor function in humans, as described earlier, are closer in design to those devised for animals, and in fact, many of the laboratory animal methods are direct analogs of those created originally for humans; however, most techniques currently used for assessing and predicting what is designated as *achievement*, such as the acquisition of specialized knowledge, still come from the neuropsychological tradition, such as intelligence tests that partly attain such aims and may serve diagnostic functions as well. Disturbances of subjective state, such as depression and irritability, are frequently assessed using neuropsychological tools. Not only may they sometimes foreshadow overt toxicity, but they can also constitute important data in themselves. As elaborated in the succeeding text, as the questions being pursued become more focused on more specific behavioral deficits, reliance on experimental psychology approaches suited to both human and nonhuman subjects and to single behavioral domains is increasing.

Certain functional variables have assumed a special role in human testing, partly because of the frequency with which they have been reported, partly because tools for their assessment are available, and partly because they are deemed critical qualities. Only a few of the methods are reviewed here, and they are meant to serve as illustrations.

Clinical Neuropsychological Test Batteries

Early studies of occupationally and environmentally exposed populations largely involved the utilization of screening batteries,[233] with multiple different tests available to ascertain a range of behavioral functions based on the uncertainty about the correlations between specific effects and specific agents. Criteria for test selection, whether in batteries or used individually, include standardization, clinical evidence of sensitivity, selectivity, and test validity, that is, the degree to which a test score reflects what the test was designed to measure. Validity can be determined in different ways because of its many dimensions, but each is defined essentially by the criterion just described. Sensitive tests reflect earlier manifestations of toxicity or efficacy at low doses; tests with a high degree of specificity are less responsive to conditions not relevant to those the test was designed to detect. Another criterion is reliability or reproducibility. A test that fails to yield consistent results from one administration to the next will bury true differences in excessive variability. A related problem is standardization, which refers to the population from which the norms for certain kinds of tests are derived. The original Stanford–Binet intelligence test, for example, was developed and standardized on a white, middle-class sample of children, so the intelligence quotient (IQ) scores calculated from it probably are misleading when the test is given to black children from other sociocultural strata. The mode of framing the experimental question will also help guide choice. If the aim is screening or determining whether toxicity is detectable at a particular exposure level, functional breadth is accorded more importance than selectivity. If the aim is to clarify the nature of the functional deficits or their mechanisms, selectivity will receive greater emphasis.

It is important to note that sensitivity cannot be judged on an absolute scale in human studies, however. Only relative sensitivity, derived from a comparison of different measures, is within our grasp. Unlike the situation in animal studies, we rarely are in a position to exert control over exposure levels, studies with human volunteers being the exception. If one test instrument discriminates an exposed from an unexposed population more definitively than another, we may term it more sensitive. Such results do not indicate whether it could identify a population with even lower exposures. Further, the exposure level at which the effects are observed is not necessarily the lowest level of effect. One approach, advocated by Kennedy et al.,[234] calibrates the test battery by relating deficits in computerized performance tests to graded doses of alcohol.

Accumulated experience, coupled with theory and tradition, has led most investigators to select neuropsychological tests for batteries that encompass a fairly common set of behavioral domains. These may include tests of memory, of simple motor function, of complex cognitive performance, of attention and vigilance, and of mood or subjective state. Not all categories may appear in any single study, because some investigations are directed at one or a small cluster of functions. A common choice for inclusion in a test battery

TABLE 38.6
Subtests of the WAIS—Revised

Verbal	
Information	Questions of a general nature
Comprehension	Interpretation, judgment
Arithmetic	Numerical calculations
Similarities	Comparisons of nouns
Digit span	Repetition of digit sequences
Vocabulary	Word definitions
Performance	
Digit–symbol	Symbol–number coding
Picture completion	Identify missing portions
Block design	Duplication of patterns
Picture arrangement	Construct narrative sequence
Object assembly	Assemble jigsaw puzzle

has been the Wechsler Adult Intelligence Scale (WAIS), an attractive feature of which is its relatively careful standardization[235] and large body of accumulated experience. The WAIS is actually a battery of tests in itself and was designed to assess a broad sample of functions. It consists of 11 subtests assigned to verbal and performance categories to yield two major subscores (Table 38.6). This division is somewhat arbitrary because the two categories show considerable functional overlap and high correlations. Many investigators have chosen certain WAIS subtests rather than the complete assemblage for incorporation into their own repertoire of tests.

Vocabulary tests are examples of the verbal category. An example of the performance category is the digit–symbol substitution test of the WAIS, which requires entering symbols that are paired with numbers (digits) according to a specified code into appropriate blanks on a form. All of the subscales can be combined to yield a full-scale IQ score that correlates highly with academic achievement. The full-scale score, because it is a global score composed of the 11 subscale scores, is unlikely to provide much information about the precise areas of impaired function arising from exposure to a particular toxic agent. For this reason, some investigators have simply adopted certain subscales of the WAIS to assay-specific functions; however, the pattern itself is often useful for diagnosis, and techniques have been developed to explicitly relate the profile of performance to diagnosis.[236]

Two of the more widely used test batteries in worksite research are shown in Tables 38.7 and 38.8. The WHO neurobehavioral core test battery consists of a small number of components selected because of broad use and because they were not reliant on instruments or techniques that would be unavailable in developing countries. To verify the applicability of the WHO battery in different settings, Anger[233] compared performance in ten countries representing diverse populations. In general, the results from country to country were remarkably consistent. A major objection to the WHO battery[230] is that its emphasis on traditional manual and paper-and-pencil tests, largely to avoid complicated technical

TABLE 38.7
Neurobehavioral Evaluation System (NES3)

Functional Domain	Core Test
Motor speed, steadiness	Aiming; placing dots in circle
Attention	Simple reaction time
Perceptual–motor	WAIS digit–symbol
Manual dexterity	Santa Ana test
Visual memory	Benton visual retention test
Auditory memory	WAIS digit span

TABLE 38.8
Neurobehavioral Evaluation System (NES3)

Vocabulary	Tracing
Continuous performance test, letters	Symbol–digit
Continuous performance test, animal	Pattern recognition
Auditory digit span	Line orientation matching
Visual span	Incomplete figures
Paced auditory serial addition test	List learning
Sequencing B	List delayed recall
Diamond naming	Pattern memory
Finger tapping	POMS

equipment, especially computer-based administration and scoring, diminishes its sensitivity to neurotoxic effects.

One development in computerized testing, pioneered by Anger and colleagues,[237] uses both computer technology and operant behavior principles to deliver instructions to subjects in novel ways. Reliance upon language to deliver instructions can be an impediment to the use of neurobehavioral tests internationally because of the problem of translation. To avoid such problems, a set of preliminary instructions is provided so the subject's responses are operantly shaped by techniques such as successive approximation to the criterion response to conform to the test requirements. After the subject masters the test procedure, he or she is then ready for the actual test. Even under these circumstances, which are designed to minimize educational and cultural contributions, such variables still exert a pronounced effect. Another study[238] compared subjects of European descent with American Indian, African-American, and Latin-American populations on two consensus neurotoxicity test batteries. One was the WHO collection (Table 38.7), and the other one was assembled by the Agency for Toxic Substances and Disease Registry (ATSDR). Education accounted for the most variance in the tests studied, followed by cultural group. Years of education and cultural group had 13%–25% shared variance on the cognitive tests, suggesting that these factors should be controlled during the design phase of a study rather than in the statistical analysis. The authors stress that failure to adequately control and analyze these variables could lead to inaccurate conclusions about the association between poor performance and neurotoxic insult.

The neurobehavioral evaluation system (NES), devised originally by Letz and Baker[239] to exploit the potential of computer administration and scoring,[71] has been among the most widely used of the current batteries (Table 38.8). It embodies three main categories of tests: psychomotor, memory and learning, and cognitive (executive function). It is a much more ambitious battery than the WHO collection and has been translated into several languages other than English. In its current form (NES3), it includes 18 tests of functions such as reaction time, motor coordination, and simple cognition.[240] Other groups have devised batteries of their own, based on personal preferences, theories, or unique aims, but the NES and WHO formats offer the most extensive databases.

Experimental Psychological Batteries

Experimental psychological-based tests based on approaches that can be used widely across species have gained increasing use in behavioral evaluation. One such example is the Cambridge neuropsychological testing automated battery (CANTAB), a computer-automated battery of neuropsychological tests designed for accurate, sensitive cognitive assessment.[241] It includes tests of memory, attention, and executive function administered via a touch screen, many identical to those used in the experimental psychology domain. Studies carried out to date have shown its sensitivity to a variety of neurodegenerative diseases and behavioral dysfunctions, including Alzheimer's, Parkinson's, and Huntington's diseases, attention deficit disorder, autism, depression, and schizophrenia, as well as age-related deficits in cognition. Moreover, its utility extends from children as young as 4 years old through seniors, and its potential for use in neurotoxicology has been suggested.[241] Studies demonstrate the sensitivity of CANTAB in revealing deficits in spatial memory and reversal learning in lead-exposed children as young as 4–5 years of age.[242] Indeed, this battery has great utility for elaborating specific behavioral deficits related to chemical exposures in human studies. The fact that many of these tests are the same as those used in an extensive experimental animal literature allows extrapolation from a rich base of experimental studies that includes neurobiological and neurochemical substrates of specific functional deficits. In addition, definition of specific deficits in human populations in response to neurotoxicant exposure using more specific and precise tests of behavioral domains can also be used to refine animal models and for the development of behavioral therapeutic approaches.

Memory

Complaints of impaired memory surface frequently in workers exposed to neurotoxic agents and, according to a survey by Anger,[233] are the functions most often assessed in worksite research. Memory disorders also appear in adults with focal brain damage and in patients suffering from degenerative neurological diseases. Given the prominence of such complaints in patients and the central role accorded memory in psychological theory and in neuroscience, it is not surprising that numerous tests and techniques are available for the study of this function. A frequent distinction is made between immediate or working or short-term memory and remote or reference or long-term memory. Such distinctions, and the more elaborate ones offered by current workers, often are difficult to apply to specific experimental conditions, and it has even been argued that the term *memory* itself has grown too vague to be useful scientifically. Often, terms borrowed from computer technology, such as *storage* and *retrieval*, are alleged to account for memory. But, if memory, as noted earlier, is defined by responses based on earlier experiences, this more neutral and empirical definition is a better platform from which to launch useful experiments, and the examples that follow are presented from an empirical standpoint for that reason.

One widely used test is the WAIS assay of short-term memory known as *digit span*. Traditionally, the examiner calls out a series of numbers that the patient or subject is asked to repeat. Smith et al.,[243] like many other current investigators, adapted the digit-span technique to the computer and presented the numbers, one at a time, on a display terminal. Their subject population consisted of workers from two mercury-cell chloralkali plants whose urinary mercury concentrations had been monitored repeatedly. The lists of digits were presented with ascending length, beginning with three digits. If the worker then recited the list correctly, a list with four digits was presented, and so on. Errors on two successive presentations of the same list length halted the test, and the worker's score was noted as the length of the previously correct list. No relationship with mercury excretion was observed, primarily because the standard WAIS procedure proved to be unreliable, yielding a correlation between successive administrations of 0.36. To overcome this inherent flaw, the investigators modified the procedure so they could use probit analysis to estimate the 50% threshold span. With this procedure, the reliability coefficient rose to 0.85, and regression analysis showed a significant correlation between this measure of digit span and urinary mercury concentration.

Hanninen[70] also noted that the digit-span subtest of the WAIS can produce ambivalent results; however, Baker et al.,[244] in a study of foundry workers exposed to lead, obtained a significant relationship between blood lead values and performance on a version of the digit-span test that required the subjects to repeat the list backward. It is possible that this result arose not so much from the inability of workers to remember the list but from intruding factors such as the fatigue, depression, and confusion detected by an inventory of subjective state. Complaints of cognitive difficulties and lowered scores on memory tests may also stem from sources such as depression and the inability to concentrate.[245] These potential confounds need to be addressed in interpreting behavioral outcomes. Variations of the digit-span procedure that are said to test verbal memory include letter span and word span. One contribution made possible by computer-based batteries is a restructuring of tests such as digit span

to offer adaptive procedures in which the presentation of test items is contingent on the performance of the subject, as in programmed instruction.

In the Benton visual retention test, a test of nonverbal memory, a common component of many neuropsychological test batteries,[246] the subject is asked to reproduce a three-figure design. Scoring systems have been developed to quantify fidelity and to correlate types of distortions with different kinds of brain damage. The test is sensitive to brain lesions that lead to neglect of one side of the body,[247] but such deficits are uncommon in neurotoxicology. Another variant, the Graham–Kendall memory-for-designs test,[245] consists of a series of 15 geometric designs presented to the patient one at a time for 5 s. The patient is then asked to copy the design, and the reproduction is scored for various kinds of errors.

The ability to memorize lists of words or nonsense syllables is commonly assessed in neuropsychological test batteries. The Wechsler Memory Scale®[248] contains a paired-associate learning task consisting of 10 word pairs. Some, such as baby–cries, are relatively simple; others, such as cabbage–pen, are less transparent. The list is read three times. After each repetition, a test trial is conducted during which the examiner calls out the first word and the subject is required to say the second word of the pair. This kind of learning paradigm has a long history in experimental psychology, and many different kinds of items, including pictures and nonsense syllables, have been used as materials. Adverse effects of food dyes on young children were demonstrated by the paired-associate learning of number and zoo (animal) combinations.[249]

As part of their performance battery used to test lead workers, Williamson and Teo[250] also examined paired-associate learning of five pairs of three-letter words. The subject was required to write down the second member of the pair after the first member of the pair was presented. This sequence was repeated until the subject was able to identify all five pairs. Lead-exposed workers recalled fewer items than controls after the first presentation (mean of 0.72 vs. 2.19), required more trials to reach criterion (mean of 3.74 vs. 2.81), and had a higher incidence of individuals failing to reach criterion (64.2% vs. 13.8%).

Although it may have been sensible in the beginning to choose methods for assessing memory function that were based on widespread diagnostic acceptance, new concepts, data, and techniques are emerging at a rapid rate, including the efficacy and utility of experimental psychology approaches. Sahakian et al.[251] relied on complex delayed matching-to-sample stimuli, presented by computer displays in the CANTAB battery and described earlier, to evaluate memory deficits in Alzheimer's disease. In this paradigm, subjects are first shown a *sample* stimulus, which is then removed. The subject must choose the sample from among several stimuli subsequently presented after a delay that matches the original sample. Since then, such techniques have been increasingly used to determine sensitivity for detecting Alzheimer's disease and mild cognitive impairment, thought to be a precursor to Alzheimer's disease. An accumulating literature has continued to demonstrate the sensitivity and specificity of the CANTAB delayed matching-to-sample paradigm in such cases.[252] Recently, using multiple linear regression analyses adjusted for age, education, and smoking status, a significant association of higher calcaneal bone lead levels with increasing memory impairments on the CANTAB delayed matching-to-sample paradigm was reported in a cohort of 47 healthy subjects ranging from 55 to 67 years of age.[253] In addition, the CANTAB version of episodic memory, paired-associate learning, has likewise been shown to be sensitive to these impairments.[252,254,255] In this paired-associate learning test, the subject is shown a screen on which a set of boxes are displayed (up to 8). The boxes are opened, one at a time, and contain a stimulus pattern. After all the boxes have been opened, each of the stimulus patterns is presented to the subject, one at a time, and the subject must recall the location of the box that contained that stimulus pattern. As previously noted, these emerging trends are bound to influence behavioral toxicology and eventually to displace or augment the traditional approaches that so far have dominated the literature.

Attention

Attention is a behavioral construct that includes three diagnostic categories: hyperactivity, inattention, and impulsivity, all of which require different behavioral approaches. Reaction time measures, frequently used to measure sustained attention, are relatively simple to implement and generate straightforward values that do not require elaborate clinical interpretation. Basic reaction time paradigms can be modified to provide an immense range of complexity in the assessment of many kinds of stimulus variables. A frequent variation is to compare reaction times under conditions where alterations in responding are required in accordance with the location or other properties of the stimulus. Reaction time measures seem to reflect both acute and subchronic toxicities resulting from volatile organic solvent exposure. Experiments with workers exposed to styrene, a solvent widely used in fabricating fiberglass boat hulls, called for the experimenters to visit the plant on Friday, familiarize the workers with the reaction time situation, and deliver a questionnaire about work history and personal habits.[256] On the succeeding Monday, simple reaction times were measured with a portable device at the beginning and end of the shift, and urine was collected by each subject until bedtime. Blood samples were drawn at the end of the shift. Following the suggestions of Gamberale and Kjellberg,[257] the rate of stimulus presentation was fairly high (16 trials per minute), a decision based on findings that such high rates lead to a quicker decline in performance.

Men exposed to styrene began the shift with slower reaction times than did men in a reference group, but by the end of the day, the groups did not differ. Within the exposed group, however, those men with the highest blood levels of styrene showed no improvement and displayed the greatest deterioration in ratings of alertness and exhaustion. In a subsequent study, the investigators observed substantial correlations between urinary mandelic acid (a styrene metabolite) and

reaction time performance on Monday morning, indicating that, even with a 60 h period away from the plant, the effects of exposure were still evident in performance. Exposure levels, it should be noted, did not exceed the British TLV® of 100 ppm.

Needleman et al.[258] demonstrated the sensitivity of reaction time measures to an index of cumulative lead exposure in children by comparing levels of dentine lead in the highest and lowest deciles of a distribution obtained from two Boston suburbs based on more than 2000 children. They used simple reaction times but varied the interval between the ready signal, which alerts the subject to the next stimulus, and the presentation of the stimulus. On two blocks of trials, the interval was specified as 3 s; on two other blocks, it was specified as 12 s. These values were chosen to probe for the possible influence of distractibility. The longer interval produced longer reaction times in both groups, but for both intervals, the children with the higher lead levels responded more slowly than those with the lower levels. A subsequent study by Yule et al.[259] confirmed these results in British children with even lower lead levels, while a study from Germany adds another dimension of support to these findings.[148] In addition to confirming the relationship between tooth lead and reaction time performance, these investigators also observed that, when the children were categorized by social class, the data of those from the less-advantaged group showed an even greater correlation with tooth lead than the group as a whole.

Despite a large number of publications featuring or including such measures, however, the power of reaction time measures to detect neurotoxicity remains only superficially exploited. Gamberale et al.[230] and Iregren and Gamberale[260] demonstrated that, with computerized testing and analysis, the sensitivity of such measures can be enhanced and can even contribute to the differentiation of various diagnostic groups, such as those suffering from solvent-induced deficits. One of the limitations of reaction time measures is the common practice of ignoring stimulus properties such as intensity. Almost never is the brightness of a stimulus light or the properties of a stimulus tone reported, even in studies of agents known to affect vision or hearing. Because reaction time is sensitive enough to stimulus intensity to be used as a psychophysical measure,[261] ignoring stimulus characteristics can lead to the suspicion of confounding.

Several different measures have been used to examine sustained attention. The most frequently used is the continuous performance test or some variant thereof, which is a more complex version of reaction time that can be made to be relatively complex and tasking and utilized in a parametrically appropriate fashion across species. Basically, the subject is instructed to watch for the occurrence of specific targets over time using computerized presentation. These can range from a single number or tone or may require detection of a sequential series of digits. Further, they include measures beyond simple reaction time, including accuracy of detection, omission errors, and commission errors as well as the ability to use measures of signal detection theory. Such tests have been found to be influenced by low-level lead exposure in children, leading to decreases in number of correct detections and increased numbers of omission errors,[262,263] findings consistent with studies in experimental animals.[264]

Highway driving and piloting aircraft are situations that require competence in motor performance and in attention and are often stated to require *vigilance*. Some examples are provided by power stations and chemical process plants where remaining alert enough to respond to infrequent or slowly changing signals is critical and is challenged by shift-work schedules that induce chronic sleep disruption. In the laboratory, these functions can be examined in detail because of the available instrumentation and time. In field testing, where simpler, often portable equipment is mandated, and where workers must be allowed time from their jobs, performance assessment is usually directed toward global screening for adverse effects rather than determining definitive answers about the parameters of dysfunction.

A prototype vigilance task is the clock test devised by Mackworth, which has served as the criterion task in many studies with drugs.[265] It is arranged so a pointer on a clock face moves 1° every second, except for occasional deflections of two steps. The subject's task is to respond to these infrequent events by pressing a key. Typically, the frequency of detections falls sharply after the first 30 min but can be counteracted by administering drugs, such as the amphetamines, that promote alertness. Dick et al.[266] included the clock test in a battery to evaluate experimental exposures to toluene, methyl ethyl ketone, and ethanol. Exposure to 100 ppm toluene (the TLV) for 4 h lowered the proportion of correct detections. Ethanol also impaired performance at a dose of 0.8 mL/kg.

More complex vigilance performance, which may demand a certain amount of coordination skills, also offers unexplored potential. In a series of classic studies, Payne and Hauty[267] reported the effects of various drugs and parametric variations on the school of aviation medicine multidimensional pursuit test,[268] which was designed originally as a tool for pilot selection. The subject was seated in a simulated aircraft cockpit and asked to monitor four dials. The pointers on the dials drifted irregularly from their null positions, and the subject's task was to restore them to null by manipulating a control stick, rudder pedals, and a throttle control. With a typical group of subjects, total time at null reached asymptotic values after about 1 h of practice, but with further testing began a gradual decline. The rate of decline was enhanced by drugs with sedative actions and retarded by drugs such as the amphetamines, even at remarkably low-dose levels. The unusual sensitivity of this task to drugs suggests that it, or a contemporary analog based on computer technology, deserves to be evaluated in behavioral toxicology.

The multidimensional pursuit test is not simply watchkeeping, because it also embodies the type of visually directed motor performance called tracking. Mackay et al.[269] examined the acute effects of carbon monoxide on a tracking task. Subjects controlled the position of a cross displayed on an oscilloscope screen by moving a joystick and were required to maintain the cross within a square target that

moved continuously around the screen in random patterns. During a single test session, they performed on five 1 min trials. Carbon monoxide significantly increased error amplitude and decreased time on target during the 2 h exposure period. Tracking tasks are now included in a number of test batteries; for example, one of the tests used in studies of methylmercury-exposed children required the subjects to follow a sine-wave display by appropriate movements of a joystick.[270]

More typically than not, performance demands occur in settings requiring more than one task to be monitored and responded to concurrently. Driving, piloting, air traffic control, and scanning and adjusting medical monitoring equipment provide examples. Most neurobehavioral test systems strive to examine one function at a time, making extrapolation to multitasking situations somewhat uncertain. A study conducted by Rahill et al.[271] employed a multitasking scheme to evaluate the performance effects of exposure to 100 ppm of toluene (see Figure 38.27). Significantly lower composite performance scores were obtained by the subjects during toluene exposure.

A third domain of attention deficit is impulsivity, also referred to as inability to manage delayed gratification and loss of self-control. Delay of reward paradigms is one measure of impulsivity and has been used across species. These paradigms essentially provide the subject with a choice between a small reward following a short delay and a large reward but only after a longer delay. Surprisingly, although validated for ADHD,[272] such paradigms have yet to be frequently employed in neurotoxicology. Another connotation for impulsivity is inability to inhibit responding. This is frequently evaluated using time-based schedules of reinforcement (see Figures 38.20 and 38.21), again with applicability across species, although to date used primarily with experimental animals. For example, on a DRL schedule, responses must be separated by *t* seconds in order to obtain a reward; thus, impulsive or premature responding will impair performance and decrease total number of rewards obtained. Such studies have revealed impairments in response inhibition in response to lead exposures as well as polychlorinated biphenyls (PCBs).[264]

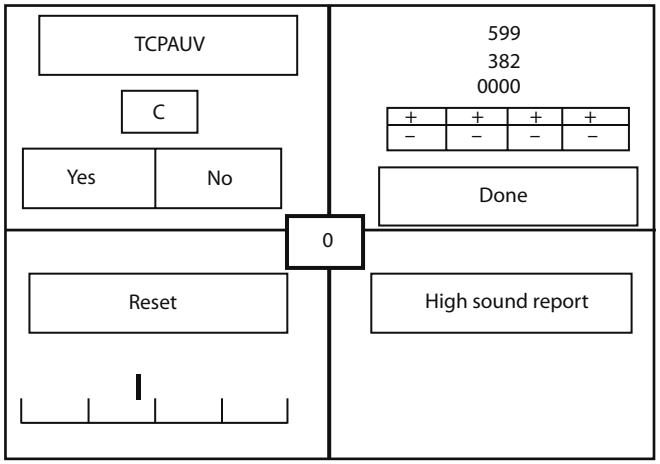

FIGURE 38.27 Multitasking performance test (SYNWORK). (Top left) A series of letters appears on the screen, then it is removed. In the following box, letters are flashed in sequence, and the subject is required to indicate whether or not the letter belongs to the original set. (Top right) The subject calculates the sum of the two upper numbers and, using the mouse, scans through the numerals 0–9 to place them in the appropriate positions in the third number. (Lower left) The cursor drifts toward the end of the scale, and the subject is required, by mouse clicks, to prevent it from reaching the end. (Lower right) A series of tones is presented through a headset. Intermittently, a louder tone is inserted. The subject is asked to respond when the louder tone is detected. The box in the center of the screen displays the subject's numerical score for that session. The SYNWORK task proved sensitive to toluene exposure. (From Rahill, A.A. et al., *Aviat. Space Environ. Med.*, 67, 640, 1996. With permission.)

Subjective State

Certain clusters of symptoms seem to be characteristic of different toxic exposures. Mercury vapor induces a cluster so well known that it earned the label *erethism* (Table 38.9). Chronic solvent exposure is associated with complaints of tiredness, depression, and confusion. Roels et al.[52] and Mergler and Baldwin[273] found excessive fatigue to dominate the list of subjective complaints among manganese workers. Measurement of such vague symptoms is not a task to be undertaken without an appreciation of the principles of psychometrics. The earlier discussion of validity and reliability probably applies more directly to symptom measurement than to almost any other criterion because accessory criteria are so difficult to define. Memory, for example, can be assessed by enough different techniques to provide some index of consistency. A complaint of depression is not as readily confirmed. To simply construct a questionnaire based on what the investigator believes are the most conspicuous symptoms associated with a particular exposure is a virtual guarantee of uninterpretable data. For this reason, neuropsychologists rely on standardized instruments such as the Beck depression inventory.[274]

An example of the arduous process required to design and develop a quantitative measure of specific symptoms is described by Goldberg,[275] who aimed to construct a scale sensitive to minor psychiatric illness. The evolution of the questionnaire proceeded through several steps: selection of items, including careful editing of wording; decisions about the form of the response (i.e., whether to ask for a *yes–no*

TABLE 38.9
Symptoms of Erethism

Hyperirritability
Blushes easily
Labile temperament
Avoids friends and public places
Timid, shy
Depressed, despondent
Insomnia
Fatigue

response or to ask for some form of rating such as a scale from *infrequent* to *always*); selecting criterion groups and then determining which items of the inventory discriminate among them (a measure of validity); and assessing the consistency of responses within individuals (reliability). Some of these stages may be repeated and items added, deleted, or modified. Some items may carry an excessive burden of social desirability and may not be answered with total candor. Several other well-designed inventories of subjective state are available that also offer considerable data about validity and reliability. Some were constructed, such as the Goldberg questionnaire, to detect mild psychiatric dysfunction.

Some were developed to measure the acute effects of drugs or to follow the course of drug therapy. The profile of mood states (POMS) is a self-rating scale designed to measure subjective responses to various therapeutic or experimental maneuvers.[276] It consists of 65 adjectives that the subject judges on a 5-point rating scale that has been tested on both psychiatric patients and normals. A factor analysis yielded the following major factors: tension–anxiety, depression–dejection, anxiety–hostility, vigor–activity, fatigue–inertia, and confusion–bewilderment. Baker et al.[244] included the POMS in a battery given to lead foundry workers and observed marked elevations in several of these factors in workers with the higher blood lead levels. The SCL-90[277] was devised as a self-report rating scale for psychiatric outpatients and, like those already referred to, provides a set of dimensions (factors) extracted from the entire 90-item scale. Its designers cite it as particularly useful in clinical drug trials undertaken to evaluate psychoactive agents. Anyone contemplating the framing of an ad hoc questionnaire should note the care taken in the construction of the SCL-90. For example, to make the wording of the items accessible to the widest variety of subjects, the designers of the inventory turned to the Thorndike–Lorge *word book*, which provides frequency counts obtained from materials such as newspapers for 30,000 words. It allowed them to equate the vocabulary levels of the nine factors and to select the most basic word levels to express the item accurately.

One example of the data that may be extracted with a properly designed inventory was provided by Levin et al..[278] They compared 24 control subjects matched by age and education with 24 subjects who had been exposed to organophosphate insecticides in recent weeks. The subjects were evaluated by a structured interview, a depression inventory, and the Taylor manifest anxiety scale consisting of 50 true–false items derived from the Minnesota multiphasic personality inventory, which enjoys wide use in psychiatric diagnosis. These items include both psychological and somatic manifestations of anxiety. The structured interview and the depression scale yielded no differences between the two groups, but several items on the Taylor scale provided a clear separation. These are listed in Table 38.10 and support the view that organophosphate exposure may induce subclinical psychological effects that would not be detected by conventional medical surveillance or even by inventories and questionnaires that did not contain specific kinds of symptom items. The 13 commercial applicators in the exposed group (the others were farmers) accounted for the bulk of the difference; their plasma cholinesterase levels, however, fell within the normal range, suggesting that this exposure measure is not an accurate reflection of central cholinergic status.

TABLE 38.10
Items on the Taylor Manifest Anxiety Scale Sensitive to Differences in Organophosphate Insecticide Exposure

I work under a great deal of tension.
My sleep is fitful and disturbed.
I have periods of such restlessness that I cannot sit long in a chair.
I believe that I am no more nervous than most other people.
I feel anxiety about something or someone almost all the time.
I am usually calm and not easily upset.
It makes me nervous to have to wait.
I practically never blush.

TABLE 38.11
Persistent Neurological Sequelae of Acute Organophosphate Poisoning

Test Name	100 Cases	100 Controls	p-Value
WAIS verbal IQ	105.40	111.86	0.001
WAIS performance	108.41	110.13	0.242
WAIS full scale	107.50	111.77	0.001
Impairment rating	1.07	0.91	0.001
Halstead index	0.30	0.23	0.020
Pegboard	148.34	137.96	0.002
Card sorting	17.07	12.91	0.001

A more ominous legacy of organophosphate exposure is now undergoing examination. Earlier probes of the aftermath of acute organophosphate poisoning had suggested persisting neurotoxic consequences, but Savage et al.[279] were the first to combine well-documented exposures and neuropsychological measures. They reported on 100 individuals who had experienced a poisoning episode 1 year earlier and on 100 matched controls. Although clinical chemistries and medical examinations indicated normal function in the exposed group, psychological testing indicated residual deficits as measured by several WAIS subtests and other indices (Table 38.11). Subsequent reports by others have confirmed these findings.[280]

Developmental Assessment

Most of the tests described earlier were devised to assess adults. Many questions about behavioral toxicity, however, arise from the possible impact on early development and involve testing during infancy or childhood, which has been the domain of behavioral teratology. Methylmercury, lead,

and PCBs are examples of substances closely connected with developmental disturbances. Although the essential functions that should be evaluated remain the same throughout the life cycle, early developmental stages can pose several unique problems. One is that young children have not yet acquired or developed the full behavioral and functional repertoire of adults. Another is that early childhood is a period of rapid change with marked individual differences in rate of development, one of the factors that dilutes the ability of tests administered at that time to predict later capacities. For these and associated reasons, investigations of developmental toxicity have to adopt approaches that sometimes differ markedly from those suitable for adults. Infancy is the most difficult stage to evaluate because of the plasticity of infant behavior.

The most common scale of infant development is the Brazelton Neonatal Behavioral Assessment Scale,[281] which usually is administered within the first 3 days after birth. It consists of two subscales. One includes items reflecting habituation, motor activity and control, stimulus responsiveness, and similar responses among 27 behavioral items. The other is comprised of 21 neurological items based on elicited reflexes. Although Brazelton scores are not highly correlated with tests of later function, they have proven capable of reflecting the effects of obstetrical medications.[282]

One of the best examples of how the Brazelton scale might be used in a study of developmental toxicology is provided by Streissguth et al.,[283] who reported their findings on a cohort of about 500 children, selected at birth, to determine the consequences of various levels of maternal alcohol consumption. The mothers of these children were interviewed during the fifth month of pregnancy to obtain data on this and other relevant drug practices, as well as on social, educational, and demographic variables. Habituation in the infant was a special focus of this investigation because of observations indicating that neonates with a clinically overt fetal alcohol syndrome failed to display a response decrement to repeated auditory stimulation. A factor analysis of scores (a procedure that reduced the 27 original Brazelton scores to a smaller number of dependent variables) yielded 6 independent factors. After a multiple-regression analysis, which adjusted for possibly confounding contributions from smoking, caffeine, maternal nutrition, and so on, maternal alcohol consumption during pregnancy remained a significant determinant of poorer habituation and increased low arousal in the infants. The care taken by these investigators to ensure that the examiners were kept uninformed of the other data and the efforts made to train and preserve consistency in examiner ratings can serve as a model for such studies.

The ultimate question for investigators is how well measures obtained during infancy predict later outcomes such as intelligence test scores or school status. One of the more promising techniques, the Fagan test of infant intelligence, measures visual recognition memory.[284] The infant is held so it faces a display with two screens, behind which an observer can record the amount of time the infant's gaze is directed to each screen. On an individual trial, a particular visual stimulus, such as a face, is projected on one of the screens. After the infant has had time to examine the stimulus and after a brief interval, the previous stimulus and a new one are presented simultaneously. The observer measures the amount of time spent by the infant in examining the novel stimulus compared to the one displayed earlier. The novel object recognition test described earlier and used in rodents represents an analog of the Fagan test. Infants who score higher on later developmental tests such as the Stanford–Binet tend to spend less time viewing the old stimulus. In studies exploring the developmental neurotoxicity of PCBs, the Fagan test revealed a significant relationship between umbilical cord PCB levels (attributed to maternal consumption of tainted Great Lakes fish) and psychological development.[285] Gunderson et al.[286] found that infant monkeys exposed prenatally to methylmercury spent more time than controls gazing at the previously displayed stimulus.

For later stages of infancy, encompassing a range from 2 to 30 months of age, the Bayley Scales of Infant Development[287] are among the most popular because of the care taken in their standardization and their ability to be used for repeated testing. The Bayley items are arranged chronologically and divided into three subscales: mental, motor, and behavioral. The mental scale is composed of 163 items, ranging from responses to visual and auditory stimuli to abilities such as naming objects. A notable application to toxicology was suggested in studies relating cord blood lead concentrations to performance, summarized by Bellinger and Needleman.[288] At 6, 12, 18, and 24 months of age, even after correcting statistically for many potential confounding variables such as maternal age and intelligence, a significant relationship between cord blood lead and scores on the mental development index emerged. Children in the group with the highest cord blood lead values (a mean of 14.6 μg/dL, compared to 6.5 for the middle group and 1.8 for the low group) attained the lowest scores at all ages tested. At 24 months of age, the difference between the lowest and highest groups reached 8%, although by that time, all three groups had converged toward the same mean blood level of about 7 μg/dL. Similar findings have since emerged from other prospective studies in Cincinnati[289] and Australia.[290]

At later ages, the number and variety of instruments available for psychological assessment are overwhelming. Most investigators choosing a battery of tests will typically include one of the intelligence tests designed for children. The Wechsler Intelligence Scale for Children–Revised (WISC-R)[291] is the dominant choice for children older than 6 years of age. In addition to its demonstrated reliability, it is attractive because, like the WAIS, it provides separate verbal and performance subscales that include several individual tasks. These offer a profile of the child's abilities across a variety of functional components. For younger children, the Wechsler Preschool and Primary Scale of Intelligence (WPPSI)[292] yields the same subscale advantages as the WISC and is basically an extension of the WISC for ages 4–6.5 years. Another instrument suitable for younger children, the McCarthy Scales of Children's Abilities,[293] also extends into

early school years, spanning 2.5–8.5 years of age. It provides five separate scales that can be combined into what is called a *general cognitive index*, similar to an IQ. All three of these, plus the older Stanford–Binet, have been used to assess the effects of lead. Needleman et al.[258] included the WISC-R in their landmark study, which indicated lowered performance on IQ tests in those children whose tooth lead concentrations fell at the upper portions of the distribution. Their results have since been confirmed by other investigators, including some from outside the United States who also used the WISC-R or an adaptation of it to measure intellectual function. This is one illustration of how reliance on a standardized test allowed different investigations to be combined with some degree of confidence.

Needleman[294] and Bellinger and Needleman,[288] in two companion articles, discuss the strategy for using such scores in a context where many potential confounding variables should be considered. Some examples, besides obvious factors such as race, birth weight, and length of gestation, are maternal age, birth order, parental education, family social class, and quality of the rearing environment. Even when these and other variables were compensated for in the statistical analysis, cord blood lead values remained significantly associated with reduced scores on the mental development index of the Bayley scales. Parallel conclusions have been drawn by other groups.

As in adult epidemiological studies, most investigations try to assemble comprehensive test batteries to try to determine relationships between neurotoxic exposure across a broad spectrum of functional outcomes. For the evaluation of the cohort of another large prospective methylmercury study,[270] in the Faroe Islands of the North Atlantic, investigators chose a neuropsychological test battery for 7-year olds consisting of the following components: finger tapping, hand–eye coordination, tactual performance test (shape discrimination when blindfolded), continuous performance test (vigilance and reaction time), WISC-R (digit spans, similarities, and block designs), bender visual motor gestalt test, Boston naming test, California verbal learning test (children), and a pictorial analog of the POMS survey. They also included a chart that is used to measure visual contrast sensitivity and several neurophysiological measures. Their analyses pointed to mercury-related deficits primarily in the domains of language, attention, and memory, but they also observed deficits in visuospatial and motor functions. Subsequent studies of this cohort have indicated that coexposures to PCBs from consumption of whale blubber[295] could be contributing to these deficits.

Evaluations of the Seychelles cohort, where PCBs are not present, have so far failed to demonstrate any adverse developmental outcomes due to methylmercury.[296] For this assessment, the test battery has included the McCarthy Scales of Children's Abilities (the general cognitive index), the Preschool Language Scale for measuring expressive and receptive language, two subtests of the Woodcock–Johnson series designed to measure reading and arithmetic achievement, the Bender–Gestalt test, and the Child Behavior Checklist designed to assess social and adaptive behaviours.

TABLE 38.12
ATSDR Test Battery

Vineland adaptive behavior scales	Kaufman brief intelligence test
Parenting stress index	Story memory
Personality inventory for children	Finger tapping
Henderson environmental learning process scale	Divided attention test
Family resources scale	Visual–motor integration
Visual acuity, contrast sensitivity	Purdue pegboard
Contrast sensitivity pretest	Verbal cancellation
Vibration II	Story memory delay

These two groups of investigators selected somewhat different arrays of instruments for these two parallel investigations because they were pursuing somewhat different questions and because they were evaluating different age ranges and stages of maturity. All investigators agree that no one test battery will be suitable for all purposes or populations. Selection will be guided by the questions asked, the population studied, and the investigator's inclinations. Often, designers of test batteries attempt to provide researchers with a menu of tests, so to speak. One example is the battery assembled[225] to assist the ATSDR, which is responsible for evaluating health risks at hazardous waste sites in the United States. The prototype test battery consists of the components shown in Table 38.12. The entire battery, because it is designed for screening populations rather than for the intensive appraisal of small groups or individuals, requires only about 1 h for children. The first five items refer to information secured from the parent.

Emerging Issues

Like other components of contemporary toxicology, behavioral toxicology is undergoing almost continuous change. Its literature has grown almost exponentially, and the range of techniques applied to its problems has expanded over a broader spectrum of behavior and neuroscience. Compare, for example, the first U.S. book on the topic[297] with a comprehensive survey published almost 20 years later.[298] The range of topics is not only broader but now more firmly based on a comprehensive literature. Especially for human assessment, however, greater efforts are necessary to extend the range, relevance, and specificity of test procedures. Although standardization and an extensive history of use are advantages, they should not limit investigators. Advances in test development are at least as crucial to behavioral toxicology as the analysis of toxic effects themselves. Contrast, for example, the relative crudeness of sensory and motor testing in most test batteries with the precise, elegant methods described in earlier sections of this chapter that have been applied to animals.

Even cognitive function, which has received the bulk of attention from neuropsychology, is only superficially addressed in most test batteries. Among the more promising advances in this area are those stemming from the translation of rigorous

laboratory test procedures into practical test methods suitable for clinical and even field evaluations, such as CANTAB, described previously,[241,254,255] and an operant battery for measuring complex performance in children[299] that, like the items on intelligence tests, reflects developmental maturity. Both have the advantage of direct comparability with the techniques used for assessment in animals with the associated literature on neurobiological substrates. In addition, because of the flexibility afforded by computer technology, both can introduce increasingly difficult versions of the same basic test to avoid ceilings on performance. Finally, because they do not require verbal responses, problems of language translation and illiteracy can be bypassed. As more data have become available, the ability to more precisely specify hypotheses with respect to predicted functional deficits should become possible, allowing the use of increasingly sophisticated and specific methods in both human and experimental studies.

QUESTIONS

38.1 New behavior can be generated in two basic ways: operant conditioning and respondent conditioning. They differ in (1) the type of response, (2) the conditioning procedure, and (3) the criteria for judging the strength of the conditioned response. How would you use each type of conditioning to evaluate impaired learning capacity? What potential confounds must be considered?

38.2 As a test of memory difficulties, design a procedure to test whether a rat can remember which of two levers (a left and a right, say) it had pressed 10 s earlier.

38.3 Workers in a machine shop, where tools are constantly being degreased, have been complaining that, away from work, their friends and families are telling them that they are confused about identifying colors. Could there be some validity to their complaints? How would you test them?

38.4 The Food Quality Protection Act of 1996 is a product of growing concern over vulnerability of children to environmental chemicals. What components of a neuropsychological test battery might be used to evaluate whether children living in a community located near a waste dump might be suffering adverse neurobehavioral effects?

KEYWORDS

Behavior, Operant conditioning, Respondent conditioning, Motor function, Sensory function, Learning, Memory, Attention, Schedule-controlled behavior, Screening batteries, Stimulus properties of chemicals

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39 Organelles as Tools in Toxicology
In Vitro and In Vivo Approaches

Bruce A. Fowler, Joseph R. Landolph, Jr., Kathleen Sullivan, and A. Wallace Hayes

CONTENTS

INTRODUCTION

As the interdisciplinary science of cell biology has evolved, it has become clear that not only are cells composed of discrete organelle systems that carry out essential functions for cellular survival but also these organelle systems interact with each other in a cooperative manner. Toxic chemicals that preferentially damage specific organelle systems may also damage cellular homeostasis and viability in a secondary manner by disrupting the relationships between intracellular organelle systems, thus further exacerbating cell injury processes leading to cell death or neoplastic transformation. Prior versions of this chapter[1] have focused on chemical toxicity to major target organelles and available methods for measuring these events. The present chapter takes a more *organelle systems toxicology* approach and, wherever possible, also examines chemically induced disruption of physical and functional relationships that must exist between organelle systems for maintaining homeostasis and that are disrupted by chemically induced toxicity and contribute to processes of cell injury and cell death. Data from both in vivo and in vitro studies will be considered in an effort to show how inclusion of an integrated organelle systems approach adds further insights into understanding basic mechanisms of cell injury/cell death and the central role of organelles in these processes. It is also important to note the recent appreciation from cell biology studies that organelles such as mitochondria are not only physically dynamic on an individual basis to divide and fuse but also appear to move about in the cytoplasm of the cell, suggesting a more macrointernal dynamic organization that contributes to interorganelle cooperation. Disruption of interorganelle cooperation during the initiation of toxic cell injury undoubtedly contributes to subsequent deleterious events ultimately resulting in cellular demise or neoplastic cell transformation. Understanding these phenomena is a complex task, but fortunately the advent of computer-assisted models[2,3] should help to better document and appreciate the dynamic nature of the subcellular organization and interorganelle interactions.

IN VITRO AND IN VIVO TEST SYSTEMS: GENERAL CONSIDERATIONS

It is important to consider the source and state of cells from which organelles are evaluated. Factors such as the composition of cellular growth medium for in vitro studies and nutritional status, age, and gender for in vivo studies need to be considered and documented, since these factors may influence the responses of intracellular organelles and systems regulating those organelles to insults from toxic chemicals.

In Vitro Systems

There are many commercial cell culture systems available to investigators, and it is important to consider constituents in the cell medium for chemical exposures. For example, responses to chemical exposures in media containing fetal calf serum versus media containing only basic salts may be very different due to differences in cellular uptake of the chemical under study.

In Vivo Systems

Similarly, responses of animals on semipurified diets to chemicals may be very different to those on general laboratory animal chow, due to differences in chemical uptake and the potential presence of contaminating chemicals, antibiotics, and excess nutrients in the general laboratory chow that may vary from batch to batch. The degree to which such dietary differences may impact cellular responses to toxic chemicals will depend on a number of factors, such as experimental design and study endpoints. Similar cautions should be made concerning strain, age, and gender of the animals from which organelles are derived. As with any experiment, the parameters of study should be specified in the greatest detail possible since these differences may impact organelle responsiveness.

INTRACELLULAR ORGANELLES

Cell Membranes

In order for toxic agents to produce intracellular toxicity, they must be transported across the cell membrane that is a dynamic structure whose characteristics vary with cell type. This organelle is not a static structure but plays an important role in the transport of toxic agents. For example, methylmercury by amino acid transporters,[4] lead by pinocytosis following external membrane surface coat binding,[5] and endocytic vesicular uptake of protein/ligand-bound toxic molecules such as cadmium bound to metallothionein or incorporated into nanomaterials[6] are common mechanisms of uptake and lead to intracellular accumulation of toxic agents. It is important to note that the cell membrane itself is not an inert organelle and may itself become a target for chemical exposures to agents such as glyphosate[7] and phospholipase A_2[8] that disrupt cell membrane permeability and hence cellular ability to regulate both essential and nonessential chemical entities leading to cell injury and cell death. Techniques for assessing alterations in cell membrane integrity include transendothelial monolayer electrical resistance (TER)[8,9] and dextran Transwell permeability assay[8,10] (please see the following). Acute exposure to agents such as mercury ion[11] and chromate (Cr^{6+})[12] appears to damage directly this organelle via oxidation of essential membrane proteins and other types of molecules.[13,14] The point to this discussion is that the cell membrane is a dynamic organelle that plays a number of essential roles in maintaining the viability of the cell by regulating the uptake of both essential and toxic chemical entities. Disruption of this organelle by chemical toxicity can lead to cell death by a number of mechanisms including altered maintenance of electrolyte balance, uptake of essential molecules, reduced excretion of metabolites, and activation of the FAS receptor, leading to induction of the caspase system and cell death via apoptosis.[14–17]

Selected Techniques

TER is a summary adaptation from Garcia et al.[10] The reader is referred to this primary reference for further details:

1. Artery endothelial cells (ECs) are grown to confluency in standard EC growth media at 37°C and in a 5% CO_2 atmosphere.
2. Gold counter electrodes (1 cm^2) are attached to a phase lock-in amplifier.
3. TER is measured with an electrical cell–substrate impedance sensing system from Applied Biophysics Inc., Troy, New York.
4. A current is applied across the electrode by a 4000 Hz ac voltage source (AMP 1 V, 1 M Ω, 1 μA).
5. Measurements are conducted for 30 min.
6. Increased TER is observed if cells spread out across the electrodes and reach a maximal value at confluence.
7. If the cells round up, lose attachment, or contract, there is a loss of TER.
8. Values of TER are pooled at regular intervals and plotted against time.

Dextran Transwell permeability assay is a summary adaptation from Dudek et al.[9] The reader is referred to this primary reference for further details:

1. ECs are grown to confluence under standard conditions as described previously on confluent polycarbonate filters—vascular permeability assay kit from Millipore (Billerica, MA).
2. Cells are exposed to the agent of interest for 1 h.
3. FITC-labeled dextran (60 kDa) is loaded into the luminal compartment of the kit Transwell inserts for 2 h.
4. The movement of labeled dextran across the filter to the external compartment is monitored by relative fluorescence (485 nm) excitation/530 nm emission and data reported as arbitrary FUs.

Mitochondria

Ultrastructural Techniques

The various ultrastructural techniques for evaluating mitochondria have been described in the fifth edition of this book[1] to which the reader is referred for details.

Biochemical Procedures

In recent years, there have been a number of advances to the biochemical procedures described in the fifth edition of this book,[1] including high-throughput automation and computer analyses of combined metabolic measurements of mitochondrial function, which link mitochondrial respiration[18] with glycolytic parameters (Seahorse Biologicals). In addition, great progress has been made in understanding the roles of mitochondria in apoptosis, as discussed in the following.

Combined Metabolic Measurements

Seahorse Biochemicals[19] applied this technology for screening a number of inhibitors of mitochondrial respiration in relation to nephrotoxic potential.

High-Throughput Mitochondrial Toxicity Screening with Fluorescent Probes

High-throughput mitochondrial toxicity has also been evaluated by the use of fluorescence probes (A65N-1)[11] and high-throughput 96-well plates with automated fluorescent plate reading systems, which compared favorably with conventional polarographic techniques. Altered mitochondrial respiratory function rates[20] including respiratory control[21] are important elements in mitochondrial generation of reactive oxygen species (ROS) from damage to essential components of the mitochondrial respirome complexes, such as cytochrome *c* oxidase[22] that may further damage other cellular components and exacerbate cell death processes[21] as discussed in the following. The central roles of mitochondria as generators of ROS and the regulatory roles of ROS in guiding redox-regulated cellular signaling processes[22–24] and cross talk with other intracellular sources of ROS, such as the NADPH oxidases, have only recently been appreciated. The implications of the understanding of these complex systems are obvious from the perspective of mechanistic toxicology and highlight the need to revisit reductionistic molecular data in a broader context.

Mitochondria and Programmed Cell Death (Apoptosis)

One of the major outcomes of mitochondrial toxicity is the central role this organelle plays in triggering the apoptotic response. Studies by Jeong and Seol[25] have highlighted the central role of regulating intracellular calcium (Ca^{2+}) in relation to Ca^{2+} signaling and the roles of the apoptotic regulatory proteins in the Bcl-2 protein family in regulating the dynamic Ca^{2+} relationships between mitochondria and the endoplasmic reticulum (ER). In addition, these authors noted the importance of mitochondrial morphology in relation to mitochondrial fission and numbers of mitochondria in the development of the apoptotic response. Studies by Chacko et al.[26] have shown that the outer mitochondrial membrane voltage-dependent channel-1 (VDAC-1) plays a major role in regulating the TRAIL-mediated apoptosis via control of mitochondrial caspase-8 activation. Martel et al.[27] studied fatty liver disease in ob/ob mice fed high-fat diets and reported altered outer mitochondrial membrane permeability, posttranslational modification of VDAC, and alteration of NADH oxidase as early mitochondrial effects leading to a number of alterations in mitochondrial functions, including phosphorylation of VDAC by GSK3. The level of VDAC phosphorylation correlated with the severity of steatosis in patients. The authors conclude that VDAC can act as an early marker for lipid toxicity, since its phosphorylation

status controls the permeability of the outer mitochondrial membrane. Kavanagh et al.[28] reported that control of the actin cytoskeleton dynamics by the $p57^{KIP2}$ protein played a major role in the proapoptotic effect on the mitochondria. Bhattacharyya et al.[29] also studied hepatocytes and reported iron induction of hepatocyte death by MAPK activation and a mitochondria-dependent apoptotic pathway, which was attenuated by addition of glycine to the cell culture medium. Hussain et al.[30] demonstrated that cerium dioxide nanoparticles induced apoptosis and autophagy in human peripheral blood monocytes with hallmark effects on BAX, loss of mitochondrial membrane potential, and DNA fragmentation. Other recent studies by McGill et al.[31] on acetaminophen liver toxicity in mice and humans showed that this drug produced both mitochondrial damage and nuclear DNA fragmentation without increases in serum caspase-3 or cleaved caspase-3 activities but that these activities were elevated in TNF-induced apoptosis. These data suggested that although APAP produced liver toxicity including mitochondrial damage and DNA fragmentation, cell death in humans was most likely by mechanisms involving necrosis. The involvement of other caspases in this model was not evaluated.

Mitochondrial Fission and Fusion Processes in Cell Injury

In recent years, there has been a growing appreciation of the importance of mitochondrial fission and fusion processes and the molecular signaling pathways regulating these physical changes as well as mitochondrial motility in mediating cell injury and mitochondria-based diseases. Indeed, from the perspective of toxicology, swollen and pleiomorphic mitochondria have been noted in liver cells of mice exposed to arsenic in drinking water[32] (Figures 39.1 and 39.2) and ethanol-fed rats.[33] Mitochondrial motility has recently been evaluated in living vascular ECs[34] and associated with mitochondrial fission and linked to mitochondrial bioenergetics. Taken together, these studies provide further support for the intrinsic linkage between dynamic changes in mitochondrial structure and biochemical function following chemical exposures.

Studies in neuronal cell systems have highlighted differences in swelling responsiveness in mitochondria from astrocytes and cortical neuronal cells in culture in situ[35] using optimized spatial filtering of mitochondrial fluorescence to assess changes in membranes as a function of swelling, fission, or fusion. This technique generates a *thinness ratio* (TR) for the mitochondrial membrane fluorescence in which measurements were sensitive to mitochondrial swelling but not fission or fusion of mitochondria. The technique demonstrated marked differences in mitochondrial swelling during uncoupling between astrocytes and neuronal cells. The balance between mitochondrial fission and fusion appears to be an important factor in defining a new mechanism of neurodegeneration from a variety of insults related to Alzheimer's disease, Huntington's disease, and stroke.[36] Studies point to the role of dynamin-related GTPases and the protein Drp1

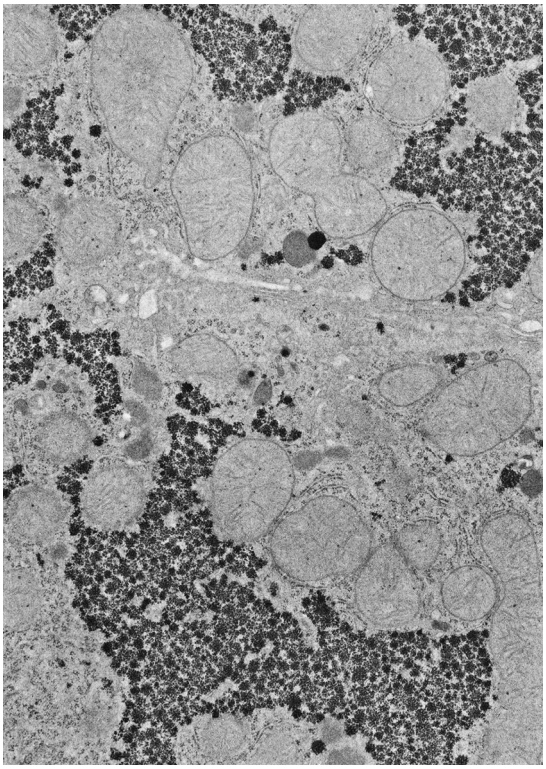


FIGURE 39.1 Hepatocyte from a mouse given the 40 ppm arsenate dose level showing swollen mitochondria. (From Fowler, B.A. and Woods, J.S., *Toxicol. Appl. Pharmacol*., 50, 177, 1979.)

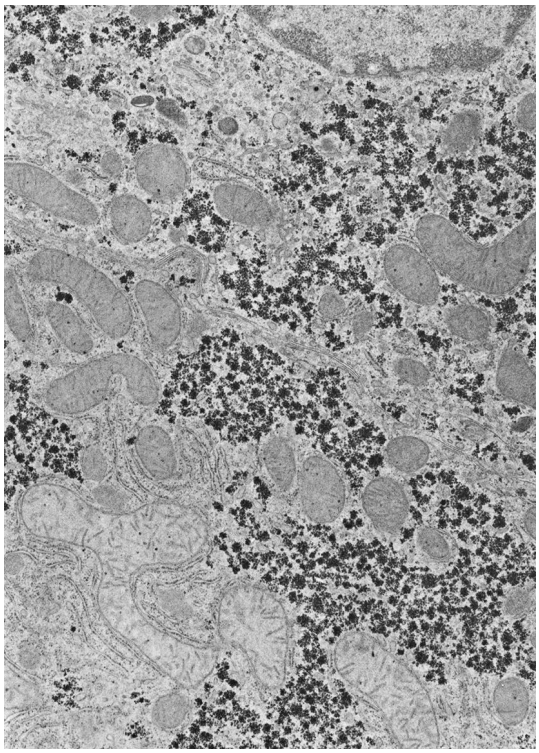


FIGURE 39.2 Hepatocyte from a mouse given the 85 ppm arsenate dose level showing mitochondria with normal appearance and swollen polymorphic mitochondria. (From Fowler, B.A. and Woods, J.S., *Toxicol. Appl. Pharmacol*., 50, 177, 1979.)

in particular in mediating mitochondrial fission processes. In neuronal cells, Drp1 appears to be positively regulated by Bcl-xL to affect mitochondrial function with regard to formation of synapses.[37] Other work[38] has demonstrated that Drp1 activity could be increased by the binding of a mutant Huntington protein resulting in increased mitochondrial fragmentation, altered mitochondrial transport processes, and neuronal cell death. By studying inhibition of mitochondrial energy production associated with fragmentation, the physical structure of mitochondria has been demonstrated to increase formation of ROS and lead to cell death of neuronal cells by an NMDA/ROS-dependent pathway.[39] The release of proapoptotic factors such as cytochrome *c* from physically altered mitochondria[40] may result in the triggering of the caspase cascade, resulting in apoptotic cell death.[41–43]

From the previous discussion, it is clear that the mitochondria are dynamic organelles, both structurally and biochemically, that perform a number of functions for the cell that are highly sensitive to perturbations by chemical agents. The consequences of these disturbances may be profound on many levels and may ultimately lead to cell death via apoptosis.

Biochemical Procedures

There are a number of new biochemical assays for evaluating the biological activities of mitochondria.

In Situ Swelling and Contraction Assays

This biophysical procedure is a summary adaptation from Gerencser et al.[35] and the reader is referred to this chapter for in-depth details:

1. Rat neuronal astrocyte and neurons are placed in cell culture on cover slips under standard culture conditions.
2. Time-lapse fluorescence microscopy is conducted on astrocyte and neuronal cell cultures in a medium containing 5.4 mM KCl, 150 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.9 mM NaH_2PO_4, and 5.6 mM glucose in a 20 mM HEPES buffer (pH 7.4) at 37°C.
3. Astrocytes are evaluated using a Nikon Diaphot 200 inverted microscope with a 75 W xenon arc lamp. This basic system is augmented with a 535/20 exciter, 570LP emitter, and a 560LP dichroic mirror. Photographs are taken with a cooled digital camera operated by MetaFluor 3.5 software from Molecular Devices, Downingtown, PA.
4. Primary neuronal cultures are evaluated using a laser scanning confocal microscope and studies conducted with the 543 line of a 5 mW HeNe laser at <7% power fitted with an HFT 488/543 dichroic mirror and 560LP emission filter.
5. Studies on mixed neuronal cultures are conducted using an inverted microscope with a lambda LS Xe arc light source (175 W and ND of 0.3).
6. Data are collected using a cooled digital CCD camera.
7. Z-stacks of seven planes with 0.8 μM spacing are recorded at 1 min intervals and time points of 0, 5, and 90 min.
8. The studies are conducted with the entire microscope stage at 37°C in an air environment. Acquisition of images is managed by a computer program (MetaMorph 6.3 from Molecular Devices).
9. The TR is calculated as the ratio of average fluorescence intensities for organelle regions from HBP-filtered images and LBP-filtered images.

Lysosome/Proteasome Systems

As with other intracellular systems, a better understanding of lysosome structure and function has emerged over the last decade and is now expanded to include the concept of the proteasome, which involves tagging of damaged or dysfunctional proteins by ubiquitin for degradation and recycling of amino acids in autophagosomes. There is growing evidence that autophagosomes have some unique functional properties and may produce constitutive ROS that exacerbate oxidative stress-mediated cell injury.[44,45] Studies by Dogra and Mukhopadhyay[46] have shown that the drug methyl *N*-(phenyl sulfanyl-1H-benzimidazol-2-YI) carbamate produces a marked inhibition of the ubiquitin–proteasome pathway, which can produce a strong inhibitory effect on cancer cells in vitro. The cell killing effect was associated with induction of stress on the ER, decreased mitochondrial membrane potential, increased formation of ROS, release of cytochrome *c*, and death of the cells by apoptosis. Other recent studies by Ivanova et al.[47] demonstrated apparent lysosomal destabilization in Caco-2 cells by the mycotoxin enniatin B, resulting in cell death by a nonapoptotic cell death pathway that occurred prior to cell cycle arrest in the G2/M phase and attendant increased production of ROS and development of apoptosis or necrosis.

The biogenesis, cargo selectivity, and directional movement of autophagosomes are also highly regulated by intracellular signals and the ubiquitin tagging of targets. The movement of autophagosomes within the cell may be driven by the motor protein dynein[48] and directionally controlled by the cellular cytoskeletal components of microtubules and F-actin.[49]

As with the other organelles discussed in this chapter, lysosomes play a number of roles in normal biology of the cell but may also become intracellular targets for toxicity by accumulating toxic materials, damaged organelles via autophagocytosis, and generation of ROS during degradative processing of deposited materials by lysosomal proteases. Inhibition of lysosomal function by agents such as cadmium[6] can have profound physiological consequences due to altered intracellular processing of proteins.

Ultrastructural Techniques

The ultrastructural techniques used to visualize and quantify lysosomes in situ have been extensively described in the 5th edition of this book.[1] The reader is referred to this source for details.

Measurement of ER Stress and ROS Activity

The following methods are adapted and summarized from Dogra and Mukhopadhyay[46] and the reader is referred to this primary publication for further details.

Measurement of ER Stress Using the Secreted Alkaline Phosphatase Assay Method

1. For these studies, 293 T cells are placed in 96-well plates and transfected 1 day later with pSEAP-con-plasmid obtained from Clontech (Palo Alto, California). This plasmid constitutively expresses secreted alkaline phosphatase (SEAP).
2. At 24 h posttransfection, cells are either left unexposed or treated with the chemicals of interest in fresh media containing 1% fetal bovine serum.
3. After 24 h, 10 μL of the culture media is extracted and incubated with 4-MUP as the substrate according to the Clontech protocol.
4. This protocol involves mixing 10 μL of culture media with 15 μL of 5× SEAP assay buffer containing 0.5 M Tris at pH 9.0 containing 0.5% bovine serum albumin (BSA) in a final volume of 50 μL in a 96-well plate and incubated for 30 min at 65°C to inactivate endogenous SEAP.
5. The plate is placed on ice for 2 min and then 25 μL of 1 mM 4-methylumbelliferyl phosphate substrate is added to each well.
6. The assay mixture is incubated for 2 h at 37°C and SEAP activity is monitored using a PE-fluorescent plate reader with excitation at 355 nm and emission at 460 nm.
7. Analyses are conducted in triplicate.

ROS Activity

1. A549 cells are grown on cover slips in 35 mm culture dishes and exposed to the chemical of interest, or 10 μM MG132 for 4 h with NAC added 2 h prior to addition of the chemical of interest.
2. In measurement of ROS after incubation, the cells are washed and incubated with 1 μM dichlorofluorescein-DA at 37°C for 1 h in serum-free media without phenol red dye.
3. The cells are washed twice and then illuminated with a 100 W mercury lamp for viewing with a Nikon fluorescent microscope with a FITC filter or using a PE fluorimeter with excitation at 485 nm and emission at 530 nm for quantitative studies.

Endoplasmic Reticulum

Ultrastructural Techniques

Visualization of the ER of both rough (RER) and smooth (SER) ER by electron microscopy including ultrastructural morphometry[50] has been extensively described in the fifth edition of this volume. Only a few recent publications related to toxicology are described here.[51–53] Ultrastructural alterations of the ER in hepatocytes in animals or cell cultures exposed to carcinogens[51] and pancreatic cells[52] from animals treated with ethanol have demonstrated similar structural changes with regard to degranulation/fragmentation of the RER, proliferation of SER, and dilation of ER membranes. Similar effects have also been reported for acridine cholinesterase inhibitors following in vitro exposures. It is important to note that these structural changes in the ER are commonly associated with alterations in biochemical functions such as those described in the following.

Biochemical Procedures for Phase I and Phase II Microsomal Metabolism

A number of new assays and biochemical procedures have been developed in recent years that have helped to further elucidate the intimate relationships between the structure of the ER and various aspects of biochemical functionality, including Phase I cytochrome P-450-based enzymatic processes, Phase II conjugation/deconjugation enzymatic reactions, and more recently the roles of acid hydrolase proteasomal degradation of ubiquitin-tagged proteins.

Preparation of Human Liver Microsomes

The separation of SER- and RER-enriched membrane fractions by differential centrifugation techniques has been previously described in the fifth edition of this book,[1] and the reader is referred there for details.

Microsomal Activation/Deactivation Systems

Microsomal Flavin-Containing Monooxygenases

Microsomal Cytochrome P-450

There is an extensive literature[54–57] on chemical-induced effects on the activities of cytochrome P-450 monooxygenases that is outside the scope of this chapter, but this literature is reviewed in an article by Guengerich[58] as described in the following.

P-450-dependent (Phase I) enzymes[58] have been demonstrated over the last 50 years to play a number of important roles in chemical toxicology, including both the bioactivation of organic molecules to more toxic chemical species and the detoxification of organic molecules into less toxic chemical species. As noted by Guengerich,[58] there is still much to be learned about this important cellular metabolic system, including characterization of *orphan* cytochrome P-450s, kinetics, animal–human extrapolations, and other aspects of these molecules related to chemical toxicity risk assessments and development of cancer. In this regard, Mohutsky et al.[54] discuss the issue of induction of hepatic drug-metabolizing enzymes in relation to preclinical and clinical risk assessments. This aspect of the cytochrome P-450s is clearly important for preventing interactions between drugs where more than one drug is being administered in a clinical setting,

which is often the case. There are a number of approaches to dealing with this issue from a risk management perspective, including altered dose levels of the drugs involved, dose timing, consideration of the age, gender, genotyping of the patient, and discontinuation of one of the drugs. The situation is complex but not without promise as we move toward more personalized medical treatments.

Evaluating Microsomal Function

Microsomal Conjugative Enzymes

In addition to the cytochrome P-450 enzyme activities associated with the ER, there are a number of linked conjugating enzyme activities that deal with reactive metabolites from cytochrome P-450 action for excretion. These enzyme functions have been studied in a wide variety of toxicology studies.[59–61]

Phase II Enzyme Systems

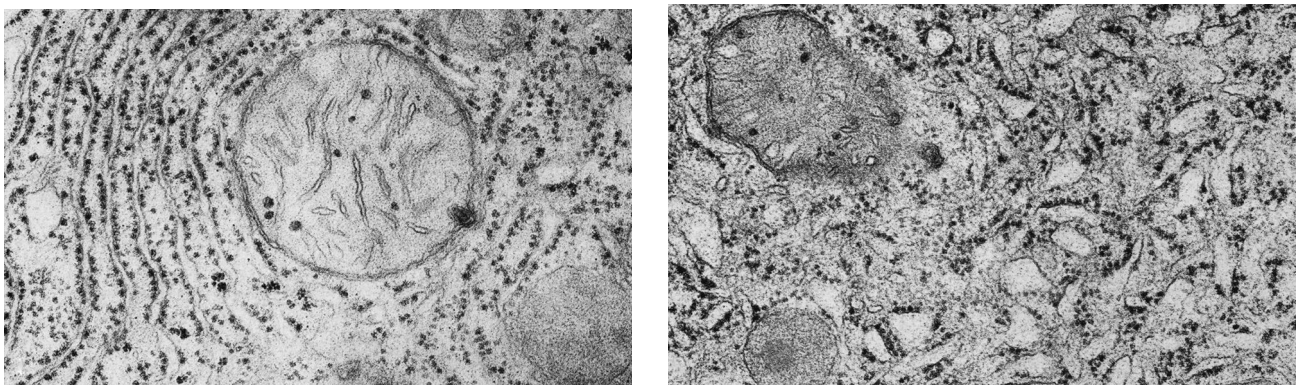
The presence of conjugation/deconjugation enzymes in the ER that further metabolize products of Phase I metabolism so they can be more readily excreted has been appreciated for many years (see fifth edition[1] for discussion).

These include a number of conjugating enzymes like glucuronyl transferase but also hydrolytic enzymes, such as acid phosphatase, beta glucuronidase, and sulfatases, and a number of proteases such as the cathepsins. In recent years, a great deal of interest has been focused on the roles of ER-based proteases, known collectively as the proteasome, and lysosomes in the recycling of amino acids from damaged proteins as part of both normal protein turnover and the impact of chemically induced cell death pathways on these processes. Proteasome degradation of ubiquitin-tagged proteins has been a major focus. It is important to note that damaged ER proteins can also be dislocated from the ER membranes into the cytosol[62] for degradation by the proteasomes and visualized by live cell imaging techniques. The mechanisms by which proteins move to and from the ER are highly complex with a number of specific chemical (cholesterol content) and molecular factors, such as chaperone proteins and dibasic amino acid regions of transported protein involved.[63–65]

As with the other organelles discussed in this chapter, there is an ever-increasing understanding of the dynamic nature and complexity of functions performed by this organelle system, both directly and in concert with other organelles, such as the mitochondria and cellular proteasome/lysosome systems. It should be noted that a close physical relationship is frequently observed between the ER and organelles, such as the mitochondria (Figure 39.3), which would facilitate transport of essential metabolic products, such as heme, produced by the mitochondria for incorporation into ER hemoproteins such as cytochrome P-450 and proteins/enzymes synthesized by the ER and chaperoned into the mitochondria. Chemically induced disruption of these physical linkages between the ER and organelles such as the mitochondria (Figure 39.3) has profound and differential consequences on biochemical functions in these organelle systems.[50]

Peroxisomes

The peroxisomes are single-membrane-bound organelles that have been studied by electron microscopy and are known to contain catalase, D-amino acid oxidases, and lipid oxidases. The morphology of these organelles varies by animal species, and a crystalline structure is sometimes observed in peroxisomes of some species. In recent years, induction of peroxisomes via the peroxisome proliferator pathway receptor (PPAR) has been linked to the development of certain types of cancers. Hence, there is extensive scientific interest in the biological function of these organelles in mediating carcinogenic processes. Kudo et al.[66] demonstrated that peroxisome proliferation in livers of mice fed 8–2 telomer alcohol produced peroxisome proliferation in a dose- and time-dependent manner, which was associated with perfluorooctanoic acid (PFOA), a metabolite of 8–2 telomer alcohol. The authors conclude that the metabolite PFOA is responsible for the observed peroxisomal proliferation effects in mice from 8–2 telomer alcohol exposure. Other studies[67] have demonstrated that the peroxisome proliferator-activated



(a) (b)

FIGURE 39.3 Higher magnification of hepatocyte from control rat (a) and indium-injected rat (b) (40 mg/kg) after 16 h showing fragmentation and dilation of ER with loss of ribosomes into cytoplasm. (From Fowler, B.A. et al., *Lab Invest*., 48, 471, 1983.)

receptor (PPAR) in mucosal cells can be regulated in part by a CCAAT/enhancer-binding protein homologous protein (CHOP) under cellular translational stress. CHOP was shown to have increased expression and nuclear translocation in human intestinal epithelial cells following exposure to ribotoxic agents such as deoxynivalenol and anisomycin. CHOP is a positive regulator of IL-8, but this effect was inversely related to expression of the PPARy. PPARy is a negative regulator of IL-8 mRNA, which is in turn linked to the mRNA protein HuR. CHOP is a positive regulator of HuR protein translocation from nuclei. The overall conclusion is that functional interrelationships between CHOP and PPARy may be important mechanistic linkages between ribosomal toxic stresses and proinflammatory production of cytokines such as IL-8.

The metabolic activity of the peroxisomes generates ROS[68] in relation to lipotoxicity, but the presence of catalase in this organelle also suggests that it may play a role in protecting the cellular processes from ROS damage as well. On the other hand, peroxisomes are also known to be generators of ROS, which are presumably usually neutralized by the action of catalase. However, this balance may be altered by exposure to some chemicals or drugs. The proliferation of peroxisomes in cells has been linked to the development of certain kinds of cancer.

The role of peroxisomes in mediating chemical toxicity and carcinogenicity is a currently active area of research.[68–70]

Fluorescent Hydrogen Peroxide-Sensitive Proteins

Application of HyPer proteins[69] for analysis of H_2O_2 generation from peroxisomes and mitochondria is a relatively new approach[68] for localizing H_2O_2 generation in an organelle-specific manner and involves some interesting molecular biology approaches. The following is a summary adaptation of the methods from Elsner et al.[68] for this purpose. The reader is referred to this primary reference for further details:

1. HyPer vector cloning is conducted using the cDNA for the H_2O_2-sensitive protein (HyPer protein–pHyPer-dMito obtained from Evrogen, Moscow, Russia). The cDNA is subcloned into a viral transfer plasmid (pLenti6/V5-MCS obtained from Invitrogen). The cDNA is excised from the pHyPer-dMito plasmid using the NheI and NotI restriction sites. The cDNA is then blunted and ligated into the EcoRv site of the pLenti6/V5 protein. In order to construct expression vector for the HyPer-peroxisome protein, PTS1 is connected to the 3′ end of the HyPer cDNA via a polymerase chain reaction (PCR) using a composite primers as expression of the HyPer-peroxisome proteins is conducted as previously reported[68] by transfecting lentivirus particles and harvesting the virus particles by ultracentrifugation for 2 h at 70,000 ×*g*. Virus titers are confirmed via TaqMan qPCR as specified in reference[68] and the pHyPer-dCyto plasmid as a template. The HyPer-peroxisome cDNA is subcloned in the Xbal/Bsp site of the plasmid pLenti6/V5-MCS.
2. Rat primary tissue culture cells are infected with lentivirus using an MOI of 10:1. The cells are selected for expression of the HyPer proteins using blasticidin at 1 μmol/L.
3. HyPer protein analysis of intracellular hydrogen peroxide generation is conducted using RINm5F cells, or primary rat cells that overexpress the HyPer are seeded onto black 24-well glass-bottom plates (Greiner, Frickenhausen, Germany).
4. The cells are cultured for 24 h and then exposed to palmitic acid for an additional 24 h.
5. Live cells are imaged using an inverted microscope fitted with a CFP–YFP dual filter with excitation bands at 427 and 504 nm and an emission band at 520 nm.
6. The microscope is fitted with CellR software for analytical imaging.
7. H_2O_2 production is measured via changes in the fluorescence ratios of the RINm5F, RINm5F-Cr, RIMm5FMitoCat, and INS-1E cells that overexpress the HyPer proteins and are quantified with a spectrofluorimeter. The fluorescence ratios are measured before and after treatment with palmitic acid or the chemical/drug of interest for 24 h.

Nuclei

The nuclear compartment of the cell is a primary repository of the cellular genome. In addition, it performs a number functions for the cell and as such may become an intracellular target for toxicity. This organelle may also show dynamic changes in size and shape, such as the formation of giant nuclei in response to agents such as ectoposide,[71] which was associated with G2/M arrest and severe DNA damage. In addition, these organelles may become intracellular storage sites for aggregated or damaged proteins such as amyloid fibrils[72] and intranuclear inclusion bodies from lead (Figure 39.4),[73] bismuth,[74] and mercury–selenium-containing complexes (Figure 39.5a and 39.5b),[75] which are formed as metal–protein structures. An important point is that nuclei as structures can be target organelles for chemical toxicity. Like the other organelles discussed previously, they play a number of dynamic roles in the biology of the cell, including acting not only as repositories for genetic materials and physical sites of DNA replication but also as dynamic intracellular storage sites for drugs, metals, and damaged proteins.

Recently, using live cell imaging techniques, transient ruptures of the nuclear envelope have been reported in human cancer cells during interphase.[76] The observed biological/structural consequences of these effects were incorrect localization of nuclear and cytoplasmic proteins. Formation of micronuclei-like structures and loss of chromatin material from the nuclei was observed and incorrect formation of intermediate support filaments in the nuclei. The overall effect of these physical changes would be an alteration of the cancer cell genomic structure, which could impact the behavior of the cancer cells.

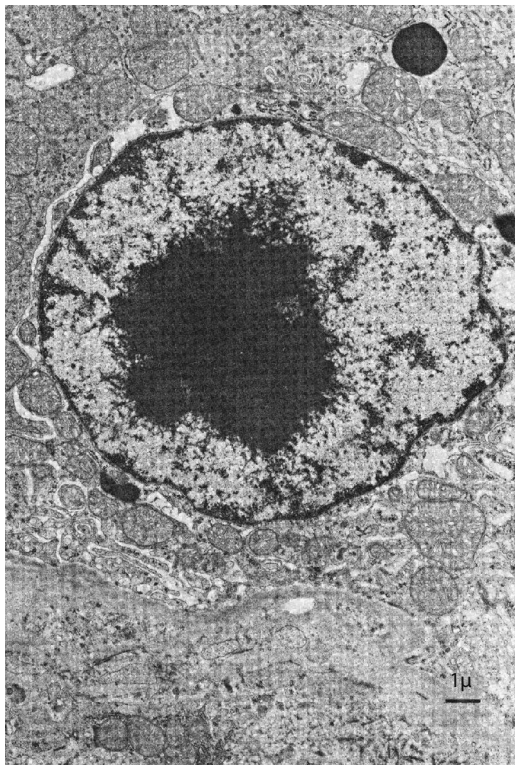


FIGURE 39.4 Proximal tubule cell from a rat exposed to lead in drinking water, showing large intranuclear inclusion body. (From Fowler, B.A., Ultrastructural and biochemical localization of organelle damage from nephrotoxic agents, in Porter, G.A., ed., *Nephrotoxic Mechanisms: Drugs and Environmental Toxins*, Plenum Press, New York, 1982, pp. 315–330.)

Increasingly, sophisticated approaches have evolved in recent (e.g., postgenomic) years for tracking altered genomic elements,[77] including automated image modeling approaches for the subsequent subcellular localization patterns of gene products[78] or sophisticated proteomic techniques[79] and statistical evaluation using image analysis databases.[80] The point is to highlight the ever-increasing sophistication and integration of scientific approaches, including bioinformatics, to generate more holistic answers to complex scientific problems. The application of these approaches for addressing pressing toxicological issues and translating information for risk assessment purposes is now a major area of focus in the toxicology community.

Cytoplasm

The cytoplasm is the overall milieu in which the various organelle systems reside, but it also is a dynamic compartment that is the site of numerous molecular functions and processes. From the perspective of toxicology, localization of antioxidant cellular defense systems, such as reduced glutathione (GSH), which is inducible and usually present in millimolar concentrations, is an important feature in preventing ROS-induced cell injury. For toxic metals such as cadmium (Cd), inducible metal-binding proteins such as metallothionein (MT) have been shown to play a central role in the biological transport, cellular uptake, and toxicity of the element. A global conceptual diagram is presented that attempts to link some known aspects of MT handling of Cd with possible linkages to mechanisms of cell death via apoptotic pathways. This framework may be particularly useful in helping to explain some of the low-dose effects of Cd in target cell populations

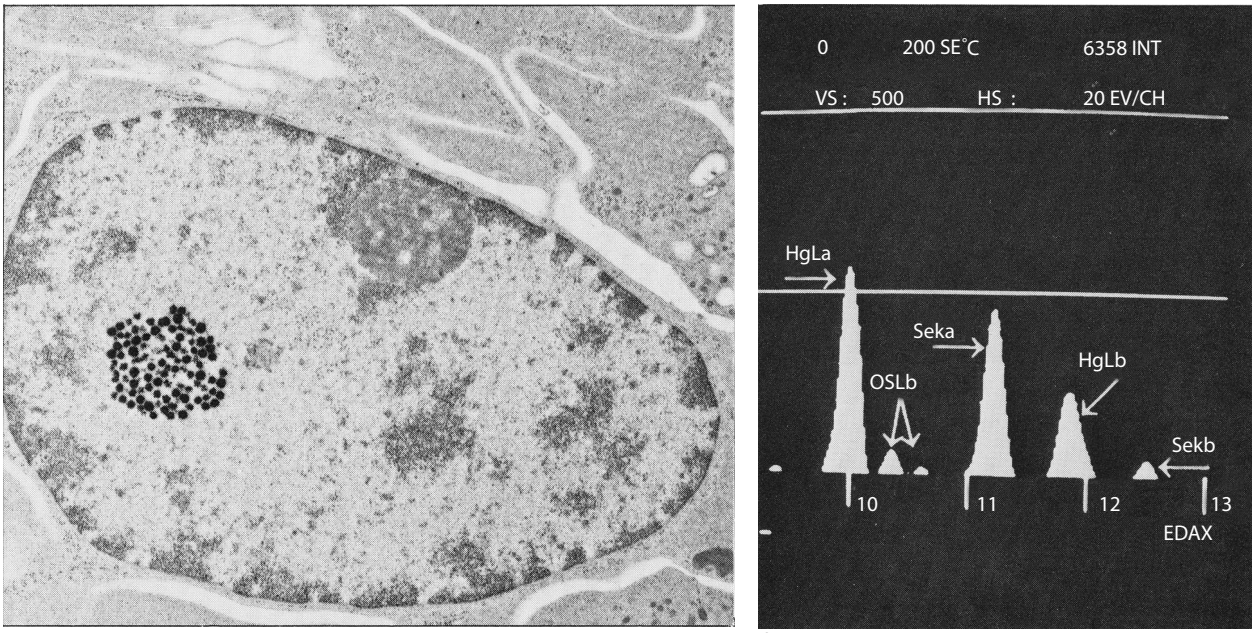


FIGURE 39.5 (a) Electron micrograph of nuclear inclusion in a proximal tubule of a rat following $HgCl_2$ and Na_2SeO_4 treatment. (b) Energy dispersive x-ray microanalysis spectrum of inclusion similar to (a). Peaks visible are HgLα, OsLα, and SeKα. (From Carmichael, N.G. and Fowler, B.A., *J. Environ. Pathol. Toxicol.*, 3, 399, 1980.)

with apparently limited metallothionein synthesis capabilities, such as ECs of the blood vasculature.[81–83] It may also be helpful in understanding the effects of low-dose Cd exposure on multiple cell types in relation to the roles of Cd in the aging process.[82] The importance of MT as a protective regulatory molecule, which is largely localized in the cytosol, is not to be underestimated. Other protective proteins, which are largely localized in the cytosol, are able to enter or be present inside the subcellular organelles, such as the stress or (HSP) proteins. They play a number of roles in mediating cell injury/cell death, chaperoning newly synthesized proteins into organelles such as the mitochondria and peroxisomes, and refolding or transporting damaged proteins to the proteasome/lysosome system for degradation or transport out of the cell (Figure 39.6).[84] Ubiquitin tagging of proteins for proteasomal degradation is a particular aspect of this general phenomenon.

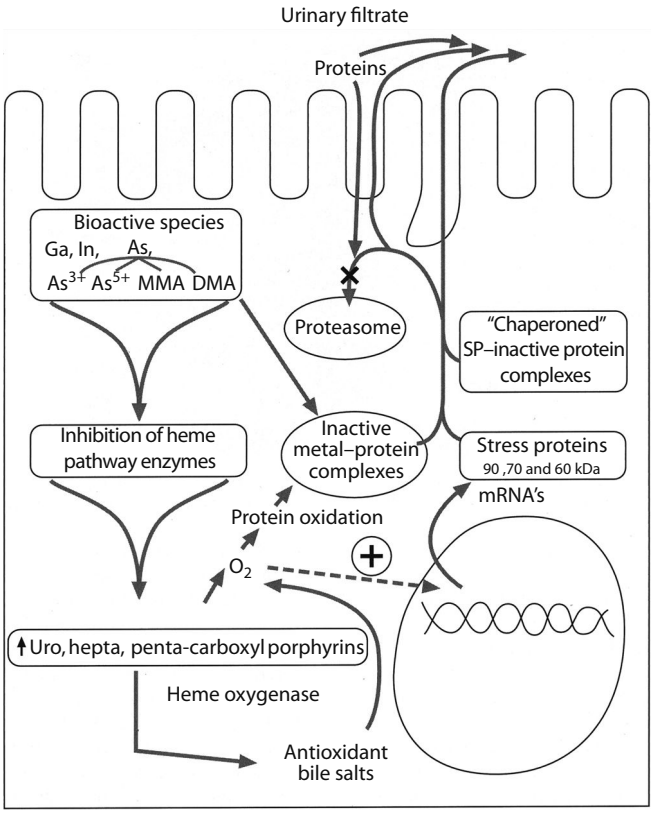


FIGURE 39.6 The hypothesized mechanisms by which GaAs and InAs produce cell injury in renal proximal tubule cells are shown in relation to alterations in the heme biosynthetic pathway and proteotoxicity and proteinuria. These findings are shown in relation to the stress protein response and the hypothesized decrease in chaperoning of reabsorbed proteins from the urinary filtrate for proteasomic degradation and export of damaged intracellular proteins into the urinary filtrate to produce the observed GaAs- and InAs-specific proteinuria patterns. The magnitude of the tubular proteinuria may be due to decreased reabsorption of filtered proteins, but the observed specificity may be the result of damaged intracellular proteins chaperoned out of the tubule cells at the apical membrane surface perhaps by ubiquitin. (From Fowler, B.A. et al., *Toxicol. Appl. Pharmacol.*, 206, 121, 2005.)

The cytosolic compartment plays an important and dynamic role in mediating fundamental processes of chemical toxicity. A full, detailed discussion of these functions is beyond the scope of this chapter. However, it can be anticipated that as tools of modern toxicology evolve, a better understanding of the complex pathway from chemical insult at the cellular level to overt cell death will continue to emerge.

Alterations in the Cytoskeleton during Chemically Induced Cytotoxicity to and Morphological and Neoplastic Cell Transformation of Mammalian Cells

The development of antibodies to cytoskeletal components, which are conjugated to fluorophores, now allows investigators to visualize cytoskeletal components of mammalian cells. Specifically, microtubules can now easily be decorated and visualized by staining cells with antibodies to α-tubulin or to β-tubulin, which are then conjugated to fluorescein isothiocyanate, and then visualizing the cells by confocal microscopy. In this manner, one can visualize disruption of microtubules when cells are treated with various toxic or carcinogenic chemicals that disrupt microtubules.[85] In addition, microfilaments in cells can be decorated and visualized when the cells are stained with phalloidin, a fungal toxin that binds to microfilaments, which has been conjugated to fluorophores, and visualized by confocal fluorescent microscopy.[85] In this way, damage to the microfilaments by specific chemical toxins can also be demonstrated.

Secondly, a number of cell culture systems now have been developed in which investigators can study chemically induced morphological and neoplastic cell transformation. In one of these, C3H/10T1/2 Cl 8 (10T1/2) mouse embryo cells, carcinogenic insoluble nickel (Ni) compounds—nickel subsulfide, green nickel oxide, black nickel oxide, and crystalline nickel sulfide—and samples of Ni refinery dust have been shown to induce morphological and neoplastic transformation.[86,87] Foci of Ni-transformed cells have been ring-cloned from these experiments, biologically characterized, and expanded into transformed cell lines.[86,87] The techniques of decorating microtubules with fluorophores conjugated to antibody directed against α-tubulin or β-tubulin and visualizing microtubules by confocal microscopy have been used to show that in transformed 10T1/2 mouse embryo cell lines derived from nickel (Ni) compound–induced transformed foci, there are higher steady-state levels of microtubules,[85] due to the amplification of the ect-2 proto-oncogene and overexpression of ect-2 mRNA and protein.[88–90] Amplification of the ect-2 oncogene and overexpression of ect-2 mRNA and protein in Ni-transformed and in 3-methylcholanthrene (MCA)-transformed 10T1/2 mouse cell lines[90] will lead to higher steady-state levels of the protein complex, RhoA–GTP, which binds to the caps of microtubules, and will lead to higher steady-state levels of RhoA–GTP-microtubule cap complexes.[85,90] This, in turn, will lead to synthesis of more microtubules, which in turn will lead to higher steady-state levels of microtubules in the Ni^{2+}- and MCA-transformed

cell lines. Higher steady-state levels of microtubules in chemically transformed cells next lead to larger cells with aberrant shapes and nonhomogeneous distribution of microtubules in the transformed cell lines.[85] This in turn will lead to changes in gene expression in the Ni^{2+}- and MCA-transformed cell lines (DeSilva-Pehl and Landolph, manuscript in preparation).

Further, due to the silencing of the β-centaurin-2 gene that our Joseph R. Landolph's (JRL's) laboratory found in Ni^{2+} ion-transformed 10T1/2 cell lines,[85,91] signals that should ordinarily be sent to the ADP-ribosylation factor (ARF) protein, which ordinarily causes the microfilaments to disaggregate,[85] are not sent to the ARF. Hence, the microfilaments in Ni^{2+} ion–transformed 10T1/2 mouse embryo cell lines lacking expression of β-centaurin-2 protein will fail to disaggregate. Since additional microfilaments will continue to be synthesized, this leads to higher steady-state levels of microfilaments in Ni-transformed cell lines[85] (DeSilva-Pehl and Landolph, manuscript in preparation). Again, this leads to a nonhomogeneous distribution of microfilaments in the Ni-transformed cells, to larger cells, and to cells with aberrant cell shapes.[85] These consequences of overexpression of the microfilaments will contribute to alterations in gene expression in the Ni-transformed cell lines[91] (DeSilva-Pehl and Landolph, manuscript in preparation).

Use of mRNA Differential Display to Analyze Aberrations in Gene Expression in Chemically Induced Morphological and Neoplastic Cell Transformation

Our JRL's laboratory has recently been using random access primer (RAP)–PCR mRNA differential display to study the alterations in gene expression that occur in transformed cell lines derived from Ni compound–induced foci and MCA-induced foci in 10T1/2 mouse embryo cell lines. With RAP–PCR mRNA differential display, we found that 10 genes are differentially expressed in Ni-transformed and in MCA-transformed 10T1/2 cells compared to nontransformed 10T1/2 cells when 8% of the total mRNA was analyzed.[88,89] Therefore, by extrapolation, in 100% of the mRNA, 130 genes would be differentially expressed between nontransformed 10T1/2 cells and Ni^{2+} ion–transformed or in MCA-transformed 10T1/2 cell lines.[88,89] Among these differentially expressed genes, we found that the epithelial cell transforming factor 2 (ect-2) proto oncogene, the calnexin gene, and the Wdr1 stress response gene were overexpressed in the transformed cell lines compared to their expression in nontransformed 10T1/2 cells.[88–90] The ect-2 proto-oncogene was amplified, and higher steady-state levels of ect-2 mRNA and protein were expressed in Ni^{2+} ion–transformed and in MCA-transformed 10T1/2 cell lines compared to their expression in nontransformed 10T1/2 cell lines.[90] In addition, we found that the vitamin D receptor-interacting protein (DRIP80), the β-centaurin-2 gene, the insulin-like growth factor receptor gene #1 (IGFR1), and the flavin adenine dinucleotide (FAD) synthetase gene were expressed in nontransformed 10T1/2 cells but were not expressed at detectable levels in Ni^{2+} ion transformed and MCA-transformed 10T1/2 cell lines.[88,89,91] The small nuclear RNA activating complex protein (SNAP C3) was expressed in nontransformed 10T1/2 cells but was significantly underexpressed in Ni^{2+} ion–transformed and in MCA-transformed 10T1/2 cells lines.[88,89]

Our current working model is that approximately 15 primary genes are altered in expression in Ni^{2+} ion–transformed and in MCA-transformed 10T1/2 cell lines. For each gene altered in expression, this will cause further nine genes (130/15) to be altered in expression.[88] Approximately 3/8 of these genes will be either mutated or amplified and/or overexpressed, and this will lead to overexpression of $3/8 \times 15 \times 9 = 50$ additional genes. Five-eighths of these primary genes will become quiescent transcriptionally in the transformed cell lines. This will lead to loss of expression of, or underexpression of, $5/8 \times 15 \times 9 = 84$ additional genes. Therefore, alterations (mutations or methylations of the promoters/inactivating mutations) in 15 primary genes will lead to aberrations in expression of 130 genes total.[88,89] This will substantially perturb gene expression and cellular physiology, leading to induction and maintenance of morphological and neoplastic cell transformation.[88,89] Hence, the use of RAP–PCR mRNA differential display can lead to significant insight into the carcinogen (Ni^{2+} ion and MCA)-induced perturbations in gene expression that lead to induction of and maintenance of the transformed phenotypes of morphological transformation, anchorage independence, and tumorigenicity in Ni^{2+} ion–transformed and in MCA-transformed 10T1/2 mouse embryo cells.[88,89,91]

QUESTIONS

39.1 How can an increase of appreciation organelle interactions improve the quality of scientific answers to pressing questions in toxicology?

39.2 How is understanding on the basic processes governing interorganelle interactions or trafficking of value to mechanism or mode of action risk assessment improved?

39.3 How can modern image analysis tools of intracellular organelles within intact cells provide improved interpretation of data from putative genomic, proteomic, and metabolomic biomarker suites?

39.4 What information does use of fluorescent antibodies to microtubules and microfilaments give us into the alterations in physiology that occur in chemically transformed cell lines?

39.5 What insight does use of RAP–PCR differential display give into perturbations in gene expression and cellular physiology of Ni^{2+} ion- and MCA-transformed 10T1/2 cell lines?

39.6 How can alterations in expression of 15 genes lead to further alterations in 130 genes in chemically transformed cell lines?

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40 Analysis and Characterization of Enzymes and Nucleic Acids Relevant to Toxicology

F. Peter Guengerich

CONTENTS

INTRODUCTION

More than 30 years have elapsed since I prepared this chapter for the first edition.[1] The book has been very successful, and the original chapter has been revised five times since then.[2–5] Many things have changed since the first version. Toxicology has become a more mechanistic field. In this sixth edition, the general discussion has been extensively modified. Some of the basic methods have been retained, because they are still utilized widely, without extensive changes, or they serve as prototypes for other assays. Several of the procedures have been changed in this sixth version, in order to reflect perceived needs in readers' laboratories. In some cases, detailed protocols have been deleted because commercial manufacturers routinely supply detailed instructions; in those cases, some general guidance about the basis of the system will be provided. Readers are also referred to some details previously covered in earlier editions of the chapter and book.

BIOACTIVATION AND DETOXICATION

What can be learned by studying enzymes? A general paradigm for the relationship of enzymes to toxicology issues is shown in Figure 40.1. A chemical can be directly transformed to an inactive product. An alternative enzymatic reaction is conversion to a *biological enzymatic intermediate*, such as the epoxide shown in the example. This reactive compound can be hydrolyzed or conjugated (possibly enzymatically in either case), yielding detoxicated product. Some of the reactive product may also react with tissue nucleophiles. Most of the reactions are with *nontargets*, which is good, although these products obscure the searches toward understanding mechanisms. The reactions with *targets* for toxicity are the detrimental ones. Sixty-five years have elapsed since the concept of ultimate toxic forms was first developed.[6] Such activation is now widely accepted as the first step leading to the toxic and carcinogenic effects of many chemicals.

The classification of the enzymes of interest is somewhat subjective, but the list provided in Figure 40.2 would probably be generally agreed on, with some possible changes. The list is based on the original work of Jakoby[8] and was utilized in two later monographs.[7,9] The reader is referred to this list. Many of these enzymes exist in medium to large gene families and are inducible. This chapter will focus on the P450 enzymes as general models for many of these enzymes, and many of the assays presented should, at least in principle, be useful for many of these enzyme systems.

The basic principles of the chemistry involved in toxicity are relatively straightforward. Most bioactivation reactions (*reactive intermediates*) can be explained in terms of either (1) the irreversible reaction of an electrophile with a tissue nucleophile or (2) propagation of free radicals (Figure 40.3). The enzymatic steps leading to the generation of electrophiles are myriad but have been extensively studied.[10,11] The following principles govern these reactions.[12,13] (1) The basic reactions involve simple chemistry, for example, reactions of electrophiles with nucleophiles and free-radical propagation. (2) The first metabolic product or the most obvious chemical prospect may not be the reactant. (3) The stability of reactive products influences sites and patterns of damage. A short time ($t_{1/2}$ of 1 s) may be considerable in a cell, and some reactive

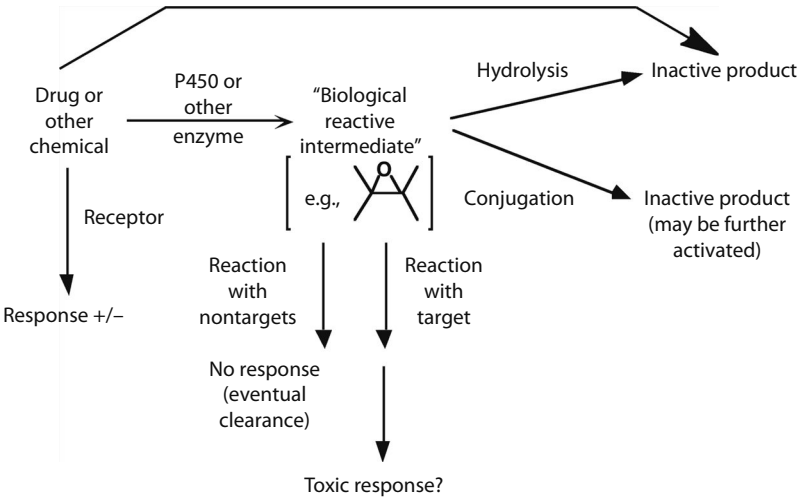


FIGURE 40.1 General paradigm for the role of enzymatic bioactivation and detoxication.

molecules are long lived ($t_{1/2}$ of min to h). (4) In vitro systems are models, good for elucidating details. However, only some results, not all, apply in vivo. (5) The dose is an issue or more properly, *the* issue, a concept traced to Paracelsus.[14] (6) Covalent binding can be an index of toxicity, but exceptions exist even after considerations of dose. Other issues (in addition to covalent binding) are receptor-mediated events (esp. signaling), ability to repair (DNA and protein) damage, cell proliferation, and immune responses.

Events leading to radical initiation involve some type of one-electron chemistry, and radical propagation is often the result of free metal ions in biological systems. An important component of the radical chemistry is oxidative damage, resulting from the production of partially reduced oxygen species. In many cases, the effects of oxidative damage are not readily distinguished from alkylation and reactions of electrophiles. These and other events, including direct interactions of either parent chemicals or products with cellular

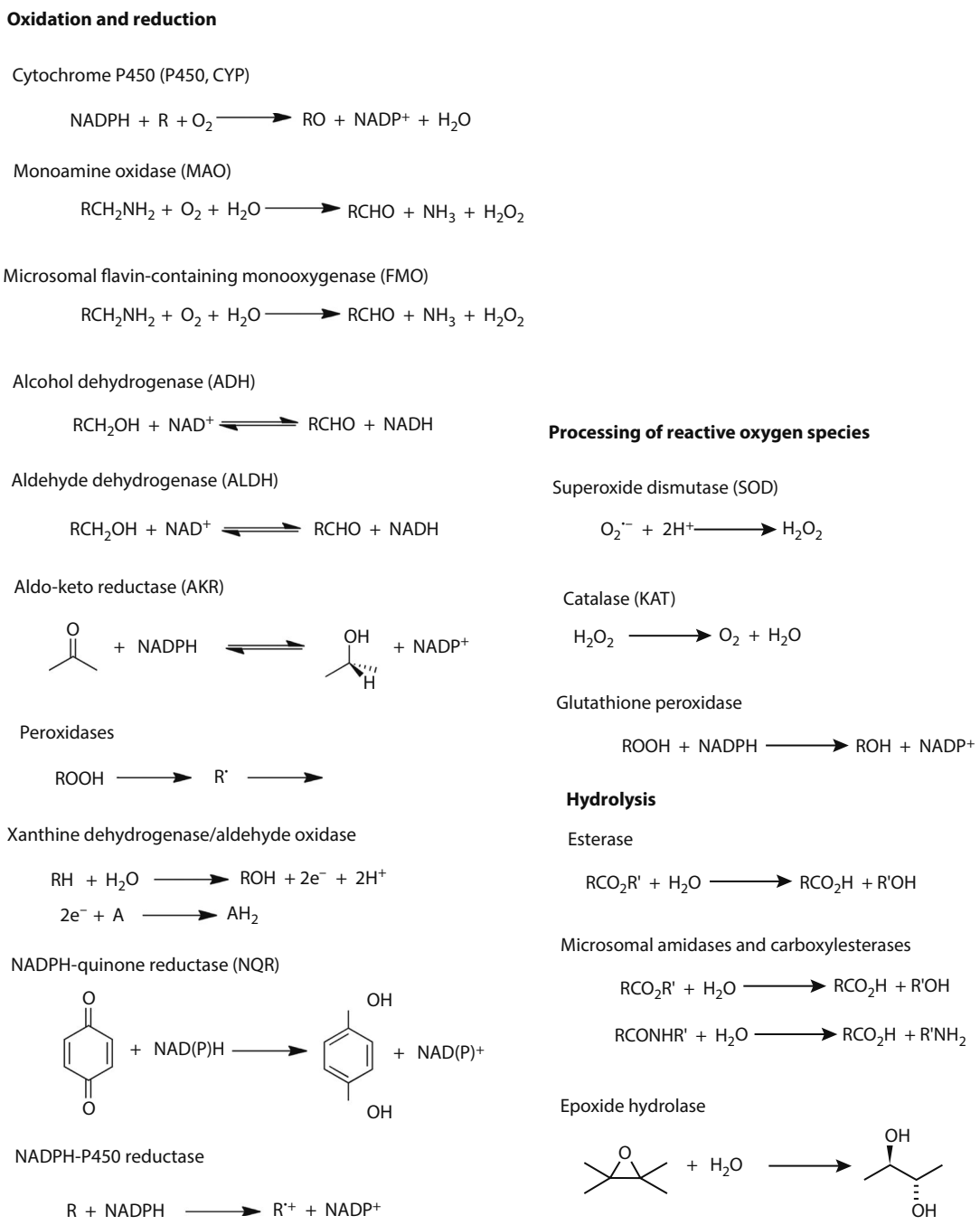
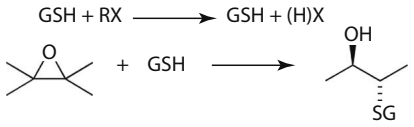


FIGURE 40.2 Enzymes involved in metabolism and relevant to toxicology. (From Guengerich, F.P., Introduction and historical perspective, in: Guengerich, F.P., ed., *Biotransformation*, McQueen, C.A., series ed., *Comprehensive Toxicology*, Vol. 4, 2nd edn., Elsevier, Oxford, U.K., pp. 1–7, 2010.)

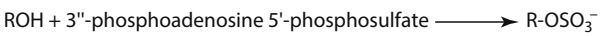
(*continued*)

Conjugation

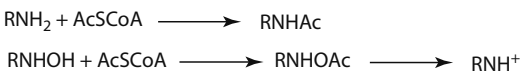
GSH transferases (GST)



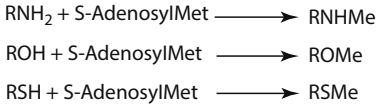
Sulfotransferases (SULT)



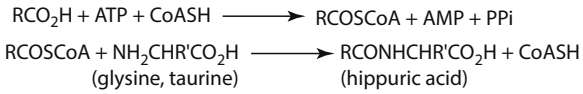
N-Acetytransferase (NAT)



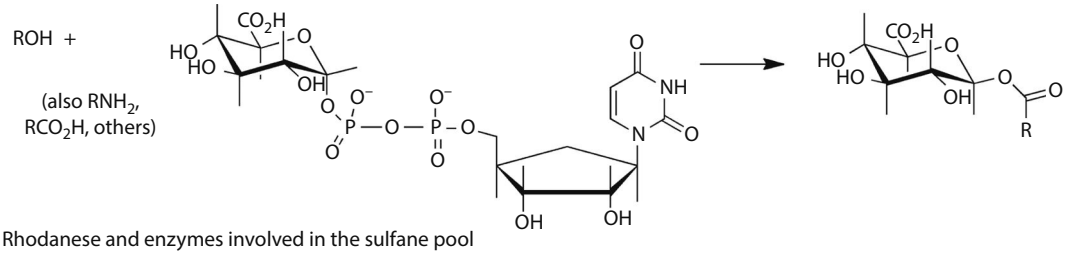
Methyltransferases



Enzymes involved in formation of amide bonds



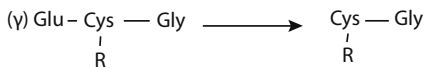
UDP glucuronosyl transferase (UGT)



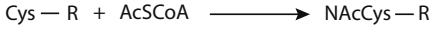
Rhodanese and enzymes involved in the sulfane pool

Enzymes involved in processing of GSH conjugates

γ-Glutamyl transpeptidase



Cys conjugate acetyl transferase



Cys conjugate β-lyase

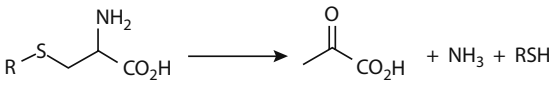


FIGURE 40.2 (continued) Enzymes involved in metabolism and relevant to toxicology. (From Guengerich, F.P., Introduction and historical perspective, in: Guengerich, F.P., ed., *Biotransformation*, McQueen, C.A., series ed., *Comprehensive Toxicology*, Vol. 4, 2nd edn., Elsevier, Oxford, U.K., pp. 1–7, 2010.)

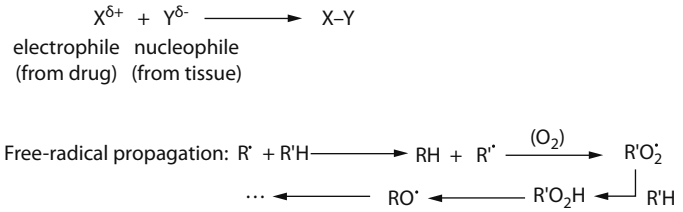


FIGURE 40.3 Basic chemical reactions involved in chemical toxicity: reaction of an electrophile with a nucleophile; free-radical propagation.

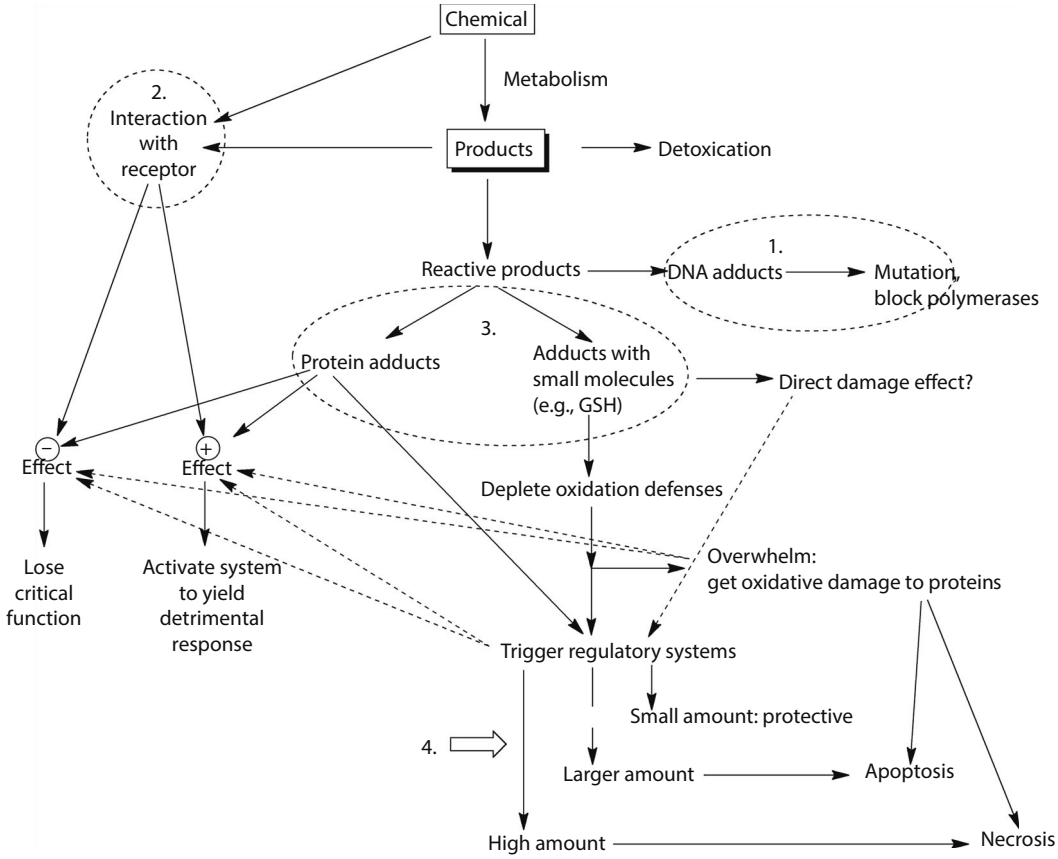


FIGURE 40.4 Cellular events related to the potential toxicity of chemicals.[13] See text for discussion. The steps indicate points for medium- to high-throughput assays: (1) genotoxicity, (2) interaction with known receptors, (3) covalent binding to proteins or surrogate nucleophiles, and (4) events related to disruption of normal cellular function.

receptor systems, are elaborated in the complex global view of the present knowledge of events related to toxicity in Figure 40.4.[13,15] Covalent binding of metabolites is only one of the ways of disrupting the networks in a cell. Some direct effects on receptors can produce similar outcomes. The numbers indicate the events that would be useful to monitor: (1) Genotoxicity tests are relatively well established and have been used in preliminary screening for >30 years. (2) Many receptor assays are now routinely done (e.g., AhR, PPARα). Most of the guilt is by association, in that most of the details related to toxicity are still vague. (3) Recently, assays have been developed to monitor the reaction of electrophiles with small thiols, with the hope of predicting toxicity in medium- to high-throughput assays.[10] (4) The real goal is to develop biomarkers and assays that will prevent events in this area related to toxicity. Some assays are in use, but this area would appear to have the greatest potential.[16]

Some examples of bioactivation processes follow. The first is the classic case of acetaminophen (paracetamol). Although this analgesic and antipyretic is used safely every day by many people, overdoses (accidents, attempted suicides) result in a large fraction of the cases of acute liver failure.[17] The chemistry related to acetaminophen is rather simple (Figure 40.5). Small amounts are efficiently conjugated by uridine glucuronosyl transferases (UGTs) or sulfotransferases (SULTs) to yield products that are readily eliminated. However, cytochrome P450 (P450) enzymes are involved in minor oxidation pathways. The catechol product does not appear to be a

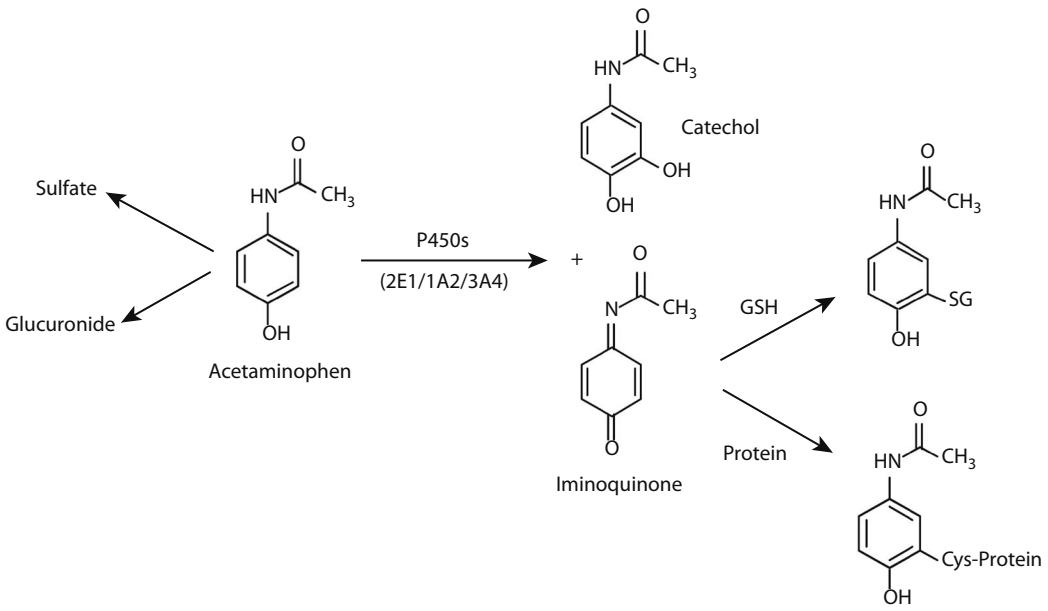


FIGURE 40.5 Pathways involved in metabolism of acetaminophen. The conjugates are formed by the action of SULTs and UGTs. For reference to the oxidations, see[18,19].

problem but the iminoquinone is. GSH reacts with the iminoquinone and blocks its reaction with proteins. Thus, at low concentrations of acetaminophen, very little tissue damage results. When the concentration of acetaminophen increases, the sulfation and glucuronidation pathways are overwhelmed and, in some cases, GSH is depleted and more of the iminoquinone reacts with tissues.[20] The extent of covalent binding is well correlated with parameters of toxicity.[21] Interestingly, the *meta* isomer of acetaminophen is much less toxic but gives the same level of total covalent binding.[22,23] The protein targets for the two isomers are not identical,[24] but exactly which targets for acetaminophen are most relevant to toxicity, if any are, is still not clear. Recently, a number of pharmaceutical companies have utilized in vitro (and in vivo) screens for covalent binding.[10] Although a strong case can be made for the role of bioactivation and covalent binding in the toxicity of many drugs—for example, [25,26]—exactly what the impact of these screens has been is not easy to discern. Several recent studies have involved analyses of the covalent binding of older drugs of established hepatotoxicity (and lack of toxicity).[27–30] The results show generally higher levels of covalent binding with known hepatotoxins, but there is wide variability, even after making appropriate corrections for dose, other rates of metabolism, etc. An overall conclusion, perhaps not surprising, is that the level of covalent binding is one contributor to toxicity but not the only one. The possibility exists that covalent binding to a very select set of proteins may be most reliable in making predictions, but that remains to be established and can be considered in the context of biomarkers.[31] One point to keep in mind is that a number of useful marketed drugs function by covalently binding their targets.[32]

The second example is the prototypic polycyclic aromatic hydrocarbon (PAH) benzo[*a*]pyrene (Figure 40.6). This carcinogen, known since the 1930s,[33] is oxidized to an epoxide. This first epoxide is not unusually reactive and is readily hydrolyzed by microsomal epoxide hydrolase to yield a dihydrodiol. The dihydrodiol is an excellent substrate for some P450s (i.e., human P450s 1A1 and 1B1). The resulting diol epoxide is quite reactive and can either react with H_2O directly, be conjugated by GSH transferases, or react with DNA.

One example, provided in Figure 40.7, is the metabolism of aflatoxin (AF)B_1. Over the course of a decade of research in this and other laboratories, the enzymes involved in nearly every step of AFB_1 metabolism have been identified, and the numerical value of the k_{cat}/K_m is given in Figure 40.7 (for nonenzymatic processes, the estimated second-order rate constant is given). These are the parameters that should be operative at the low AFB_1 concentration encountered in relevant human exposures, although some caveats must be considered when substrate concentrations begin to approximate the enzyme concentrations. As we consider this work in a global text, P450s can activate or detoxicate AFB_1 (Figure 40.7). GST M1 has a protective role, although not being as effective as in some species (e.g., mice). Some aldo-keto reductases (AKRs) protect against protein (but not DNA) damage. Epoxide hydrolase is too inherently slow to provide much catalytic protection of DNA from the 8,9-*exo* epoxide, although enough of the enzyme might scavenge the epoxide, acting as a reagent.[36]

An analysis of interest is the activation of chemical carcinogens (Figure 40.8A).[37] The most striking aspect is the dominant role of the P450 enzymes. Interestingly, beyond these, the AKR enzymes have a role that exceeds that of any other enzyme group, driven by their reported roles in PAH activation to quinones.[38] Of the P450s, six P450s—1A1, 1A2, 1B1, 2A6, 2E1, and 3A4—account for 77% of the reported activations (Figure 40.8B).[37] The Family 1 enzymes are prominent in the activation of PAHs and heterocyclic amines, plus arylamines and a variety of other compounds. The values for

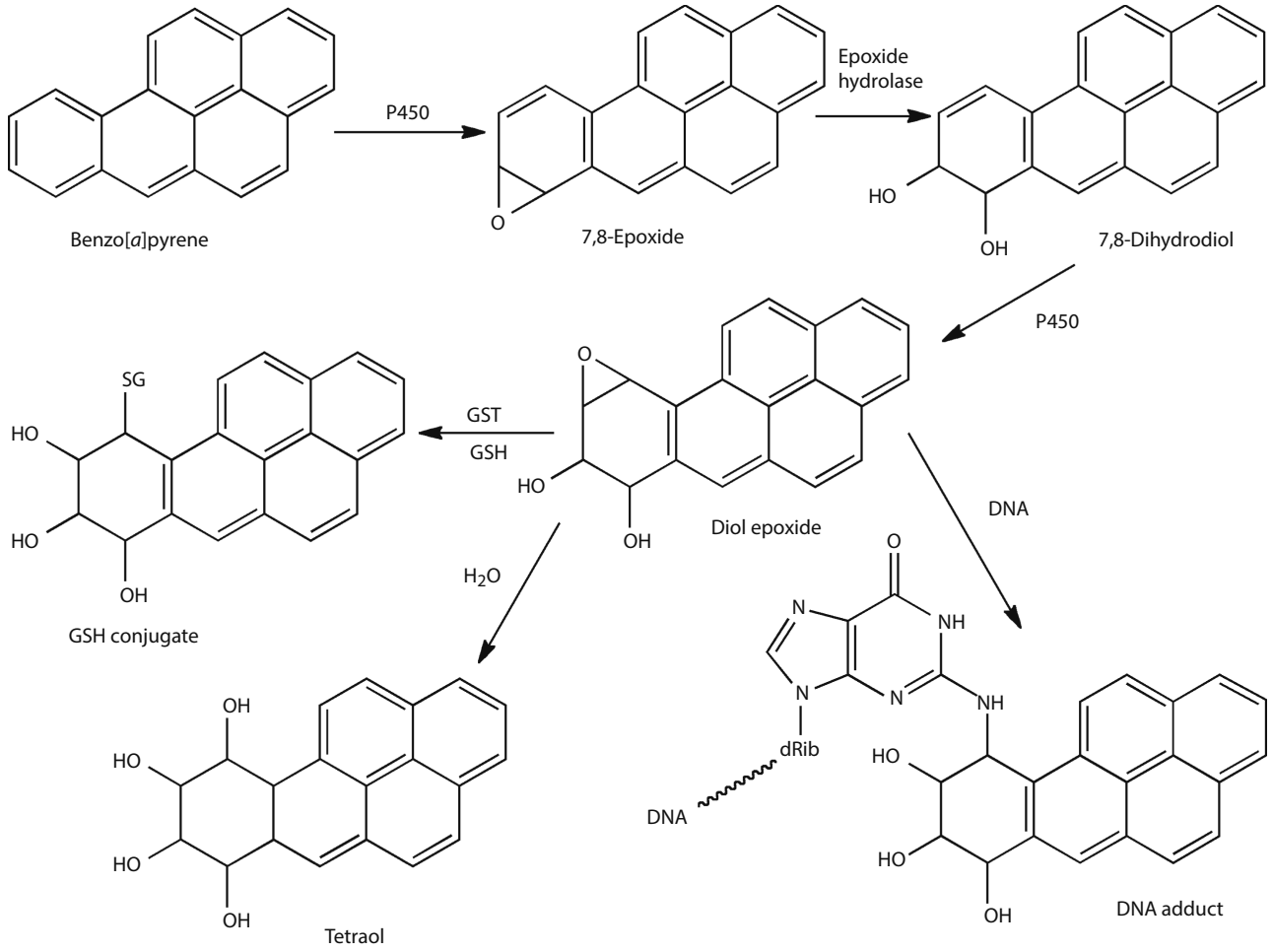


FIGURE 40.6 Major reactions involved in the conversion of the PAH benzo[*a*]pyrene to a reactive diol epoxide, plus reactions of the diol epoxide, plus reactions of the diol epoxide with H_2O, DNA, and GSH.

P450s 2A6 and 2E1 are driven by their roles in the metabolism of *N*-nitrosamines and a variety of low M_r commodity chemicals, including several vinyl monomers.[39] Another analysis involves the type of reactions involved in bioactivation reactions (Figure 40.9). As seen there, 11 reactions account for 94% of the total, each representing 5%–15%. The *O*-acetylation and *O*-sulfonation conjugations collectively account for 22%. Reductions constitute 16%. Most of the other reactions are oxidations, together accounting for ~56%. Of these, *N*-hydroxylation was the most prominent.[37]

Knowledge about the overall pathway can lead to logical intervention strategies. For instance, chlorophyllin can be used to absorb AFB_1, the initial substrate.[40] Oltipraz inhibits P450 oxidation.[41] Oltipraz and other antioxidant response (ARE) inducers (Keap1 modifiers) induce GSTs and AKR enzymes involved in detoxication.[42]

P450 AS A PARADIGM FOR ENZYMES INVOLVED IN TOXICOLOGY

By far the most common mechanism for bioactivation of chemicals is mixed-function oxidation by P450. This enzyme system was really discovered in the late 1950s and has attracted a great deal of interest ever since. A number of enzyme forms exist within each species; some are tissue specific as well as species specific. The different forms preferentially oxidize different substrates, and this specificity contributes to the preferential bioactivation and detoxication of chemicals by different enzyme forms.

With a general catalytic mechanism involving abstraction of electrons or hydrogen atoms followed by oxygen rebound,[43] one can explain the apparently diverse oxidative reactions catalyzed by P450, which can be classified as carbon hydroxylation, heteroatom oxygenation, heteroatom release (dealkylation), epoxidation, oxidative group migration, and other related events (Figure 40.10).[11,44] These basic mechanisms can also explain the suicidal inactivation observed with substrates such as olefins, cyclopropylamines, and aminobenzotriazole; variation of structural features has allowed the selective inactivation of individual enzymes by mechanism-based (suicide) inhibitors.[45,46] P450 also reduces some compounds such as azo dyes, CCl_4, and *N*-oxides.[11,47]

The total number of P450 substrates easily runs into the thousands.[37,47] The broad specificity is due in part to the existence of multiple forms, but even a single purified form

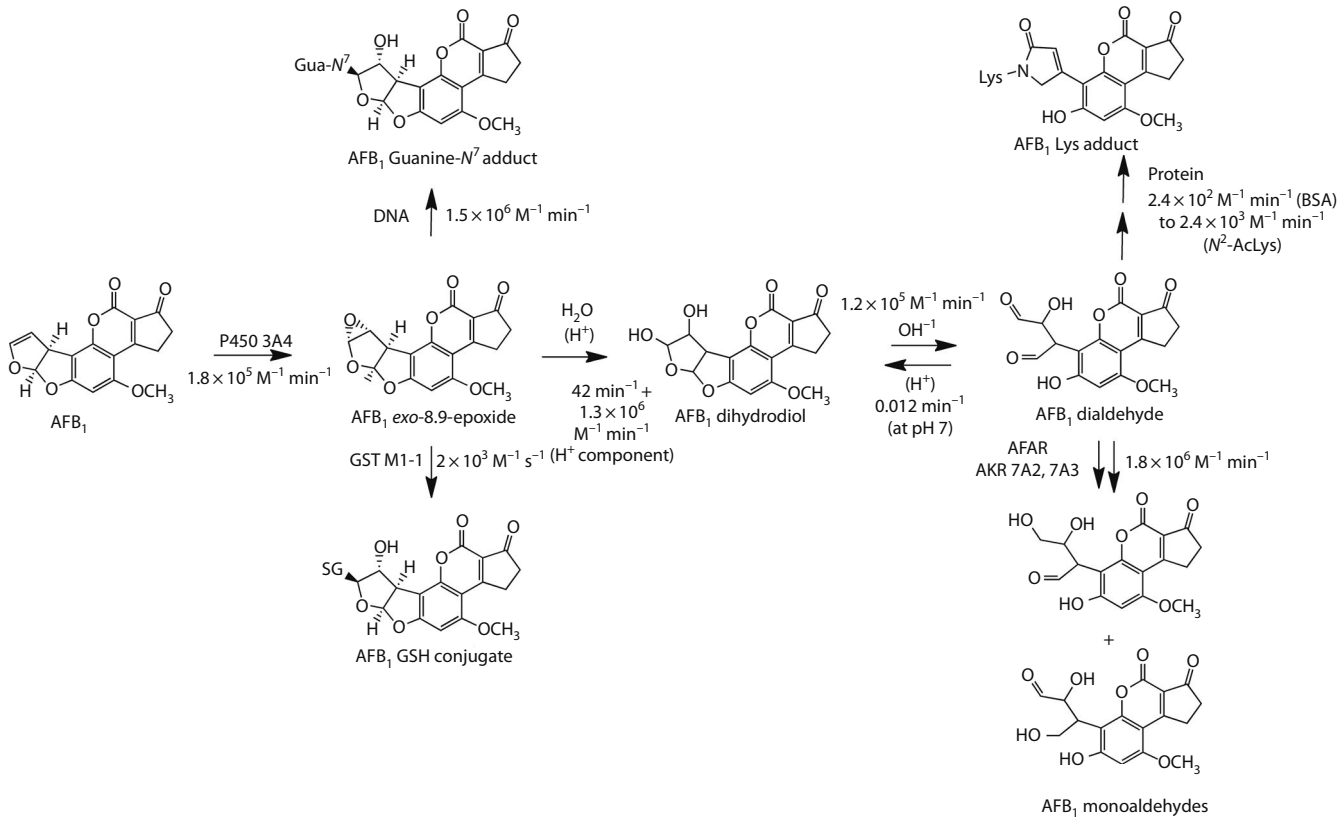


FIGURE 40.7 Reactions involved in the metabolism of AFB_1 in the human liver. The major human enzymes involved in each step are indicated. The values indicated are either second-order rate constants (for chemical reactions) or k_{cat}/K_m values for the purified enzymes. (From Guengerich, F.P. and Johnson, W.W., *Drug Metab. Rev.*, 31, 141, 1999; Guengerich, F.P. et al., *Chem. Res. Toxicol.*, 15, 780, 2002.)

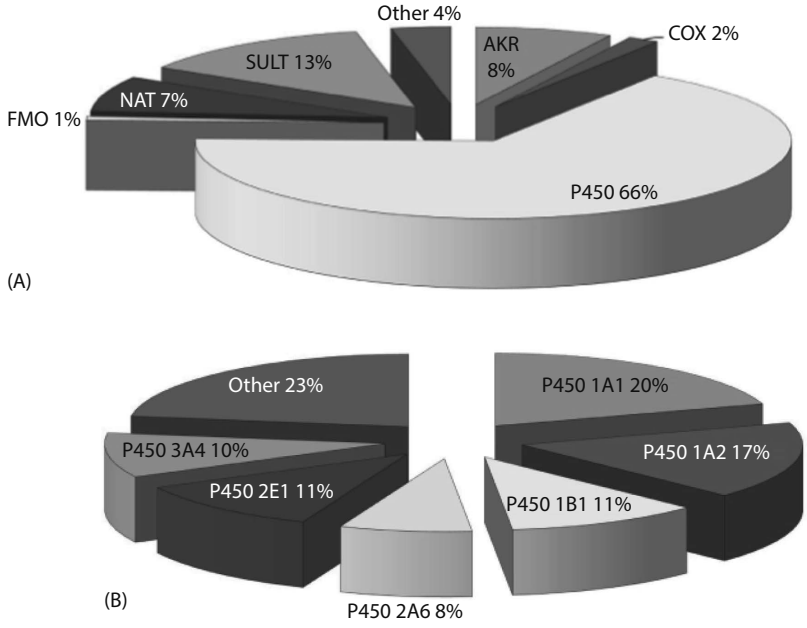


FIGURE 40.8 Enzyme contributions to activation of carcinogens. (From Rendic, S. and Guengerich, F.P., *Chem. Res. Toxicol.*, 25, 1316, 2012.) (A) Fractions of activation reactions attributed to groups of enzymes. The analysis is based on 713 reactions. Abbreviations not previously defined in the text are COX (cyclooxygenase or prostaglandin H synthase), NAT (*N*-acetyltransferase), and FMO (microsomal FMO). (B) Fractions of P450 activation reactions attributed to individual human P450 enzymes (from a total of 473 reactions considered).

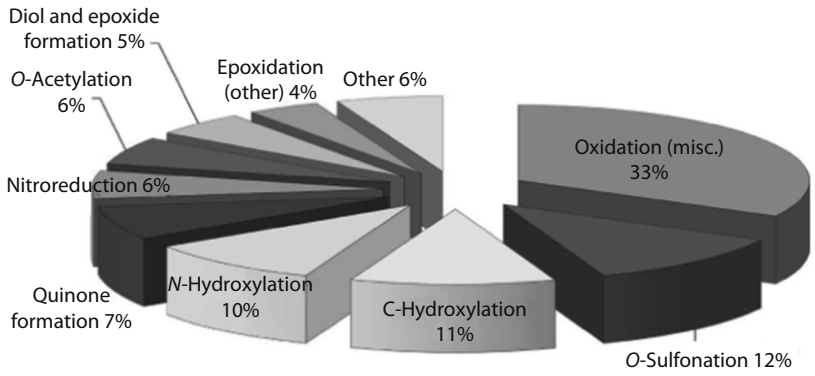


FIGURE 40.9 Analysis of types of activation reactions (total of 799 reactions). (From Rendic, S. and Guengerich, F.P., *Chem. Res. Toxicol.*, 25, 1316, 2012.)

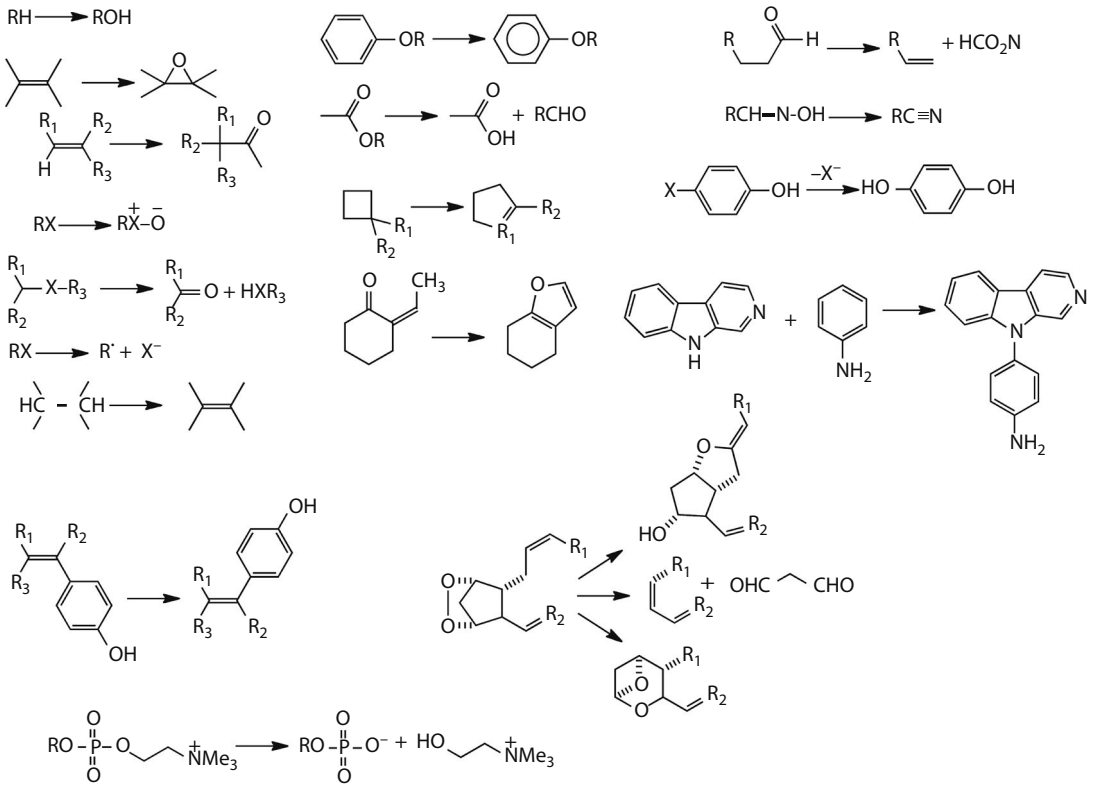


FIGURE 40.10 Some P450 reactions.[11] See also [364,365].

(e.g., human P450 3A4) has been shown to oxidize >1000 different substrates.[48,49] The active site must be large enough to accommodate all of these. However, a number of larger substrates such as warfarin, testosterone, and debrisoquine and other drugs are stereo- and regioselectively oxidized, indicating that the binding sites do really have some distinct features. The smaller substrates apparently fit into these sites and the sites of oxidation on them are maybe governed more by chemical than spatial (physical) properties, although this hypothesis needs to be tested further.

One view is that most P450 enzymes exist for the metabolism of specific endogenous substrates such as fatty acids, steroids, and eicosanoids; however, others believe that the purpose of these enzymes is the clearance of ingested foreign chemicals (terpenes, alkaloids, pyrolysis products, etc.).[50] There is validity in both viewpoints and good cases for individual enzymes with each function may be found (Table 40.1).

One of the major reasons for the widespread study of P450 is that these enzymes are involved in the activation and detoxication of xenobiotics. However, much of the early evidence was circumstantial. The literature is filled with examples in which one can directly demonstrate covalent binding of a chemical to protein or DNA with a P450 or produce mutagenesis in bacterial strains. Known P450-generated metabolites can be found bound to macromolecules in vivo, and in some cases, administration of known products of P450 oxidation

TABLE 40.1
Classification of Human P450s Based on Major Substrate Class[a]

Sterols	Xenobiotics	Fatty Acids	Eicosanoids	Vitamins	Unknown
1B1	1A1	2J2	4F2	2R1	2A7
7A1	1A2	4A11	4F3	24A1	2S1
7B1	2A6	4B1	4F8	26A1	2U1
8B1	2A13	4F12	5A1	26B1	2W1
11A1	2B6		8A1	26C1	3A43
11B1	2C8			27B1	4A22
11B2	2C9				4F11
17A1	2C18				4F22
19A1	2C19				4V2
21A2	2D6				4X1
27A1	2E1				4Z1
39A1	2F1				20A1
46A1	3A4				27C1
51A1	3A5				
	3A7				

[a] This classification is somewhat arbitrary, for example, P450s 1B1 and 27A1 could be grouped in either of the two different categories.[51]

TABLE 40.2
Marker Activities for Some Human P450s Involved in Toxicology Studies

P450	Tissue Sites	Typical Reaction
1A1	Lung, several extrahepatic sites, peripheral blood cells	Benzo[*a*]pyrene 3-hydroxylation
1A2	Liver	Caffeine N^3-demethylation
1B1	Many extrahepatic sites, including lung and kidney	17β-Estradiol 4-hydroxylation
2A6	Liver, lung, and several extrahepatic sites	Coumarin 7-hydroxylation
2A13	Nasal tissue	Activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
2B6	Liver, lung	(*S*)-Mephenytoin *N*-demethylation
2C8	Liver	Taxol 6α-hydroxylation
2C9	Liver	Tolbutamide methyl hydroxylation
2C19	Liver	(*S*)-Mephenytoin 4′-hydroxylation
2D6	Liver	Debrisoquine 4-hydroxylation
2E1	Liver, lung, other tissues	Chlorzoxazone 6-hydroxylation
3A4	Liver, small intestine	Testosterone 6β-hydroxylation
3A5	Liver, lung	Testosterone 6β-hydroxylation
3A7	Fetal liver	Testosterone 6β-hydroxylation
4A11	Liver	Fatty acid ω-hydroxylation

Source: Guengerich, F.P., Human cytochrome P450 enzymes, in: Ortiz de Montellano, P.R., ed., *Cytochrome P450*: *Structure, Mechanism, and Biochemistry*, 3rd edn., Kluwer Academic Publishers, New York, pp. 377–530, 2005.

can produce the toxicity observed with the parent substrate (e.g., fluoroxene and trifluoroacetic acid).[52] Often, the administration of chemicals that are known to induce (or inhibit) forms of P450 can increase or decrease the toxicity of a certain chemical in experimental animals. The usefulness of this approach is limited because of the ability of these chemicals to alter the levels of several cytochromes P450 concomitantly, alter the content of other enzymes, and affect physiological parameters (e.g., blood flow) that contribute to endpoints under consideration. The problem of not knowing exactly *how* metabolites exert toxic effects also obfuscates the problem. While P450s do appear to play a role in the generation of ultimate toxicants and carcinogens, efforts to show the importance of changes in the composition of individual forms on the effects are more difficult.

Today, no doubt exists concerning the existence of distinct forms of P450 in experimental animals or man. At this time, 83 gene products have been characterized in rats and 57 in humans (Table 40.1).[53] See the Internet sites http://drnelson.uthsc.edu/cytochromeP450.html (Cytochrome P450 Homepage) and http://www.imm.ki.se/CYPalleles/ (The Human Cytochrome P450 (CYP) Allele Nomenclature Database) for more current information regarding P450 genes and sequences. All of the human P450 genes are known from the Human Genome Project. The purified proteins differ in electrophoretic properties, immunochemical aspects, primary sequence, and other criteria, including catalytic specificity (Table 40.2). The genomic sequences of >18,000 P450 enzymes in different species are known. In addition, many genomic DNA sequences have been established. There is no evidence to support an earlier view that gene translocations have made P450 a huge supergene family like the immunoglobulins.

In some cases, considerable conservation of P450 structure is found between species. Small differences among the P450 proteins, however, can generate large differences in catalytic activity and substrate specificity, as shown in several notable examples. Lindberg and Negishi[54] showed that the change of a single residue (209) of mouse P450 2A5 could alter its catalytic selectivity quite dramatically.

Some human P450 enzymes were purified,[48,55] and many more have been characterized using recombinant methods.[51] These have been shown to have specificity in catalyzing the oxidation of drugs and other chemicals. In every case, some structural similarity with certain P450 forms isolated from experimental animals has been shown; however, this similarity can be misleading in ascertaining catalytic specificity in some cases.[56] As in the case of experimental animal models, immunochemical methods have been of use in establishing the roles and catalytic specificities of the human P450s.

There are several levels at which overall catalytic activity can be influenced.[57,58] Cofactor supply can be important: NADPH levels can be altered by starvation, and oxygen gradients exist in the liver. The different P450 forms also are localized preferentially in different regions of the liver (and in different cell types in liver and extrahepatic tissues). Heme is necessary as a prosthetic group for P450 and a deficiency

can lower activity. NADPH–P450 reductase is present at a level an order of magnitude lower than P450 and is needed for activity. While reconstituted P450 systems are stimulated by phospholipids, in vivo changes in lipid composition probably do not have any significant effect in most cases,[59] with the notable exception of the P450 3A family enzymes.[60,61]

In experimental animals, the major way in which activities, or at least rather specific catalytic activities, are modulated is via changes in the amounts of individual P450 forms present.[62,63] Such changes have been clearly demonstrated to involve de novo synthesis and often involve specific increases in rates of nuclear DNA transcription. The list of P450 inducers is nearly as long as the list of substrates. Only in a few cases does one see induction of only one P450; more commonly, several are induced. In some cases, the level of one of these proteins is depressed, while others are induced.[64,65] Several of the rodent liver cytochromes P450 are also regulated by steroid hormones.[66,67] Most of the P450 proteins have similar half-lives (about 24 h); however, some evidence exists for a stabilizing effect of certain classic *inducers* on mRNA stability. In the case of most cytochromes P450, evidence exists for intracellular receptors that bind the inducing ligands.[68]

Another aspect of regulation is polymorphism, which is observed with certain catalytic activities in both experimental animals and man. While most of the catalytic activities associated with P450s are affected at least somewhat by environmental factors, genetic polymorphisms are distinct and not so readily influenced by other factors. These can be produced by variations in structural genes (coding or regulatory regions) or by variations in other proteins that regulate expression. Polymorphisms have been mapped to chromosomes in rats and mice, and structural details underlying the polymorphisms have been elucidated in many cases. In humans, genetic polymorphisms related to the metabolism of certain drugs have been identified. Correlations have been made between individuals expressing individual phenotypes (or genotypes) and susceptibility to chemical carcinogenesis. Low activity of the steroidogenic P450s can lead to lethality or debilitating diseases.[51,69,70] Still another issue with human P450 is inhibition, which can be a major issue in drug–drug interactions (Table 40.3).

While the cytochromes P450 have received considerable attention, one should realize that the existence of *isozymes* (or more appropriately, *enzymes*) in a family is not unusual nor is a multigene family. These probably occur with many gene families, including some others that are involved in other aspects of metabolism of xenobiotic chemicals. For instance, at least 19 different forms of human glutathione (GSH) S-transferase (GST) exist and have been shown to be distinct gene products.[75] Again, the catalytic specificities of these enzyme forms differ and many are under differential regulatory control. Considerable evidence supports the view that many forms of UGT exist,[76] and the epoxide hydrolases are distinct gene products.[77] Distinct forms of microsomal flavin-containing monooxygenase (FMO) are found in different tissues, specifically the liver and lung.[78] However, in some of the other instances, only one gene appears to be involved, for example, NADPH–cytochrome P450 reductase.[79]

TABLE 40.3
Useful Selective Inhibitors of Human P450 Enzymes

P450 1A1	7,8-Benzoflavone
	(but see [71,73] regarding P450 1A2)
	Ellipticine
	1-(1-Propynyl)pyrene
	2-(1-Propynyl)phenanthrene
P450 1A2	7,8-Benzoflavone
	Furafylline
	Fluvoxamine
P450 1B1	7,8-Benzoflavone
	2-Ethynylpyrene
P450 2A6	Diethyldithiocarbamate (see [74])
P450 2C9	Sulfaphenazole
	Tienilic acid
P450 2D6	Quinidine
P450 2E1	Aminoacetonitrile
	4-Methylpyrazole
	Diethyldithiocarbamate (see [74])
P450 3A4	Troleandomycin
	Ketoconazole
	Gestodene

Sources: Correia, M.A., Inhibition of cytochrome P450 enzymes, in: Ortiz de Montellano, P.R., ed., *Cytochrome P450: Structure, Mechanism, and Biochemistry*, Kluwer Academic Publishers, New York, pp. 247–322, 2005; Shimada, T. et al., *Chem. Res. Toxicol*., 11, 1048, 1998; Newton, D.J. et al., *Drug Metab. Dispos*., 23, 154, 1994.

ROLES OF P450S IN RELATIONSHIP TO OTHER ENZYMES IN BIOACTIVATION AND DETOXICATION

As mentioned earlier, P450-catalyzed oxidation can result in either the bioactivation or detoxication of a potential toxicant. In general, reduction reactions catalyzed by either P450 or NADPH–P450 usually lead to more reactive products. Oxidations by other enzymes (i.e., FMOs, alcohol, and aldehyde dehydrogenases) can also result in either bioactivation or detoxication. For instance, oxidation of allylic alcohols by alcohol dehydrogenase yields acrolein derivatives, which react rapidly with *soft* nucleophiles. In classical drug metabolism, oxidation–reduction reactions have been termed *Phase I*, and conjugation reactions, which usually follow after oxidation or reduction, are termed *Phase II*.[80] However, this classification is outdated and frankly misleading[81] and it will not be used here. The majority of these conjugation processes detoxicate chemicals, and therefore, increases in the concentrations of the proteins that catalyze these reactions, or increases in the concentrations of cofactors (cosubstrate), tend to render an organism at decreased risk to protoxicants. However, many exceptions to this generalism can be found. For example, epoxide hydrolase action on benzo[*a*]pyrene 7,8-oxide leads to formation of a substrate that is efficiently converted to 7,8-dihydroxy-7,8-dihydro-9,10-oxo-benzo[*a*]pyrene (the dihydrodiol),

which reacts rapidly with DNA and is a potent mutagen and carcinogen (Figure 40.6). GSTs can activate *vic*-dihaloalkanes to yield DNA damage (*vide infra*). Glucuronides are formed by action of UGTs on hydroxylamines, which can break down in the acidic environment of the bladder to release nitrenium ions to alkylate DNA. Thus, we see that metabolic transformations must be viewed in a global manner to put the importance of individual steps into context.

What can studies on metabolic transformations tell us about the toxicity of chemicals? Comparison of the actions of a series of small industrial compounds provides some examples:

$$CH_2{=}CH{-}CN$$

$$CH_2{=}CH{-}Cl$$

$$Br{-}CH_2{-}CH_2{-}Br$$

The first, acrylonitrile, is acutely toxic and also causes several types of general toxicity problems when administered at high doses in chronic studies (i.e., nausea, weight loss, gastric disturbances). The compound is not particularly carcinogenic, causing only tumors of the forestomach, brain, and Zymbal's gland at high doses. These actions can be understood when the various pathways for acrylonitrile are measured using in vitro assays. Acrylonitrile reacts rapidly and nonenzymatically with sulfhydryls, both in proteins and in GSH (Figure 40.11). Conjugation with GSH is the major fate of acrylonitrile and renders it innocuous. Reaction with proteins is considerable and probably accounts for the toxic effects of acrylonitrile. About 10% of acrylonitrile is oxidized by P450 to its epoxide, which can (1) release cyanide (which does not appear to play a role in most toxicity), (2) be conjugated with GSH, (3) alkylate proteins, or (4) alkylate nucleic acids. The extent of the latter reaction does not appear to be very great, consistent with the relatively low tumorigenic potential of acrylonitrile.[82–84]

Vinyl chloride appears similar in structure to acrylonitrile at first glance but behaves quite differently. Only very high doses are acutely toxic and this toxicity is probably unrelated to metabolism. However, vinyl chloride is carcinogenic, causing a peculiar hemangiosarcoma that is almost unique to vinyl chloride production workers and can be reproduced in laboratory animals. Unlike acrylonitrile, vinyl chloride does not react directly with thiols and its metabolism proceeds strictly through oxidation. The epoxide 2-chlorooxirane (2-chloroethylene oxide) can react with nucleic acids to form several lesions, including 1,N^6-ethenoadenine, N^3,4-ethenocytosine, 1,N^2-ethenoguanine, 7-hydroxy-1,N^2-ethanoguanine (5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1,2-*a*] purine), N^2,3-ethenoguanine, and N^7-(2-oxoethyl)guanine.[85] Which of these is most intimately related to tumorigenesis is yet unclear, although evidence favors the etheno adducts.[86,87] The epoxide also spontaneously rearranges to form 2-chloroacetaldehyde,[88–90] which is more like acrylonitrile, reacting rapidly and nonenzymatically with GSH and protein thiols. It reacts only slowly with nucleic acids and is probably not relevant to tumor initiation. The major site of vinyl chloride oxidation is the parenchymal cells of the liver. However, hepatic tumors originate in the reticuloendothelial cells, which have little, if any, oxidation capacity. A possible explanation is that the epoxide is formed in the parenchymal cells and is stable enough to migrate to other cells (some experimental evidence supports this view)[91]; the differential susceptivity to the alkylating agent may be explained by variations in rates of DNA adduct repair among the cell types.

The next compound to consider in this series is ethylene dibromide (1,2-dibromoethane). This compound causes kidney toxicity and is carcinogenic at a number of sites. Oxidation by P450 (esp. P450 2E1)[39] yields 2-bromoacetaldehyde, which behaves in the same way as 2-chloroacetaldehyde (*vide supra*) and depletes sulfhydryls. GSH transferase-catalyzed conjugation of ethylene dibromide with GSH also occurs; the ratio of ethylene dibromide metabolized through the oxidative and conjugative pathways is ~4:1 in rats.[92] In this case, the GSH conjugate is unstable, however, because of the leaving group still present (Br) (Figure 40.12). Nonenzymatic dehydrohalogenation produces an episulfonium (thiiranium) ion,[95,96] which also has several fates. If it is hydrolyzed, *S*-(2-hydroxyethyl)GSH is formed and this innocuous product is degraded and excreted. The putative

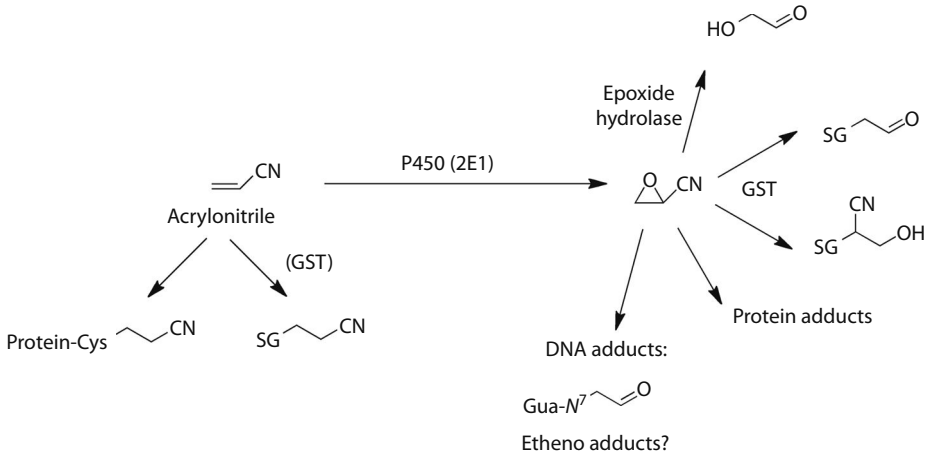


FIGURE 40.11 Proposed scheme for metabolism of acrylonitrile. (From Geiger, L.E. et al., *Cancer Res.*, 43, 3080, 1983.)

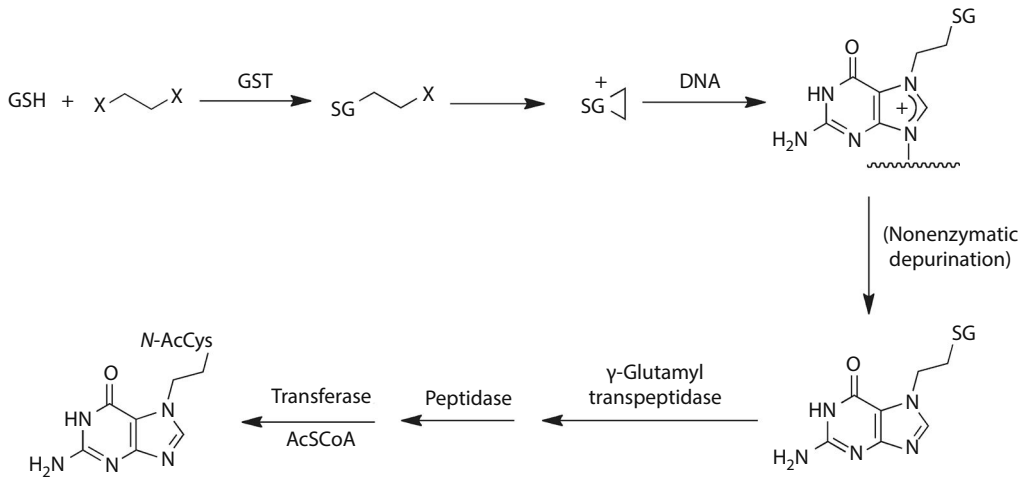


FIGURE 40.12 Scheme depicting formation of the major DNA adduct from ethylene dihalides and the degradation of the adduct. (From Inskeep, P.B. et al., *Cancer Res.*, 46, 2839, 1986; Kim, D.H. and Guengerich, F.P., *Cancer Res.*, 49, 5843, 1989.)

episulfonium ion can also react with another GSH to form the ethylene-bis GSH adduct, which is also innocuous. Another possibility is elimination to yield GSSG (oxidized GSH) plus ethylene, another mode of detoxication.[97] However, another reaction (of the episulfonium ion) occurs with DNA to yield *S*-[2-(*N*^{7}-guanyl)ethyl]GSH as the major product.[98] This GSH pathway appears to be related to carcinogenesis because in vitro DNA binding and mutagenesis are much more dependent upon cytosolic than microsomal enzymes, and in vivo studies also support this view.[93,99] Thus, we see here that GSH conjugation can become a major bioactivation pathway.

The aforementioned three compounds share some apparent features of similarity, yet further analysis indicates that they differ widely in terms of their chemical properties, the manner in which they are handled by the body and the biological effects that are exerted. Much of our understanding of these chemicals has come from in vitro studies using assays of the type that are described here. But what can we learn from studies that focus on identification and quantitation of individual enzymes?

One example involves the suppression of a particular P450 in rat liver. PAHs (e.g., 3-methylcholanthrene), β-naphthoflavone, and isosafrole induce increased synthesis of at least two forms of P450 in rat liver, P450 1A1 and P450 1A2. Increases in microsomal catalytic activities following administration of such compounds have generally been held to support the involvement of these inducible forms in a particular transformation, and in many in vivo studies alterations in acute toxicity of compounds by these inducers have been interpreted in the same terms. The levels of a particular form of P450, P450 2C11 (measured with a specific antibody), are decreased when compounds are given to rats that induce P450 1A1 and P450 1A2 (also measured immunochemically).[65] The decrease is as much as 10-fold when certain polybrominated biphenyl congeners are administered to rats.[64] P450 2C11 is male specific[66,67] and is responsible for the bulk of certain catalytic activities, including testosterone 2α-hydroxylation[67] and (in rats) generation of the most toxic products of AFB_1.[100] If formation of a reactive metabolite is mediated by P450 2C11 and neither P450 1A1 nor P450 1A2 act on the parent compound, then one might (without knowledge of the complexity of the situation) conclude that if administration of PAHs such as 3-methylcholanthrene to rats decreases toxicity and total P450 levels, P450 must have a detoxicating role in metabolism. As we see here, that view could be totally erroneous and lead to unsound predictions for other situations. The basic information underlying the phenomenon presented here, that is, the suppression of individual forms of P450, could only have been obtained with the use of purification, enzyme reconstitution, and immunochemical techniques.

Does the identification and assay of individual enzyme forms have any relevance in clinical settings? The answer is yes, and several examples will be given from the realm of drug toxicity and therapeutic effectiveness. The antituberculosis drug rifampicin is a potent enzyme inducer and appears to increase the P450 form(s) that catalyzes the A ring hydroxylation of 17α-ethynylestradiol, the major estrogenic component of oral contraceptives. Such oxidation renders the drug ineffective, and cases have been reported where rifampicin administration to women has led to unanticipated pregnancies.[101] In other clinical cases, genetic deficiency in P450 2D6 has led to the accumulation of certain drugs and the production of undesirable side effects, such as the neuropathy associated with perhexiline and captopril-induced agranulocytosis.[102] The suggestion has been made that dangers associated with chemicals in the environment may be affected by some of the same factors that influence drug clearance; for instance, individuals lacking P450 2D6 have been suggested to be less prone to tumors related to AFB_1 and cigarette smoking,[103,104] although the findings are controversial. The molecular basis of the P450 2D6 polymorphism is now known.[105] We can now understand some interindividual variations in response to potentially toxic chemicals at the level of specific sequence changes. Toward this end, methods in enzymology can be applied in the field of toxicology.

For many of the enzymes under consideration here, a number of purification and heterologous expression techniques have been developed independently, and the reader is referred to the original literature for details. In describing the general assay procedures for use with microsomal and purified fractions, an effort has been made to deal with some of those most commonly used in the author's and other laboratories. Selected examples of different types of assay procedures are given, and many of these can be adapted to other uses.

ANALYTICAL AND PREPARATIVE PROCEDURES

Preparation of Microsomal and Cytosolic Fractions

Microsomal fractions have been prepared from a variety of tissues using procedures developed for use with rat liver. The following procedure[106,107] has been found to be useful in this laboratory for the preparation of microsomes and cytosol from a variety of animal and human tissues.

Reagents (All should be at 0°C–4°C for storage and use):

1. 1.15% KCl (w/v).
2. Buffer A: 0.10 M Tris–acetate buffer (pH 7.4) containing 0.10 M KCl, 1.0 mM EDTA, and 20 mM butylated hydroxytoluene (BHT).
3. Buffer B: 0.10 M potassium pyrophosphate buffer (pH 7.4) containing 1.0 mM EDTA and 20 mM BHT.
4. Buffer C: 10 mM Tris–acetate buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v).

Rats are killed by CO_2 asphyxiation in a closed container, in line with current animal care regulations. Livers are excised and placed in cold 1.15% KCl. All subsequent steps are carried out at 0°C–4°C. The livers are trimmed of debris and washed with 1.15% KCl; if one desires, hemoglobin contamination can be lowered by perfusing livers with KCl via the portal vein. The livers are blotted and weighed, placed in four times that weight of buffer A, and minced with a scissors. The method of homogenization depends upon the scale of the preparation. If only a few livers are used, a mechanically driven Teflon-glass homogenizer (4–5 vertical passes) is preferred. For larger preparations, two 40 s bursts in a Waring blender are more efficient.

The homogenate is centrifuged at $10^4 \times g$ for 20 min and the supernatant is saved. If the yield of microsomes is a factor, the precipitate can be homogenized in buffer A again and recentrifuged to obtain additional supernatant. The supernatant is submitted to centrifugation at $10^5 \times g$ (3.5×10^4 rpm in a Beckman 45 Ti rotor) for 60 min to yield a microsomal pellet. After discarding the supernatant, a volume of buffer B equal to that of the discarded supernatant is added and microsomes are removed from the clear glycogen pellet by gentle swirling or, if necessary, with the use of a rubber policeman. The suspended microsomes are homogenized with four passes of a mechanically driven Teflon-glass homogenizer and recentrifuged at $10^5 \times g$ for 60 min; the resulting pellets are homogenized and recentrifuged (60 min at $10^5 \times g$). The pellet is homogenized (with four strokes of the Teflon-glass system) in a minimum volume of buffer C (to give 20–50 mg protein mL^{-1}) and stored at –20°C or –70°C (the latter is preferable).

Several comments are in order. BHT and EDTA are added to retard lipid peroxidation, and the pyrophosphate buffer is useful in removing hemoglobin and nucleic acids.[107] If proteases are a potential problem, as is often the case in extrahepatic tissues, phenylmethylsulfonyl fluoride (PMSF) or other protease inhibitors can be used. PMSF is unstable in water; a stock 0.10 M solution should be prepared in absolute ethanol or *n*-propanol, stored at –20°C, and added to buffers to give a final concentration of 0.10 mM immediately prior to their use. The use of dithiothreitol (DTT) has also been reported to be useful in the preparation of functional rat colon microsomes.[108]

Buffers containing 0.25 M sucrose can be substituted for buffers A and B in the procedure. Some older procedures can be applied if an ultracentrifuge is not available. Precipitation of microsomes can be done at much lower speeds when 8 mM $CaCl_2$ is added to buffers.[109] Alternatively, microsomes have been isolated using gel exclusion chromatography.[110,111] More sophisticated techniques are available for the separation of rough and smooth endoplasmic reticulum and Golgi apparatus fractions. Some workers prefer to store microsomes as frozen pellets. For many enzyme activities, microsomes are functional for at least several months when stored frozen, either as pellets or homogenized in buffer C.

Essentially identical procedures are routinely used to prepare microsomes and cytosol from livers of other animals, including humans.[112] In work with human samples, it is advisable to have individual samples tested for HIV and hepatitis B (and other forms of hepatitis) if at all possible before proceeding. Personnel should be immunized against hepatitis B and C. In handling tissues, personnel should handle samples as if viruses such as HIV or hepatitis might be present, that is, that even a viral test could be in error. Good hygiene is essential, and some key practices used in this laboratory include the following: (1) delivery of all residual tissue materials to the infectious diseases division of the institution for incineration or other disposal; (2) carrying out early steps that produce aerosols (homogenization, balancing of tubes containing crude fractions) in a fume hood; (3) *absolutely no* mouth pipetting; (4) prompt disposal of blotters over which all work has been done; (5) use of disposable plastic gloves and other protective clothing; (6) disinfection of glassware, knives, etc., in an appropriate detergent (e.g., Decon LopHene Detergent Disinfectant, Fisher Scientific, Pittsburgh, PA—containing phenols); and (7) above all, use of common sense in handling potentially dangerous material.

The same procedures used for livers of various animals may be adapted to extrahepatic tissues,[113] although these are usually more resistant to homogenization. Cutting devices (e.g., Tissue-mizer) may be used, although caution is advised if catalytic activity is to be measured in samples. The effects of such procedures should be checked.

Assay of P450

The most generally used method is that of Omura and Sato,[114] which utilizes the reduced-CO versus reduced difference spectrum. The procedure used in this laboratory is outlined in the succeeding text.

Reagents:

1. 0.10 M potassium phosphate buffer (pH 7.4) containing 1.0 mM EDTA, 20% glycerol (v/v), 0.50% sodium cholate (w/v), and 0.40% Tergitol NP-10 (Sigma–Aldrich Chemical Co., St. Louis, MO), Emulgen 913 (Kao-Atlas, Tokyo, Japan), or equivalent detergent (w/v)
2. $Na_2S_2O_4$ (sodium dithionite, sodium hydrosulfite), reagent grade (keep bottle tightly closed when not in use)
3. CO gas (in pressurized tank), reagent purity; *store and use in fume hood*

Microsomes (or other preparations) are added to buffer to give a final concentration of 0.05–5 μM P450, mixed, and divided into two 1.0 mL glass or (disposable) polystyrene cuvettes (10 mm pathlength). The sample cuvette is saturated with 40–60 bubbles of CO at a rate of about 1 bubble s^{-1}. A baseline is recorded between 400 and 500 nm using a split-beam spectrophotometer. A few crystals of $Na_2S_2O_4$ (1–2 mg) are added to each cuvette; the cuvettes are covered with parafilm, inverted several times to mix the $Na_2S_2O_4$, and placed in the spectrophotometer again after checking for liquid on the sides of the cuvettes (alternatively, a *plumping* device [*add-a-mixer*, from NSG Precision Cells, Farmingdale, New York] can be used to mix the contents, without removing the cuvette from the chamber). Spectra are recorded (400–500 nm) until the 450 nm peak reaches a maximum.

The A_{490} (isosbestic point) serves as a reference point. P450 content is determined as follows (Figure 40.13):

$$\frac{\left(\Delta A_{450}-A_{490}\right)_{\text{observed}}-\left(\Delta A_{450}-A_{490}\right)_{\text{baseline}}}{0.091}=\text{nmol P450 mL}^{-1}$$

Cytochrome P420 represents denatured forms of P450 and is determined using the following formulae:

$$\left(\text{nmol P450/mL}\right)\times\left(-0.041\right)=\left(\Delta A_{420}-A_{490}\right)_{\text{theoretical}}$$

$$\frac{\left(\Delta A_{420}-A_{490}\right)_{\text{observed}}-\left(\Delta A_{420}-A_{490}\right)_{\text{theoretical}}-\left(\Delta A_{420}-A_{490}\right)_{\text{baseline}}}{0.110}$$

$$=\text{nmol cytochrome P420 mL}^{-1}$$

The extinction coefficient ($\Delta\varepsilon_{450-490}$) of 91 mM^{-1} cm^{-1} has been verified using highly purified rat and rabbit liver P450 preparations.[115,116] The second set of formulae is based upon the observation that P450 has an extinction coefficient of −41 mM^{-1} cm^{-1} ($\Delta\varepsilon_{420-490}$) in the difference spectrum

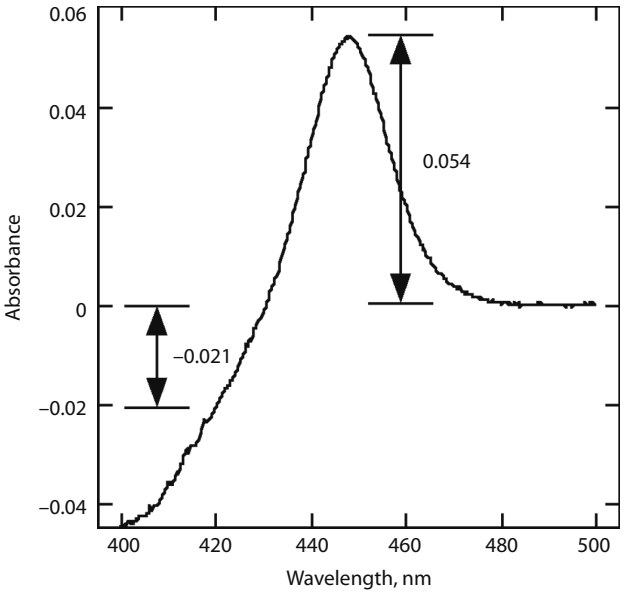


FIGURE 40.13 Calculation of P450 and P420 concentrations. A sample of rat liver P450 1A1 was diluted 10-fold with 0.1 M potassium phosphate buffer (pH 7.7) containing 1 mM EDTA, 20% glycerol (v/v), 0.2% Emulgen 913 (w/v), and 0.5% sodium cholate (w/v). The sample was divided into two cuvettes, and one was saturated with CO gas. The two cuvettes were balanced in an Aminco DW2a/OLIS spectrophotometer using the automatic baseline correction mode. The corrected baseline was recorded. After addition of $Na_2S_2O_4$ as indicated in the text, the final difference spectrum was obtained. The calculations are as follows: 0.054/0.091 = 0.59 nmol P450 mL^{-1}, 0.59 × (−0.041) = −0.024 (A_{420}), −0.024 − (−0.021) = 0.003, 0.003/0.110 = 0.03 nmol cytochrome P420 mL^{-1}, 0.59 × 10 = 5.9 nmol P450 mL^{-1}, and 0.03 × 10 = 0.3 nmol cytochrome P420 mL^{-1}. See text for further discussion.

(i.e., the A_{420} of the reduced-CO complex is less than the A_{420} of the reduced P450).[117]

While rat liver microsomes can be routinely prepared with minimal hemoglobin contamination, this is not the case for extrahepatic preparations. The basic procedure of Matsubara et al.[118] for assaying P450 in liver homogenates is then useful. In this method, two cuvettes are prepared as before but both are equilibrated with CO and the baseline is recorded. $Na_2S_2O_4$ is added *only* to the sample cuvette to obtain a reduced-CO versus oxidized-CO difference spectrum; the extinction coefficient ($\Delta\varepsilon_{450-490}$) is 106 mM^{-1} cm^{-1}. Distinguishing between methemoglobin and cytochrome P420 is difficult, although Johannesen and DePierre[119] have reported that methemoglobin can be specifically reduced by ascorbate and phenazine methosulfate.

Detergents are routinely used in the assay of P450 in this laboratory, as these solubilize the microsomal membranes to reduce light scattering and do not denature P450 in the presence of glycerol.[107] The buffer also helps prevent settling of any insoluble particles. However, some particular P450 proteins may not necessarily be stable in the presence of these detergents.[60] If one desires to carry out determinations in the absence of detergents, a spectrophotometer should be used that is capable of handling turbid solutions. The limit of

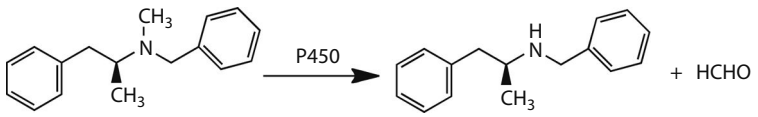


FIGURE 40.14 *d*-Benzphetamine *N*-demethylation.

detection of P450 in extrahepatic tissues is influenced more by the presence of hemoglobin than instrumental considerations. In our own laboratory, we have used Varian 635M and Cary 14, 210, and 219 spectrophotometers for such measurements in the past. Other instruments are also suitable. The Aminco DW-2a instrument has historically been popular among investigators, and we currently use a computer-updated version (On-Line Instrument Systems, Bogart, GA) in our own laboratory; this is particularly useful with bacterial cells (e.g., heterologous expression) because of the ability to handle turbid samples.

Assay of NADPH–Cytochrome *c* Reduction

This enzyme is conveniently measured by its NADPH–cytochrome *c* reduction activity.[120]

Reagents:

1. Horse heart cytochrome *c* (0.50 mM) in 10 mM potassium phosphate buffer (pH 7.7)
2. 0.30 M potassium phosphate buffer (pH 7.7)
3. 10 mM NADPH (fairly stable for <7 days at 4°C in the dark; however, for the most accurate work, solutions should be prepared fresh daily)

Pipette 80 µL of cytochrome *c* solution, the enzyme sample, and sufficient 0.30 M phosphate buffer in a 1.0 mL cuvette (10 mm pathlength) to bring the total volume to 0.99 mL. The components are mixed and preincubated at 30°C (room temperature is also acceptable for most measurements) in a recording spectrophotometer; the recorder is adjusted to zero absorbance at 550 nm (full scale 1.0; slit width 1.0 nm if possible, because of the narrow band being observed). After recording the baseline for 3 min, 10 µL of NADPH is added and the A_{550} is followed for about 3 min. Activity is calculated as follows:

$$\frac{\Delta A_{550}\,\text{min}^{-1}}{0.021} = \text{nmol cytochrome } c \text{ reduced min}^{-1}$$

An amount of enzyme should be used such that the initial ΔA_{550} does not exceed 0.2 min^{-1}. The assay is an indirect measure of NADPH–P450 reductase activity; measurement of the actual reduction of P450 activity requires anaerobic conditions and rapid reaction techniques.[120] The hydrophobic N-terminus of the reductase is required for efficient reduction of P450 but not cytochrome *c*; if no proteolysis has occurred, the two activities are closely correlated.[121]

NADPH–cytochrome *c* reduction activity is stimulated by the high salt concentration used in the assay.[120] If activity is assayed in the presence of mitochondria (or in bacteria), KCN (1.0 mM) may be added as a precautionary measure to block nonmicrosomal activity. The reduction of several other compounds, such as dichlorophenolindophenol, ferricyanide,[121] or a tetrazolium dye,[122] may also be used to assay activity. Spectrophotometers with automatic sample positioners or plate readers may be used to carry out multiple assays simultaneously.

Assays of P450-Linked Activities

Some classic and typical assays will be presented, along with prototypes of different types of methods and approaches.

Benzphetamine *N*-Demethylation

The assay of this activity (Figure 40.14) has been very popular because of its ease and sensitivity, especially with microsomes prepared from animals treated with barbiturates or similar inducers. At least three assays can be used. HCHO is released during the reaction and forms the basis for the first two of the assays.

Colorimetric Measurement of HCHO

For studies on colorimetric measurement of HCHO and other carbonyls, see Nash et al.[123–125]

Reagents:

1. 1.0 M potassium phosphate buffer, pH 7.7.
2. 10 mM $NADP^+$.
3. 1 mg = 10^3 IU yeast glucose 6-phosphate dehydrogenase mL^{-1} (dissolved in 10 mM Tris–acetate buffer [pH 7.7] containing 1.0 mM EDTA and 20% glycerol [v/v]).
4. 0.10 M glucose 6-phosphate.
5. NADPH-generating system: Mix 25 parts Reagent 2, 1 part Reagent 3, and 50 parts Reagent 4, in an amount sufficient to supply 0.15 mL (mL of total reaction)$^{-1}$; store on ice during the day of use and then discard. This mixture may need to be preincubated to the reaction temperature prior to addition; otherwise, keep on ice during the day of use, then discard. *Note*: The system may be easily checked before use, which is a good idea when using new reagents or doing a large number of experiments: add 0.10 mL of the reaction buffer, 0.05 mL of Reagent 2, 0.10 mL of Reagent 4, and 0.75 mL H_2O to a cuvette and mix. Place this in a spectrophotometer and adjust the baseline to zero. Add 2 µL of Reagent 3, preferably using a plumping device (see P450 assay given earlier), and record the change in A_{340}. The system should reach an A_{340} of 1.0–2.0 within 60 s.

6. 10 mM *d*-benzphetamine · HCl.
7. 17% aqueous $HClO_4$ (w/v, 1/4 dilution of conc. $HClO_4$).
8. Nash reagent [300 g $NH_4CH_4CO_2$, 4.0 mL acetylacetone (2,4-pentanedione), and 6.0 mL glacial CH_3CO_2H L^{-1}].

Incubations are carried out in 1.27 mL total volume and include 0.5–2 mg microsomal protein, 50 mM phosphate buffer, and 1.0 mM benzphetamine·HCl (this should be added after the other components from a 10 mM aqueous stock). Tubes are preincubated for 3 min at 37°C and then 0.23 mL of Reagent 5, the NADPH-generating system, to start incubations. After shaking the tubes (150 rpm) for 10 min at 37°C, the incubations are stopped by the addition of 0.50 mL of 17% $HClO_4$ (w/v) and chilled on ice for 5–10 min. (Because of the short incubation time, it is convenient to start and stop individual tubes every 10 s.) Tubes are centrifuged at $3 \times 10^3 \times g$ for 5 min and 1.0 mL of each supernatant is transferred to a new tube. To each of these tubes is added 0.40 mL of Nash reagent. The tubes are heated at 60°C–70°C for 20 min in a water bath (covered with aluminum foil to prevent evaporation). The tubes are cooled in a bath of tap water and A_{412} values are read versus a water blank. Both minus benzphetamine and minus NADPH-generating system blanks should be included; the A_{412} values for these should be similar and are subtracted from the experimental values. A standard curve can be prepared using HCHO; we find that such curves routinely yield factors of 460–480, which, when multiplied by net A_{412}, give the total nmol of HCHO produced. If microcuvettes are used for reading A_{412}, the entire procedure can be scaled down 10-fold if an appropriate spectrophotometer is available. The same procedure is generally applicable to many substrates that release HCHO as a consequence of oxidation by P450.

Radiometric: Extraction of $H^{14}CHO$

This procedure necessitates the use of [*N*-methyl-^{14}C]-benzphetamine but offers increased sensitivity.[116,126] This material can be synthesized from *d*-benzylamphetamine. The synthesis has been carried out in this laboratory.[127]

Assays are set up as in the colorimetric procedure, but the volume is reduced to 0.75 mL and the protein concentration may be reduced to fit the situation. Incubations are stopped by the addition of 0.25 mL 1 M NaOH and 5.0 mL $CHCl_3$ (or CH_2Cl_2). Tubes are mixed using a vortex device and centrifuged ($3 \times 10^3 \times g$ for 5 min). The aqueous upper layer is transferred to a clean tube, 5.0 mL of $CHCl_3$ is added, and the mixing, centrifugation, and transfer steps are repeated. The aforementioned step is repeated once more, and a 0.50 mL aliquot of the aqueous phase is transferred to a miniscintillation vial. The contents are neutralized by the addition of 0.50 mL of 0.10 M sodium citrate buffer (pH 6.5) to which has been added 0.060 M HCl; 5 mL of a water-miscible liquid scintillation cocktail is added. Vials are capped, mixed, and counted (10 min will usually produce satisfactory counting deviation). Blanks contain all components except NADPH or protein.

The efficiency of extraction is >95%. HCHO remains in the aqueous phase and residual substrate is extracted in the $CHCl_3$ layers at the basic pH. A similar procedure may be used in the assay of any substrate that can be labeled with a labeled methyl group that is released following oxidation. An alternate method involves trapping the labeled HCHO product as the dimedone derivative[128] or as the 2,4-dinitrophenylhydrazone or dansylhydrazone derivative and analysis by HPLC (detection with UV, fluorescence, or mass spectrometry).[129,130]

Enhancement of NADPH Oxidation or O_2 Uptake

Because of high rates of endogenous oxidase activity, these procedures are more commonly used with reconstituted enzyme systems than with microsomes.[131,132] In the oxygen electrode procedure, experiments are set up as before and a background rate of O_2 uptake is observed. The differences in the rates obtained with substrates are measured; each nmol of O_2 consumed corresponds to one nmol of substrate oxidized.[132]

The NADPH oxidation assay is carried out in a similar way. The NADPH-generating system is deleted. Incubations, containing all components except NADPH, are preincubated for 3 min at 37°C in 1.0 mL cuvettes in a recording spectrophotometer set at 340 nm (1.0 full scale absorbance). NADPH (15 μL of a 10 mM solution, prepared the same day) is added and the rate of decrease in A_{340} is observed. Blank incubations contain all components except benzphetamine. Rates are determined by dividing net ΔA_{340} min^{-1} by 0.00622 to obtain nmol NADPH oxidized min^{-1}.

The NADPH oxidation and O_2 uptake rates are higher than the rate of oxidation of the substrate because some of the electrons are used in the abortive reduction of O_2 to H_2O_2 and H_2O. The stoichiometry can be calculated.[133]

Fluorescence

7-Ethoxycoumarin O-Deethylation

This reaction (Figure 40.15) is an example of a fluorescence assay involving extraction; it is very sensitive, convenient, and applicable to a wide variety of samples.[134,135]

Reagents:

1. 30 mM 7-ethoxycoumarin (Sigma–Aldrich Chemical Co., St. Louis, MO), dissolved in CH_3OH (avoid exposure to light, store in amber glass).
2. 1.0 M potassium phosphate buffer (pH 7.4).
3. 10 mM $NADP^+$.
4. 10^3 IU yeast glucose 6-phosphate dehydrogenase mL^{-1} (dissolved in 10 mM Tris–acetate buffer [pH 7.7] containing 1 mM EDTA and 20% glycerol [v/v]).

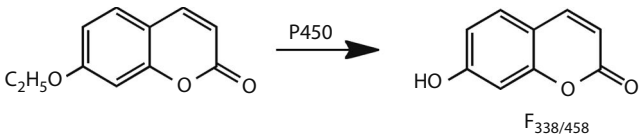


FIGURE 40.15 7-Ethoxycoumarin *O*-deethylation.

5. 0.10 M glucose 6-phosphate.
6. NADPH-generating system: Mix 25 parts Reagent 2, 1 part Reagent 3, and 50 parts Reagent 4 in an amount sufficient to supply 0.15 mL (mL of total reaction)$^{-1}$; see "Benzphetamine *N*-Demethylation" section regarding checking the system.
7. 0.20 M sodium borate (pH 9.6).
8. 1.0 mM 7-hydroxycoumarin (Sigma–Aldrich), dissolved in an aqueous solution of 0.10 N NaOH and 0.10 M NaCl (prepare fresh solution each day; avoid exposure to light).

An appropriate amount of enzyme is placed in a test tube along with 50 mM phosphate buffer, 0.30 mM 7-ethoxycoumarin, and water to bring the volume to 0.90 mL. After 3 min preincubation at 37°C, 0.15 mL of Reagent 6, the NADPH-generating system, is added per mL to start incubations. (As in the case of benzphetamine, reactions are conveniently started and stopped each 15 s.) After 5–10 min, incubations are stopped by the addition of 0.10 mL of 2.0 M HCl and 2.0 mL $CHCl_3$. Tubes are mixed and centrifuged for 5 min at $3 \times 10^3 \times g$. One milliliter of the lower $CHCl_3$ phase (containing both substrate and product) is transferred to a clean tube and 2.0 mL of 0.20 M sodium borate buffer is added. The tubes are mixed and centrifuged 5 min at $3 \times 10^3 \times g$. The upper phase, containing the phenolic product, is transferred to a new tube and fluorescence is read versus a standard curve in a fluorimeter with the excitation wavelength set at 338 nm and the emission wavelength set at 458 nm.

Benzo[a]pyrene Hydroxylation

This assay has been widely used because of its sensitivity, the widespread occurrence of this activity, and the interest in carcinogenic aspects of benzo[*a*]pyrene. This substrate is carcinogenic, light sensitive, and gives rise to many metabolites. The following procedure measures primarily the 3-hydroxy derivative (Figure 40.16) and, to a lesser extent, 9-hydroxybenzo[*a*]pyrene.[136]

Reagents:

1. 1.0 M potassium phosphate buffer (pH 7.4).
2. 10 mM $NADP^+$.
3. 10^3 IU yeast glucose 6-phosphate dehydrogenase mL^{-1} (dissolved in 10 mM Tris–acetate buffer [pH 7.7] containing 1.0 mM EDTA and 20% glycerol [v/v]).
4. NADPH-generating system: Mix 25 parts Reagent 2, 1 part Reagent 3, and 50 parts Reagent 4 in an amount sufficient to supply 0.15 mL (mL of total reaction)$^{-1}$; see "Benzphetamine *N*-Demethylation" section regarding checking the system.
5. 8.0 mM benzo[*a*]pyrene, dissolved in $(CH_3)_2CO$.
6. 6.0 mM quinine, dissolved in 0.10 M H_2SO_4.
7. 1.0 mM 3-hydroxybenzo[*a*]pyrene (this material can be obtained from the National Cancer Institute Chemical Carcinogen Reference Repository, c/o Midwest Research Institute, Kansas City, MO).

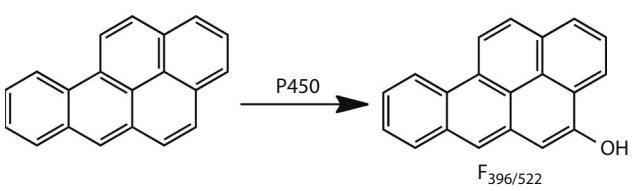


FIGURE 40.16 Benzo[*a*]pyrene 3-hydroxylation.

An appropriate amount of enzyme is placed in a test tube along with 50 mM phosphate buffer (pH 7.4), 80 μM benzo[*a*]pyrene, and sufficient water to bring the total volume to 1.0 mL. (All procedures should be carried out in dim light [or under yellow light]. Alternatively, reactions may be done in amber glass vials. Appropriate precautions should be taken to prevent exposure of skin to benzo[*a*]pyrene or its metabolites. When solid material is being handled, precautions should be taken to avoid breathing dust.) After 3 min preincubation at 37°C, 0.15 mL of Reagent 4 (NADPH-generating system) is added per mL to initiate reactions (this is conveniently done every 10–15 s). After 5–10 min, reactions are stopped by the addition of 1.0 mL of cold acetone and mixed. Hexane (3.25 mL) is added and mixing is repeated. An aliquot of the upper layer (2.0 mL) is transferred to a clean tube with a pipette and 4.0 mL of 1.0 M NaOH is added to this. After vortex mixing and centrifugation for 5 min at $3 \times 10^3 \times g$, the aqueous phase is carefully transferred to a clean tube.

Fluorescence is read with an excitation wavelength of 396 nm and emission wavelength of 522 nm. A standard curve is prepared in 0.10 M NaOH using 3-hydroxybenzo[*a*]-pyrene. Since solutions of the standard are unstable, a convenient method involves setting up the standard curve, changing the wavelength settings to 350 nm (excitation) and 450 nm (emission) without adjusting other settings and preparing a standard curve using serial dilutions of quinine sulfate. The quinine sulfate can then be used as a secondary standard in subsequent experiments and levels of 3-hydroxybenzo[*a*]pyrene can be calculated by reference to the original curves.

An alternative fluorescence procedure devised by Dehnen et al.[137] is comparable in terms of convenience and sensitivity. Other benzo[*a*]pyrene metabolism assays can be carried out with radioactive substrate to measure total polar metabolites,[138] individual metabolites (after separation by high-pressure liquid chromatography [HPLC]),[139,140] or metabolites covalently bound to protein or added nucleic acids.[141]

HPLC

Nifedipine Oxidation

A description of this assay is added to serve as an example of how HPLC methods may be utilized very effectively. Nifedipine is a widely used calcium channel blocker and oxidation by P450 renders it inactive.[142]

Nifedipine oxidation (from the dihydropyridine to the pyridine product [Figure 40.17]) is measured in the following manner. *All incubation, extraction, and other handling of samples are done in amber vials because of the light sensitivity of nifedipine solutions.* Amber glass vials

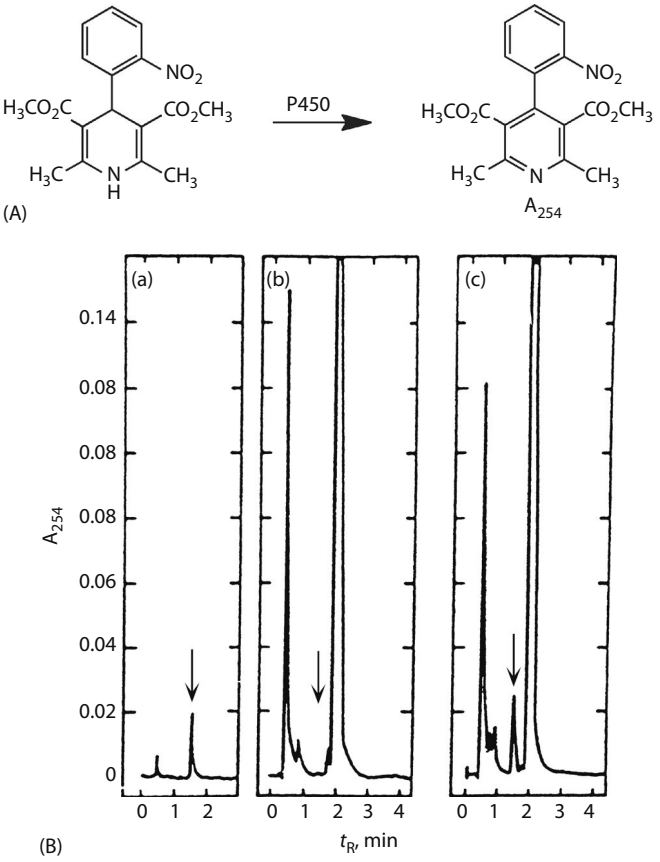


FIGURE 40.17 (A) Oxidation of nifedipine to the pyridine derivative.[142] (B) HPLC separation of the oxidized (pyridine) product of nifedipine oxidation from human liver microsomes. A typical 10 min incubation with 100 pmol of human liver microsomal P450 was done and 20 µL of the total extract was chromatographed as described. The t_R of the nifedipine oxidation product is indicated with an arrow. (a) Authentic standard of the metabolite (200 ng), (b) extract of an incubation devoid of NADPH, and (c) complete incubation. (From Guengerich, F.P. et al., *J. Biol. Chem.*, 261, 5051, 1986.)

are available from Fisher Scientific (Pittsburgh, PA). The use of aluminum foil is not a substitute (here or in other light-sensitive applications), in that it only acts as a mirror. Typical incubations include liver microsomes containing 10–100 pmol of P450, 0.10 M potassium phosphate buffer (pH 7.85), and 0.20 mM nifedipine (added from a stock solution of 20 mM in CH_3OH) in a final volume of 0.50 mL. The components are equilibrated for 3 min at 37°C and the reaction is initiated by the addition of an NADPH-generating system consisting of (final concentrations) 10 mM glucose 6-phosphate, 0.50 mM $NADP^+$, and 1.0 IU yeast glucose 6-phosphate dehydrogenase mL^{-1} (see "Benzphetamine *N*-Demethylation" section regarding using and checking the system). The reaction proceeds for 10 min at 37°C and is then quenched by the addition of 2.0 mL of CH_2Cl_2. One hundred microliters of 1 M Na_2CO_3 buffer (pH 10.5) containing 2.0 M NaCl is added to each vial. The contents of each vial are mixed using a vortex device and the two layers are separated by centrifugation at $3 \times 10^3 \times g$ for 10 min. From each lower organic layer, 1.4 mL is transferred to an amber *Reactivial* (Fisher Scientific, Pittsburgh, PA). The total extract is reduced to dryness at 23°C under an N_2 stream. The residue is dissolved in 50 µL of CH_3OH and 20 µL is injected onto an octadecylsilane (C_{18}) reversed-phase HPLC column (e.g., 6.2 mm × 80 mm; Mac-Mod, Chadds Ford, PA,[142] or suitable alternative) placed in series following an octadecylsilane guard column and 0.2 mm filter. The column is eluted with an isocratic mixture of 64% CH_3OH–36% H_2O (v/v) at a flow rate of 3.0–4.0 mL min^{-1}. Detection is at 254 nm (found to be optimal by previous scanning). Quantitation is usually done with external standards and by the use of peak heights. Alternatively, nitrendipine or another dihydropyridine[143,144] can be used as an internal standard. Typically, a 20 ng sample of the metabolite (59 pmol) yields a maximal A_{254} of about 0.015 under these conditions (in the HPLC effluent). A typical chromatogram resulting from injection of a human liver microsomal incubation extract is shown in Figure 40.16.

Assay conditions were optimized with a human liver microsomal sample.[142] Product formation was linear up to a time of 20 min, the pH optimum was 7.85 (Tris–HCl yielded lower rates than did potassium phosphate buffer), the rate of product formation per unit enzyme was constant over a range of 5–1000 nM P450, and a substrate concentration of 200 µM was optimal (K_m ~ 10 µM; substrate inhibition observed at concentrations >500 µM; no evidence for multiphasic behavior observed over the concentration range of 2–1000 µM).

When purified P450 fractions are assayed for activity, the microsomes are replaced with 20–100 pmol of P450, 250 pmol of rabbit NADPH–P450 reductase, 250 pmol cytochrome b_5, and 15 nmol of L-α-1,2-dilauroyl-*sn*-glyceryl-3-phosphocholine. These components are mixed and then incubated for 10 min at 23°C prior to addition of other materials. Examination of experimental conditions indicated that the NADPH–P450 reductase, substrate, and phospholipid concentrations used are optimal. However, product formation is not linear for more than 5 min. The use of alternate phospholipids has been found to improve the activity.[61]

The separation of the product from the substrate is very efficient with the reversed-phase system used. The only solvent components needed are CH_3OH and H_2O, thus eliminating any problems with salts. The product elutes before the substrate, enhancing sensitivity of the assay. There is no need to have more than baseline separation, and interfering peaks are absent. Thus, a short column can be used with a high flow rate and low back pressure. The total HPLC time for each assay can be <3 min. The efficiency of analysis could also be improved with the use of an automated injection system. Analysis time can also be reduced if incubations are only deproteinized and not extracted prior to HPLC analysis, but the sensitivity would be reduced.

Chlorzoxazone 6-Hydroxylation

Another HPLC-based assay of P450 activity involves measurement of the 6-hydroxylation of chlorzoxazone (Figure 40.18).

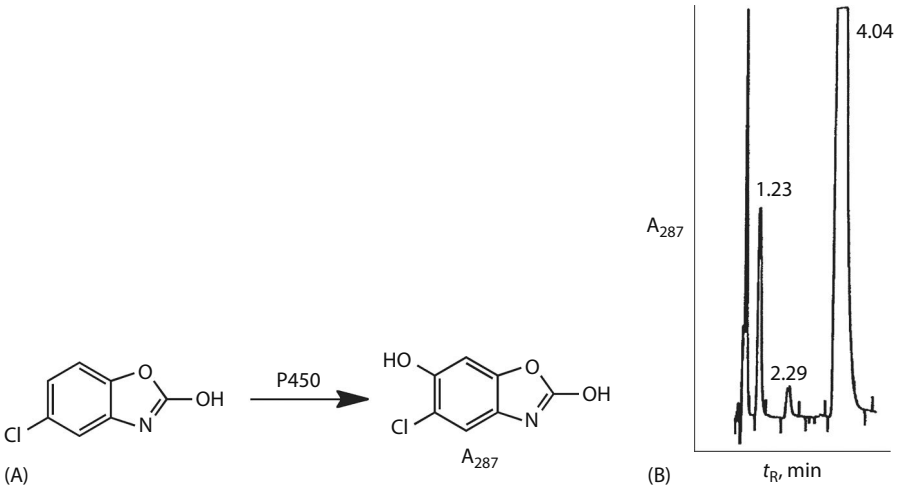


FIGURE 40.18 (A) Chlorzoxazone 6-hydroxylation. (B) HPLC separation of an extract of a chlorzoxazone incubation. See text for details. In this particular assay, 0.27 nmol of human P450 (in liver microsomes) was incubated for 10 min with 0.5 mM chlorzoxazone. The t_Rs of the individual peaks are designated: 1.23 min, 6-hydroxychlorzoxazone; 2.29 min, 5-fluorobenzoxazolinone; and 4.04 min, chlorzoxazone. (From Peter, R. et al., *Chem. Res. Toxicol.*, 3, 566, 1990.)

This reaction has been reported to be highly selective for P450 2E1 in human liver.[39,145] The assay presents considerable advantages of sensitivity, reliability, and specificity over many other traditional assays for the function of P450 2E1, for example, *N*,*N*-dimethylnitrosamine *N*-demethylation and 4-nitrophenol 2-hydroxylation. Chlorzoxazone is used as a drug ([145] and references therein) and this general procedure can be used to measure pharmacokinetic parameters in humans to assess their relative levels of P450 2E1.[146,147]

Reagents:

1. 1 M potassium phosphate buffer (pH 7.4).
2. $NADP^+$, 10 mM.
3. 10^3 IU yeast glucose 6-phosphate dehydrogenase mL^{-1} (dissolved in 10 mM Tris–acetate buffer [pH 7.4] containing 1 mM EDTA and 20% glycerol [v/v]).
4. 0.1 M glucose 6-phosphate.
5. NADPH-generating system: Mix 25 parts Reagent 2, 1 part Reagent 3, and 50 parts Reagent 4 in an amount sufficient to add 0.15 mL (mL of total reaction)$^{-1}$; see "Benzphetamine *N*-Demethylation" section regarding checking the system.
6. 50 mM chlorzoxazone in 60 mM KOH (prepare fresh daily and store on ice: dissolve 51 mg chlorzoxazone [Sigma Aldrich, St. Louis, MO] in 0.36 mL 1.0 N KOH by vigorous mixing with a vortex device or sonic bath, add 0.64 mL H_2O, continue mixing until dissolved, and then add 5.0 mL H_2O). Avoid introduction of organic solvents, because they are inhibitors of P450 2E1.[148,149]
7. 6-Hydroxychlorzoxazone [standard product; see Peter et al.[145] for synthesis].
8. Either 2-benzoxazolinone (Sigma Aldrich) or 5-fluorobenzoxazolinone[145] dissolved in 2.0% aqueous propylene glycol 400 (internal standard [optional]).

An appropriate amount of enzyme is placed in a test tube along with 50 mM potassium phosphate buffer (pH 7.4) and 0.50 mM chlorzoxazone (final incubation volume 0.50 mL). After 3 min preincubation at 37°C, 0.15 mL of Reagent 5 (the NADPH-generating system) is added per mL to start incubations. After 10–20 min at 37°C, reactions are stopped by the addition of a 25 µL of aqueous 43% H_3PO_4 (w/v). An appropriate amount of the internal standard (5.0 nmol) is also added to each tube, followed by 2.0 mL of CH_2Cl_2. The contents of each tube are mixed using a vortex device and the layers are separated by brief centrifugation ($3 \times 10^3 \times g$, 10 min). An aliquot (1.6 mL) of each lower (CH_2Cl_2) layer is transferred with a pipette to a clean test tube or conical vial (Reacti-vial, Fisher Scientific, Pittsburgh, PA) and the solvent is removed under an N_2 stream of 23°C. The residue is dissolved in 50 µL of CH_3CN (mixing on a vortex device) and 20 mL is injected onto a 6.2 mm × 80 mm Zorbax octylsilane (C_8) HPLC column (3 µm, Mac-Mod, Chadds Ford, PA)[145] (or equivalent) utilizing a solvent mixture of 32% CH_3CN (v/v) and 0.5% H_3PO_4 (w/v) in H_2O, with UV detection at 287 nm (flow rate 3.5 mL min^{-1}) (Figure 40.18). Under these conditions, each assay requires ≤5 min HPLC time. For more complex samples, the CH_3CN concentration may need to be decreased to move the produce peak away from the solvent front. It is reasonable to use as little as 5 pmol of microsomal P450 in this assay and obtain reliable results.

GC–Mass Spectrometry

N,N-Dimethylaniline N-Demethylation

Amine *N*-dealkylations are catalyzed by a number of different oxidases and are important in the disposition of drugs and other chemicals. The P450-dependent reactions have historically been of the most interest, although such reactions can also be catalyzed by peroxidases, flavoproteins, and other enzymes as well.[150] The mechanism of the reaction has been a matter of considerable interest and the reader is

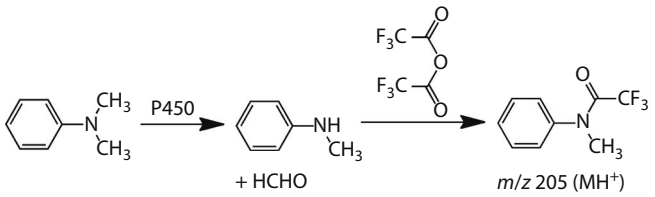


FIGURE 40.19 *N*,*N*-Dimethylaniline *N*-demethylation assay. (From Okazaki, O. and Guengerich, F.P., *J. Biol. Chem*., 268, 1546, 1993; Miwa, G.T. et al., *J. Biol. Chem*., 258, 14445, 1983.)

referred to work on this topic.[151,152] The *N*-demethylation of *N*,*N*-dimethylaniline (Figure 40.19) can be assayed by measurement of HCHO by other procedures (*vide supra*), but the assay is typical of many used for GC and GC–mass spectrometry. It was specifically used in our work to measure kinetic deuterium isotope effects.[151]

Incubations contained an enzyme, an NADPH-generating system (consisting of final concentrations of 0.50 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1.0 IU glucose 6-phosphate dehydrogenase mL^{-1}; see "Benzphetamine *N*-Demethylation" section regarding checking the system), and 1.0 mM *N*,*N*-dimethylaniline in a final volume of 1.0 mL. Reactions proceed for 10 min in vials sealed with Teflon liners (to prevent evaporation of substrate) and are quenched by the addition of 0.40 mL of 17% $HClO_4$ (w/v). After 0.10 mL of 270 μM [N^2-H_3, *ring*-$^2H_5$]*N*-methylaniline is added as an internal standard, the pH is made alkaline and the substrate, products, and perdeuterated internal standard are extracted into 2.0 mL of CH_2Cl_2 by mixing with a vortex device (the internal standard may be prepared by reaction of [*ring*-$^2H_5$]aniline with C^2H_3I[151]). The layers are separated by centrifugation ($3 \times 10^3 \times g$, 10 min) and the (lower) CH_2Cl_2 layer is removed, dried by adding anhydrous Na_2SO_4 and mixing, and then transferred to a clean vial. $(CF_3CO)_2O$ (50 μL) is added to each vial, and the vials are sealed with caps and Teflon liners and allowed to stand overnight at 4°C to form trifluoroacetanilide.

Most of the CH_2Cl_2 is removed under a stream of N_2 (≤30°C), and aliquots (~2 μL) are injected onto a 0.5 mm × 3 m SPB-1 capillary GC column connected to a mass spectrometer operating in the chemical ionization mode with He as the carrier gas and CH_4 as the ionization gas. The column temperature is programmed from 100°C to 280°C at 20° min^{-1}. The ions at *m/z* 205 and 214 (both [M+H$^+$]) are monitored for the product and internal standard. Mixtures of varying ratios of trifluoroacetanilide and the perdeutero derivative are used to prepare a standard curve.

Another example of a procedure involving derivatization of product and GC–mass spectrometry involves the conversion of ethanol to acetaldehyde by human P450 2E1 and derivatization as the oxime.[154] Another example, without derivatization, is presented for ethyl carbamate (EC) (*vide infra*). In some cases, it is impractical to use a heavy isotope as an internal standard, and product analogs must be considered. There are many procedures for GC-based methods involving flame ionization, electron capture, and other means of detection. Electron capture can be a very sensitive assay procedure when halides or nitro groups are present. The use of capillary GC columns has made packed columns nearly obsolete because of the superior resolution. However, the need to use very small injection volumes requires the need for internal standards except in cases where head space analysis is done.

Ethyl Carbamate Desaturation

Ethyl carbamate (urethane) desaturation is presented as another example of a reaction with an assay involving GC–mass spectrometry. The desaturation of EC to vinyl carbamate (VC) (Figure 40.20) had been suggested by Dahl et al.[156,157] but direct evidence for this view had been difficult to obtain. It is now realized that the oxidation of EC to VC is a very slow process and that the succeeding step, epoxide formation, is ~400 times faster (and is catalyzed by the same P450 enzyme).[155] Thus, the steady-state level of VC is very low. The procedure described in the succeeding text can be used to estimate levels of several EC metabolites—VC, 2-hydroxyethyl carbamate, and *N*-hydroxyethyl carbamate.[155] In the reference cited, a major aspect was the qualitative demonstration of these products. With this procedure, it was possible to detect one part product in 10^4 parts substrate.

Reagents:

1. 1.0 M potassium phosphate buffer (pH 7.4).
2. $NADP^+$, 10 mM.
3. 10^3 IU yeast glucose 6-phosphate dehydrogenase mL^{-1} (dissolved in 10 mM Tris–acetate buffer [pH 7.4] containing 1.0 mM EDTA and 20% glycerol [v/v]).
4. 0.10 M glucose 6-phosphate.
5. NADPH-generating system: Mix 25 parts Reagent 2, 1 part Reagent 3, and 50 parts Reagent 4 in an amount sufficient to supply 0.15 mL (mL of total reaction)$^{-1}$; see "Benzphetamine *N*-Demethylation" section regarding checking the system.
6. 0.10 M EC (urethane, Sigma Aldrich Chemical Co., St. Louis, MO; purify by sublimation—use house vacuum or a water aspirator to achieve ~15 mm Hg).
7. 2-Hydroxyethyl carbamate (formerly Pfaltz and Bauer, Stamford, CT; now listed as available from AKos Building Blocks, Steinen, Germany).

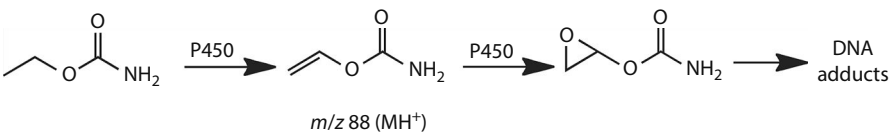


FIGURE 40.20 Desaturation of EC and epoxidation of VC. (From Guengerich, F.P. and Kim, D.-H., *Chem. Res. Toxicol*., 4, 413, 1991.)

8. *N*-Hydroxyethyl carbamate (Sigma–Aldrich, St. Louis, MO).
9. VC.[156,158]
10. Methyl carbamate (Sigma–Aldrich; purify by sublimation before use).

Note: These carbamates, particularly ethyl and vinyl, should be handled with care. They are carcinogenic and volatile. Use fume hoods, closed vials, and appropriate protective clothing.

The oxidation of EC is catalyzed primarily by P450 2E1, so care should be taken to avoid adding organic solvents, which are inhibitory.[148] The reaction is slow and a high concentration of microsomal protein is needed, so it is useful to remove glycerol (or sucrose) from microsomes by dialysis for 1–2 h versus 0.10 M potassium phosphate buffer (pH 7.4) at 4°C just prior to use. Microsomal protein (10–15 mg protein mL^{-1}) is mixed in a vial with 100 mM potassium or an equivalent amount of a reconstituted P450 system, 10 mM EC, and an NADPH-generating system consisting of (final concentrations of) 0.50 mM $NADP^+$, 1 IU glucose 6-phosphate dehydrogenase mL^{-1}, and 10 mM glucose 6-phosphate (see "Benzphetamine *N*-Demethylation" section regarding checking the system). The total volume is 1.0 mL. Each vial is sealed with a cap and Teflon liner and incubated for 2 h at 37°C.

Methyl carbamate is then added as an internal standard and the products are extracted twice into 1 mL of CH_2Cl_2 by mixing on a vortex device and the layers are separated by centrifugation ($3 \times 10^3 \times g$, 10 min). The (lower) CH_2Cl_2 layer is removed and concentrated to ≤0.1 mL under an N_2 stream at ≤23°C, with care taken to avoid concentration to dryness. This extract can either be analyzed directed by GC–mass spectrometry or, if a cleaner sample is required (e.g., for obtaining scans of individual compounds), the products can be extracted from the ~0.1 mL CH_2Cl_2 sample into 1 mL of H_2O. The products are then extracted from the 1 mL of H_2O into 2 mL of CH_2Cl_2, and the CH_2Cl_2 layer is evaporated again to ~0.1 mL. Thus, the water solubility of EC and its products can be used to advantage in this case. An aliquot (1–2 mL) of the extract is injected onto a 0.1 mm × 9 m Carbowax 20M capillary GC column with the effluent directed into a mass spectrometer operating in the chemical ionization mode with CH_4 as the carrier gas. The selectivity and sensitivity are enhanced in the chemical ionization mode, relative to electron impact, in this case. Total ion current and the ions at *m/z* 74, 88, and 106 are monitored as the column is heated with a linear temperature gradient. The actual t_R values depend upon the rate of heating, gas flow rate, etc. Under typical operating conditions, methyl carbamate (*m/z* 74) is eluted at ~4 min, VC (*m/z* 88) at 6.8 min, EC (*m/z* 90, not monitored) at 8.0 min, ethyl *N*-hydroxycarbamate (*m/z* 106) at 10.4 min, and 2-hydroxyethyl carbamate (*m/z* 106) at 12.9 min.[155] Standard curves may be prepared by plotting the ratio of detector response relative to methyl carbamate versus the concentration of each product; such curves were linear over several orders of magnitude for each of the oxidation products under consideration.[155]

The steady-state concentration of VC can be expressed as a function of the EC concentration:

$$[\mathrm{VC}] = \frac{(k_1 K_2)[\mathrm{EC}]}{V_2}$$

where

k_1 is the rate constant for oxidation of EC to VC

V_2 and K_2 are the parameters V_{max} and K_m for the oxidation of VC to form the epoxide (estimated from the rate of formation of 1,N^6-ethenoadenosine)[155]

Thus, the steady-state concentration should be relatively independent of the concentration of enzyme, if both desaturation and epoxidation are catalyzed by the same enzyme (i.e., P450 2E1 in this situation).[155]

HPLC-Fluorescence Assay: 1,N^6-Ethenoadenosine Formation

Fluorescence assays have long been regarded for their high sensitivity.[159] The discrimination of compounds provided by the use of selective excitation and emission wavelengths may be further enhanced by coupling to HPLC. In many cases, the enzyme product has intrinsic fluorescence and may be monitored. In other cases, postcolumn derivatization can be used to render products' fluorescence. The situation described here is somewhat different. A compound is present in the enzyme mixture (adenosine) that does not impede the reaction but reacts with the reaction product to generate a fluorescent product, that is, 1,N^6-etheno(ε)adenosine (Figure 40.21). The procedure has a deficiency in that it probably does not serve as a quantitative trap for any of the compounds under consideration. On the other hand, it is of considerable relevance to studies in toxicology and chemical carcinogenesis in that the same reaction serves to modify DNA to introduce a potentially mutagenic lesion. The procedure described here is based upon our own use[39,155] of methods described earlier by Leithauser et al.[158] and Rinkus and Legator.[160]

Reagents:

1. 1.0 M potassium phosphate buffer (pH 7.4).
2. 50 mM adenosine, dissolved in H_2O (heat in a bath of hot tap water or sonicate to dissolve).
3. Appropriate substrate, dissolved in H_2O (not organic solvent at ~10 × the concentration used in the assay.
4. 10 mM $NADP^+$.
5. 10^3 IU yeast glucose 6-phosphate dehydrogenase mL^{-1} (dissolved in 10 mM Tris–acetate buffer [pH 7.7] containing 1.0 mM EDTA and 20% glycerol [v/v]).
6. 0.1 M glucose 6-phosphate.
7. NADPH-generating system: Mix 25 parts Reagent 4, 1 part Reagent 5, and 50 parts Reagent 6 in an amount sufficient to supply 0.15 mL (mL of total reaction)$^{-1}$; see "Benzphetamine *N*-Demethylation" section regarding checking the system.
8. Standard solutions of 1,N^6-ε-adenosine dissolved in H_2O.
9. 0.60 M $ZnSO_4$ (in H_2O).

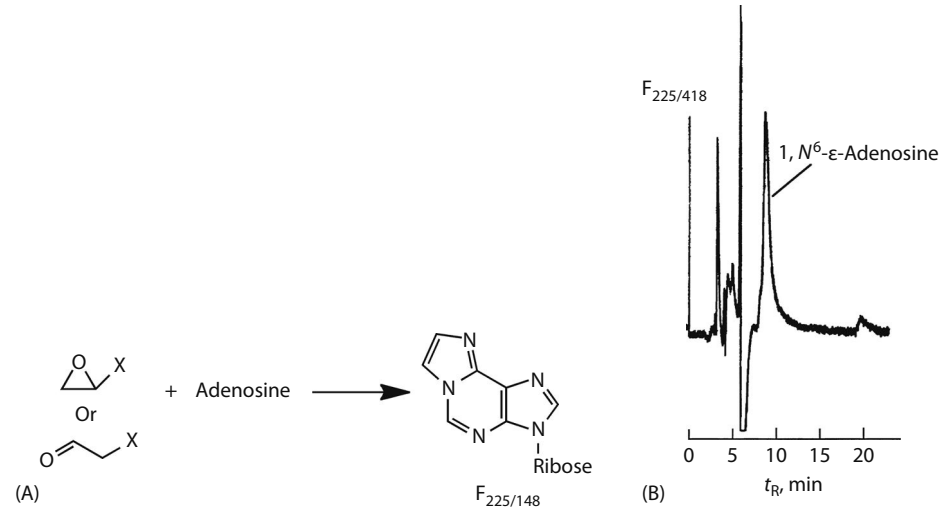


FIGURE 40.21 (A) Reaction of bifunctional electrophiles with adenosine. (B) Formation of 1,N^6-ethenoadenosine in a microsomal incubation. The incubation included 5 mg liver microsomal protein (prepared from isoniazid-treated rats) mL^{-1}, 2.0 mM *N*-methyl,*N*-vinylnitrosamine, 10 mM adenosine, and an NADPH-generating system. After an incubation time of 120 min, a 100 µL aliquot was analyzed as described in the text. (From Guengerich, F.P. et al., *Chem. Res. Toxicol.*, 4, 168, 1991.)

An appropriate amount of microsomes or purified enzyme system is placed in a glass vial (generally to provide ~2 mg microsomal protein mL^{-1}). Reagents are added to give 50 mM potassium phosphate buffer (pH 7.4), 0.50 mM $NADP^+$, 1.0 IU glucose 6-phosphate dehydrogenase mL^{-1}, and the substrate. Most of the substrates that generate products reacting with adenosine to produce 1,N^6-ε-adenosine are small organic compounds (e.g., ethylene dibromide, ethylene dichloride, vinyl chloride, vinyl bromide, acrylonitrile, VC, EC, and some nitrosamines).[39] These are usually substrates for P450 2E1 enzymes; because organic solvents are competitive inhibitors, they should be avoided.[148,149] Although these compounds are considered to be organic solvents, most are actually reasonably soluble in H_2O[39] [see Merck Index and various handbooks of physical constants of chemicals]. Many of these are rather volatile, so the vials may have to be sealed with screw caps and Teflon liners (rubber septa absorb these chemicals and should be avoided). Gases such as vinyl chloride can be added directly to the head space of such vials.

Incubations are initiated by the addition of 0.15 mL of Reagent 7, the NADPH-generating system, which is added per mL, and proceed with shaking at 37°C for a specified period of time. With some of the slower reactions (e.g., EC), Guengerich and Kim[155] and Leithauser et al.[158] have found, surprisingly, that reactions can proceed in a linear manner for up to 3 h. Reactions are stopped by the addition of $ZnSO_4$ to 30 mM. The protein is precipitated by centrifugation at $3 \times 10^3 \times g$ for 10 min, and aliquots of the supernatant are injected onto an HPLC system (octadecylsilane [C_{18}], equilibrated with 11% CH_3CN [v/v] in 50 mM $NH_4CH_3CO_2$ [pH 5.0]). With a 4.6 mm × 250 mm or a 6.2 mm × 80 mm column, as much as 250 µL of material may be injected; at a flow rate of 1.0 mL min^{-1}, the t_R of 1,N^6-ε-adenosine is typically ~8 min. The effluent passes through the flow cell of a spectrofluorimeter, with the excitation wavelength set at 225 nm and using a 418 nm emission filter. The 1,N^6-ε-adenosine peak follows a large negative peak of residual adenosine (Figure 40.21). If enough separation is not seen, then the concentration of CH_3CN should be reduced. As little as 0.5 pmol of 1,N^6-ε-adenosine can be detected using this method.

Continuous Assays

Two examples of P450-based assays are provided as examples, coumarin 7-hydroxylation and 4-nitroanisole *O*-demethylation. The continuous monitoring of a reaction, when possible, allows the investigator to determine exactly what part of the enzyme assay data to establish linearity, that is, when the enzyme is functioning under steady-state conditions and before substrate depletion, product inhibition, etc., are issues. The change in absorbance is used to calculate a rate, using the known extinction coefficient for the product. In the case of fluorescence, a known amount of the product must be used to calibrate the instrument.

4-Nitroanisole O-Demethylation

This is an assay that has been used in P450 assays for many years and uses the (yellow) absorbance of the product as the basis (Figure 40.22).[161,162]

Reagents:

1. 1 M potassium phosphate buffer (pH 7.7)
2. 10 mM $NADP^+$
3. 10^3 IU yeast glucose 6-phosphate dehydrogenase mL^{-1} (dissolved in 10 mM Tris–acetate buffer [pH 7.7] containing 1.0 mM EDTA and 20% glycerol [v/v])
4. 0.10 M glucose 6-phosphate
5. 10 mM 4-nitroanisole (in CH_3CN)

In this assay, the product 4-nitrophenol has a broad absorbance band at 400 nm. The absorbance is only seen for the

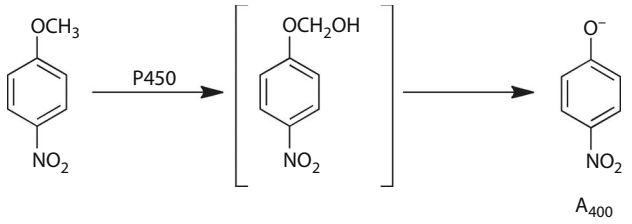


FIGURE 40.22 4-Nitroanisole *O*-demethylation.

anionic form of the product (unprotonated), so the apparent extinction coefficient is pH sensitive.

An enzyme system containing P450 and NADPH–P450 reductase is incubated in 1.0 mL of 0.1 M potassium phosphate buffer (pH 7.7), along with 100 μM 4-nitroanisole, 10 mM glucose 6-phosphate, and 150 μM $NADP^+$, in a glass or plastic cuvette in a spectrophotometer and equilibrated at 37°C (for 5 min). Absorbance can be recorded at 400 nm to be sure that no change in A_{400} occurs during this period due to turbidity, or refractive index changes. In a typical experiment, 0.25 μM rabbit P450 1A2, 0.50 μM NADPH–P450 reductase, and 50 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine were used as the enzyme system.[162] The assays can also be done with microsomes.[161] The reaction is started by the addition of 2 μL of the glucose 6-phosphate dehydrogenase solution. A convenient means of adding the last reagent to start the reaction is with a *plumping* device (see P450 determination section previously mentioned), that holds up to 50–100 μL of a reagent and can be used to add materials to cuvettes in a spectrophotometer, without withdrawing the cuvette. Doing this minimizes the *dead* time and also avoids errors due to repositioning the cuvette in the chamber. A_{400} is monitored for 1–2 min, recording the trace in the instrument. Under these conditions, $\Delta\varepsilon_{400}$ = 12.0 mM^{-1} cm^{-1}, so an A_{400} change of 0.012 corresponds to 1 nmol of product formed (in a 1.0 mL cuvette, with an optical pathlength of 10 mm).

A convenient means of analyzing the results is to do the experiments in a spectrophotometer connected to a computer. In this laboratory, the assays are usually done with a Cary 14-OLIS instrument (OLIS, Bogart, GA). The collected data can be handled to use only the linear portions of the traces and to fit the lines with regression analysis. The disappearance of substrate can also be monitored at 314 nm ($\Delta\varepsilon_{314}$ = 10.4 mM^{-1} cm^{-1}).[162]

Coumarin 7-Hydroxylation

The principle of the assay is similar to that described for the 7-ethoxycoumarin *O*-deethylation assay. Coumarin has some fluorescence, but the product 7-hydroxycoumarin has much stronger fluorescence (Figure 40.23). The fluorescence

FIGURE 40.23 Coumarin 7-hydroxylation.

($F_{390/460}$) is much stronger for the anionic form of the product, so it is pH dependent (a 390 nm wavelength is used for excitation to avoid interference from the NADPH at 340 nm). Among the human P450 enzymes, the reaction is selectively catalyzed by P450 2A6.[163,164]

Reagents:

1. 1 M potassium phosphate buffer (pH 7.7)
2. 10 mM $NADP^+$
3. 10^3 IU yeast glucose 6-phosphate dehydrogenase mL^{-1} (dissolved in 10 mM Tris–acetate buffer [pH 7.7] containing 1.0 mM EDTA and 20% glycerol [v/v])
4. 0.10 M glucose 6-phosphate
5. 5 mM coumarin, dissolved in H_2O
6. 100 μM 7-hydroxycoumarin, dissolved in H_2O

An enzyme system containing P450 2A6 and NADPH–P450 reductase is incubated in 3.0 mL of 0.10 M potassium phosphate buffer (pH 7.7) and equilibrated at 37°C for 3–5 min in the compartment of the spectrofluorimeter, along with 50 μM coumarin, 10 mM glucose 6-phosphate, and 150 μM $NADP^+$. The fluorescence excitation wavelength is set to 390 nm and the emission wavelength is 460 nm. The reading can be adjusted to zero with some instruments at this point, and the fluorescence output should be stable. The reaction is initiated by the addition of 3 μL of the glucose 6-phosphate dehydrogenase solution, and fluorescence ($F_{390/460}$) is monitored continuously for 2–3 min (Figure 40.24). The data are calibrated by adding a known amount of 7-hydroxycoumarin (also using a plumping device) and recording the change in $F_{390/460}$. A computer-linked spectrofluorimeter is useful for recording and analyzing the data. In this laboratory, an OLIS DM-45 instrument is routinely used.

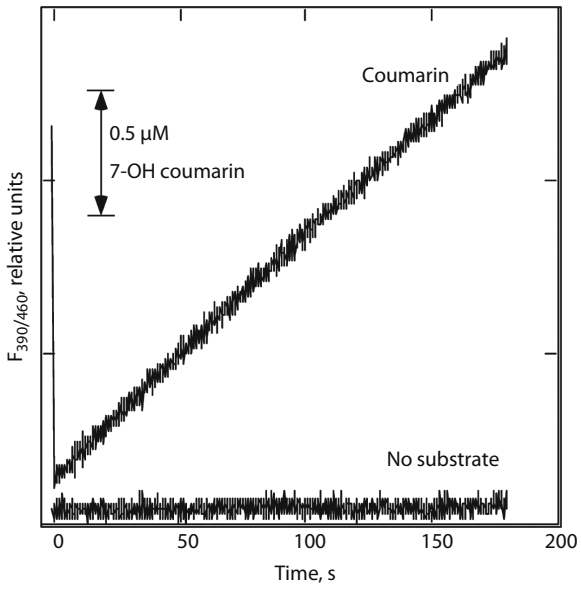


FIGURE 40.24 Continuous fluorescence traces for 7-hydroxylation of coumarin by P450 2A6.[165] An NADPH-generating system was used in both cases.

HPLC–Mass Spectrometry: Midazolam Hydroxylation

Midazolam has been increasing used as in vivo probe for P450 3A4 activity because its metabolism is essentially complete and is totally mediated by P450 3A4.[166] The directions here are for plasma samples but can be modified for in vitro assays, in that the chromatography is the same.

Reagents:

1. β-Glucuronidase type H1 from *Helix pomatia* (if sample is plasma) (Sigma–Aldrich, St. Louis, MO)
2. Diazepam
3. 2 M bicine buffer saturated with NaCl, pH 9.3
4. Methyl-*tert*-butyl ether containing 10% ethyl acetate, v/v
5. 20 mM $NH_4CH_3CO_2$
6. 20 mM $NH_4CH_3CO_2$ in CH_3CN

Midazolam and the 1′-hydroxy product (Figure 40.25) are determined via HPLC–tandem mass spectrometry. One milliliter (200 units) of β-glucuronidase type H1 from *H. pomatia* is added to 1.0 mL of plasma, diluted with 0.55 mL of H_2O containing 50 ng of diazepam. Diazepam is used as the internal standard for the study. The mixture is incubated at room temperature for 20–24 h. Add 1.0 mL of 2 M bicine buffer saturated with NaCl, pH 9.3. The mixture is extracted with 6 mL of methyl-*tert*-butyl ether containing 10% ethyl acetate (v/v). After centrifugation, the organic layer is evaporated to dryness under an N_2 stream at 60°C. Samples are dissolved in 250 µL of an equal mixture mobile phase A and mobile phase B. Samples are then mixed briefly with a vortex device, incubated for 60 s, and filtered through 2 µm Spin-X Costar centrifuge filters. A 60 µL aliquot is chromatographed, using an autosampler for injections. Gradient chromatography is performed with a 4.6 mm × 50 mm Zorbax XDB C_8 octylsilane column, using a flow rate of 0.35 mL min^{-1}. Mobile phase A consists of 20 mM $NH_4CH_3CO_2$ in H_2O and mobile phase B is 20 mM $NH_4CH_3CO_2$ in 90% CH_3CN (v/v). The gradient is initiated with equal parts of both phases and then linearly changed over 6 min to 10% phase A/90% phase B (v/v). Then the gradient is returned to the original 50:50 mixture (v/v) and equilibrated for 2 min prior to the next injection.

LC–MS–MS analysis can be performed with the use of a Finnegan 7000 TSQ or equivalent instrument with an atmospheric pressure chemical ionization (APCI) source interfaced with a Waters HPLC system. APCI settings are as follows: capillary temperature 200°C, vaporizer temperature 500°C, and current 5 µA. Collision energy is 27 eV with a collision gas pressure of 2.5 mTorr for MS–MS. Selected reaction monitoring (SRM) and retention characteristics of the various compounds are as follows: [$^{15}N_0$]-midazolam, 326–291 *m/z* and 4.4 min; [$^{15}N_0$]-1′-hydroxymidazolam, 342–324 *m/z* and 3.2 min; diazepam, 285–193 *m/z* and 5.4 min; [$^{15}N_3$]-midazolam, 329–294 *m/z*; and [$^{15}N_3$]-1′-hydroxdymidazolam, 345–327 *m/z* and 3.2 min.[166] A sample chromatogram is shown in Figure 40.25B.

Covalent Binding

Binding of Metabolites to Protein

The irreversible binding of reaction products to protein was observed 65 years ago.[6] Although no consensus is presently clear about the importance of individual targets or how such modification of proteins actually leads to death of cells, in vitro binding of chemicals to protein provides an index of bioactivation processes and can be useful in the characterization of reactive intermediates.

In general, the enzyme system under investigation is incubated with the radioactive substrate for a fixed amount of time, during which the rate of production of species binding covalently should remain constant. In practice, this is usually <1 h. Incubations are terminated and binding to protein is measured. Several approaches are available for measuring binding.

One method involves precipitation of the protein with organic solvent (C_2H_5OH or CH_3OH, >2 volumes) or aqueous Cl_3CCO_2H (5% final concentration, w/v) and collection of the pelleted material after centrifugation in any case ($10^4 \times g$, 15 min). The sensitivity of the residual substrate (and the protein adduct) to the solvent must be considered, as well as the solubility. The supernatant is decanted and more acid or solvent is added; the protein pellet is washed by vigorous mixing or homogenization. We have found that carrying out the entire procedure in stainless steel centrifuge tubes is convenient because the vessels can be centrifuged in a (Sorvall SS-34 or SA-600 or equivalent) rotor and homogenized (with a Sorvall Omni mixer) (Thermo Scientific) without the need to transfer contents. The process of homogenization, centrifugation, and decantation of supernatant is repeated several times until significant radioactivity no longer appears in the wash fractions. At that point, the protein samples are dried by heating at 60°C for 2 h. The protein is dissolved in 1.0 M NaOH (about 1–2 mL) for 1 h at 60°C. Insoluble material is removed by centrifugation and the protein in an aliquot of each sample is measured by the Lowry et al.[167] or bicinchoninic acid method (*vide infra*), [168] because the recovery is variable. A larger aliquot of each sample is added to 5–10 mL of a scintillation cocktail capable of holding H_2O, and chemiluminescence is allowed to decay overnight at room temperature in the dark prior to counting. The results are expressed in terms of nmol adduct/mg protein, with subtraction of values obtained with an inactive enzyme (e.g., without NADPH in the case of mixed-function oxidases).

Another approach is extensive dialysis of protein samples against buffer containing sodium dodecyl sulfate (SDS).[169] We have utilized this method with hydrophilic materials such as acrylonitrile[82–84] but have not obtained as reliable results with more hydrophobic materials.

A third method, currently preferred, involves the adsorption of protein to glass fiber filters, such as those used for in vitro protein translation experiments.[170] These disks can be washed with organic solvents in a shaking device to remove unbound material. We have usually used 5–8 changes of the wash solvent, with wash times of 30 min per cycle (usually

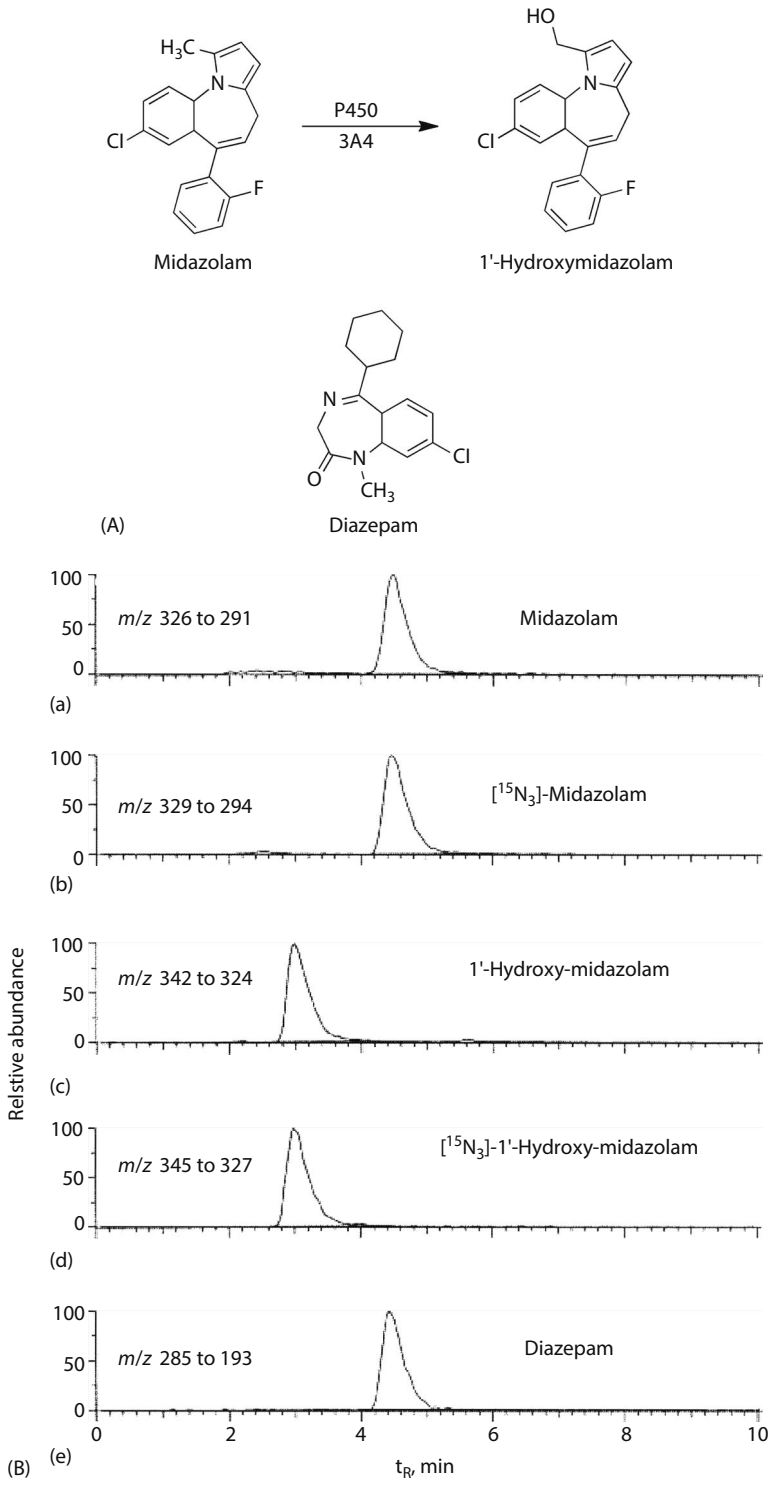


FIGURE 40.25 LC–MS scheme.[166] (A) Midazolam hydroxylation by P4503A4 to yield 1′-hydroxymidazolam and the structure of the internal standard diazepam. (B) A sample chromatogram of the midazolam hydroxylation assay with P450 3A4.[166] SRM combined with liquid chromatography gives the following retention characteristics: (a) midazolam, *m/z* 326–291 and 4.4 min; (b) [$^{15}N_3$]-midazolam *m/z* of 329–294; (c) 1′-hydroxymidazolam, *m/z* 342–324 and 3.2 min; (d) [$^{15}N_3$]-1′-hydroxymidazolam, *m/z* 345–327; and (e) diazepam (internal standard), *m/z* 285–193.

with C_2H_5OH as the solvent).[39,90] The capacity of these filters is limited, so that such an approach may not be satisfactory if considerable amounts of protein must be used. However, if satisfactory sensitivity can be achieved with the use of submilligram amounts of protein, then the method is considerably easier than the other approaches. In our experience with trichloroethylene, we have found that recovery of protein on the filters is nearly quantitative when ≤1 mg protein is used (checks on binding can be done with radiolabeled proteins).[39,90]

Binding of Metabolites to DNA

Like the binding of reaction products to protein, binding to DNA provides an index of bioactivation. One must remember that binding to naked DNA in such a system may be much higher than in vitro or in cells. Binding to protein is not to be equated with nucleic acid binding; notable examples demonstrate that different enzymes and pathways can be involved in the generation of the various types of adducts (cf. acrylonitrile, ethylene dibromide—*vide supra*).[12,171]

Calf thymus DNA has been used in many in vitro binding experiments. Herring sperm DNA is considerably cheaper and much more soluble; its fragmented nature should not cause a problem in this type of work unless one attempts to use SDS sedimentation (*vide infra*). In general, DNA is added to incubations, containing a radioactive substrate, at a concentration of ~2 mg mL^{-1}, with other components as used in standard procedures (*vide supra*).

Several methods can be used to purify the DNA for measurement of bound adducts, and the choice depends upon the situation.

One approach involves initial extraction of the aqueous solution with H_2O-saturated butanol to remove small compounds. Centrifugation ($3 \times 10^3 \times g$, 10 min) is used to separate the layers after each step. The DNA solution is mixed with an equal volume of phenol solution: (liquid phenol is first washed sequentially with equal volumes of 1.0, 0.50, and 0.02 M Tris–HCl [pH 7.4] and then 1 g of 8-hydroxyquinoline is added per liter of phenol). After mixing (with a vortex device), the layers are separated by centrifugation; DNA remains in the lower phase. The step is repeated one or two more times. NaCl is added to the DNA layer to a concentration of 0.10 M (NaCl). The DNA is precipitated by the addition of 5 volumes of cold (–20°C) C_2H_5OH and recovered by centrifugation; the material, dried under a N_2 stream, can be dissolved in any of a number of low ionic strength buffers, although shaking or other agitation may be necessary.

Another procedure that has been used as an alternative to the butanol and phenol extractions involves the sedimentation of DNA by ultracentrifugation.[172] This process is usually done following the enzymatic incubation: SDS is added to a final concentration of 1% w/v (from a stock 10% solution). The incubation buffer should not contain potassium ions because potassium dodecyl sulfate is rather insoluble. The preparations are centrifuged at $10^5 \times g$ for 16 h at 20°C to pellet the DNA. The recovery is generally good (>80%) and most proteins remain soluble and are decanted in the supernatant. Although the procedure is limited by availability of rotor places in the ultracentrifuge, the effort involved in manipulations is minimal. (*Note*: This method is not effective if herring or salmon sperm DNA is used, due to the fragmented nature. It works well with calf thymus DNA.)

Sometimes these procedures do not completely remove unbound materials. A useful procedure for further purification is hydroxylapatite chromatography. A method used in this laboratory is outlined: for a sample containing 1 mg of DNA, 1 g of dry hydroxylapatite (DNA grade, formerly Calbiochem, San Diego, CA; now EMD Chemicals, San Diego, CA) is suspended in 5 mM sodium phosphate buffer (pH 6.8) and swirled to hydrate the particles. The suspension is evacuated (at 40°C) to remove gas bubbles, using an aspirator, and poured into a column (1.6 cm diameter), where 5 mM sodium phosphate buffer (pH 6.8) is pumped through the column at a flow rate of ~2 mL min^{-1} using a peristaltic pump. The DNA is applied to the column (using the pump) and the column is sequentially eluted with 100, 200, and 300 mM sodium phosphate buffers (pH 6.8). The eluate is monitored at 260 nm and the elution buffer is not changed until A_{260} has decreased nearly to the baseline (eluted fractions are collected). Proteins are eluted with 100 mM phosphate, RNA with 200 mM phosphate, and DNA with 300 mM phosphate. Aliquots of fractions can be assayed for radioactivity after mixing with a cocktail containing a detergent and designed for aqueous samples. When large volumes of water and high phosphate concentrations are used, the conditions needed for formation of a stable gel should be checked carefully beforehand.

If concentration of peak fractions is necessary, this is conveniently achieved by removal of the salt from the pooled samples by extensive dialysis and subsequent lyophilization. In some cases, further treatment of DNA with RNase (heating at 80°C for 10 min prior to use to destroy DNase) or pronase may be desired, with subsequent recovery of DNA by phenol extraction and ethanol precipitation. Analyses for RNA can be done using the basic orcinol procedure.[173] Protein can be measured with any of several assays or, if necessary, by amino acid hydrolysis[84] (*Note*: purine decomposition leads to abnormally high levels of glycine). DNA itself can be estimated with the diphenylamine procedure,[173] with Hoechst 33258 dye using a fluorimeter,[174] or by UV spectroscopy using the approximate relationship of $E^{1\%}_{260} = 200\,cm^{-1}$.

If major DNA adducts have been identified for a particular substrate, then the best approach is to hydrolyze the DNA and measure the adduct by a specific method, for example, chromatography or immunoassay.

Other Enzyme Assays

Many of the types of enzyme assays have been covered in the list of examples. The reader is referred to Table 35.1 of an earlier version of this chapter[4] for more. Other types of assays that are not covered here include the use of a bacterial mutagenic or other genotoxicity endpoint[175,176] and radioisotope characterization (i.e., adding a radiolabeled compound to derivatize the product).[177,178]

Structural Elucidation of Enzyme Reaction Products and Development of Assays

One common problem that occurs when an enzyme acts on a drug or other chemical is that a new, undefined compound results. Of course, this can happen not only with a purified enzyme but also with crude systems. In the past, the elucidation of the structure of a new metabolite was often a heroic task and a considerable amount of material was required.

Today, the process is much more efficient, and in many situations, there are demands to identify new products quickly and develop assays for further quantitative analysis. Such situations are now routine in the pharmaceutical industry, and in our own experience, we are of the opinion that the rapid identification of reaction products will gain even more importance in academic laboratories.

A very typical situation is one in which a research incubates a chemical with an enzyme and analyzes the extracted products by HPLC, finding one or more new products, often as judged by the appearance of UV response. The challenge is to identify these.

First, consider the HPLC system. The majority of work is done with reversed-phase systems (C_8, C_{18}, phenyl, cyano, etc.) but not all. The effluent from most systems can probably be used with online UV–visible detection, but the system may be incompatible for other spectroscopy. Therefore, there is a preference for avoiding normal-phase, ion-exchange, and ion-pairing systems. Some of the salts and organic solvents may be incompatible with a mass spectrometer. The researcher will also need to decide if spectra will be acquired online or offline. In the latter case, the peaks of interest are collected manually and processed. Manual collection (*by hand*) is preferable to use of a fraction collector, unless major separations are achieved. If manual collection is done, it works best with a detector having a rapid response, for example, a simple detector with a strip-chart recorder, because many computer-based detectors have time delays for averaging and processing signals. Early in the work, the researcher will also have to decide what scale will be needed for the separation and accumulation. If a semipreparative HPLC column is needed (typically 10 mm × 250 mm), then the change should be made immediately in order to define the operating conditions. Typically, the flow rate for a 10 mm × 250 mm reversed-phase HPLC column is ≥4 mL min^{-1}, which will give a separation similar to a classical analytical column (4.6 mm × 150–250 mm) operating at 1.5–2 mL min^{-1}. In some cases, we have used 25 mm × 250 mm columns, operating at ≥10 mL min^{-1}.[179] The analytical columns are usually very appropriate for doing online UV–visible spectroscopy and mass spectrometry. If fractions are to be collected offline for NMR, then the larger columns may be in order.

Online UV–visible spectroscopy is reasonably straightforward. In this laboratory, we utilize both diode array (Hewlett-Packard, Agilent) and rapid-scanning monochromator (Thermo)-based systems. The two systems each have their own advantages in handling and displaying data. With both, the software can be used to subtract the solvent absorbance and also any peaks contributed by other eluting chemicals, for example, not completely resolved. If the sample is too concentrated, the spectrum will not be valid (e.g., if the absorbance is >1 or 2), and a more dilute sample should be analyzed. With regard to the interpretation of spectra, there are some general rules that can be of use in the interpretation of spectra.[180–182] The data should be stored in files on the hard drive of the computer, backed up (preferably to a compact disk, DVD, or flash drive rather than a magnetic diskette), and printed to a version that can be appropriately saved in a notebook. As always, all relevant information needed to repeat the work should be recorded.

Online LC–mass spectrometry is similar, in terms of the HPLC. In contrast to HPLC/UV–visible spectroscopy, HPLC columns larger than analytical cannot be used unless there is a splitter. In some cases, microbore columns are preferred. A review of mass spectrometry and complete description of all relevant aspects is beyond the scope of this review. However, almost all work of this sort will begin with either electrospray or APCI, in either the positive or negative ion mode. The preferred solvents are those composed of H_2O, CH_3OH or CH_3CN, and some $NH_4CH_3CO_2$ or a similar compatible volatile buffer. With a new compound, one will have to collect *full-scan* data because the *m/z* values are unknown. To monitor the presence of all eluted chemicals, one should follow either a UV absorbance signal (from an in-line detector) as the total ion current as a function of timed. The detected peaks can then be scrutinized to recover full spectra.

The interpretation of mass spectra is beyond the scope of this chapter. One should have some intuition as to what *m/z* values are realistic from the knowledge of the substrate. The molecular ion will be manifested as $[M+H]^+$ or $[M-H]^-$ in electrospray (M is the molecular mass), depending upon whether the positive or negative ion mode is used. One simple guide for chemicals containing the common atoms dealt with in this type of research is that an odd number for M indicates an odd number of nitrogen atoms (1, 3, 5,…) and an even number indicates either none or an even number (2, 4, 6,…). It is possible to utilize a higher-resolution instrument to acquire more mass accuracy, but linking such instruments (e.g., magnetic sector, Fourier transform ion cyclotron) to HPLC is not as common. With a resolution of $>1/10^4$, four decimal places are recorded and the atomic composition of a molecule can be established. It is possible to collect samples and submit them for such *elemental analysis* or *exact mass* determination.

In some cases, the structure of a compound can be established with reasonable certainty just from the fragmentation pattern. Modern mass spectrometers allow the fragmentation of individual ions, which can be quite useful. However, with complex molecules and in the absence of work done with stable isotopes, establishing structures of unknown compounds only by UV–visible and mass spectrometry methods is usually not a definitive process.

NMR spectroscopy is a very powerful approach to determination of chemical structures. Two basic types of information can be obtained: through-bond coupling (which atoms are attached to each other) and through-space coupling (for our purposes, stereochemistry). The simplest through-based method is a simple 1D ^1H NMR spectrum. More complex 2D methods require more sample or longer acquisition times. Some of these establish the linkages of protons and ^{13}C atoms, in a manner based on the protons and thus much more sensitive than direct analysis of the ^{13}C signals. Examples we have utilized in this laboratory include COESY, HSQC, HMBC, and CIGAR methods. The

2D through-space methods we have utilized with small molecules are NOESY and ROESY.[183,184]

NMR spectra of small molecules are usually run in deuterated solvents to avoid artifacts introduced by signal suppression methods. If HPLC peaks are collected offline, then the chemical of interest can often be extracted from the (aqueous) elute into an organic solvent (e.g., CH_2Cl_2) and concentrated for NMR analysis. If compounds cannot be extracted (e.g., nucleosides), one approach is to use a *volatile* combination of buffer salts (e.g., NH_4HCO_2, $NH_4CH_3CO_2$, *N*-ethylmorpholine) and remove the buffer by repeated lyophilization. Removing solvents and H_2O as rigorously as possible should be done in order to avoid interfering peaks. With small amounts of a chemical, the limitation to sensitivity is usually not the amount of the chemical itself but the extent of contamination by solvents, H_2O, grease, and other contaminants. Spectra are usually recorded on instruments with field strengths of 300–600 MHz. The inherent sensitivity increases as a function of the square of the field strength, so an 800 MHz instrument should provide fourfold more sensitivity than a 400 MHz system. Other factors for sensitivity are the sample volume (smaller is better) and the probe temperature. *CryoProbe* technology provides more sensitivity because of a square root relationship with the absolute temperature.

A relatively recent development is the implementation of combined LC–NMR systems (Figure 40.26). In true online systems, one of the two HPLC buffers is usually deuterated and the elution is paused when peaks are eluted (into the NMR probe) to collect spectra for longer time periods. A new development involves automated robotic systems for collecting designated HPLC peaks (detected by UV–visible or mass spectral measurements). The designated effluent is directed onto a solid-phase extraction (SPE) cartridge. (The run can be repeated to apply a multiple load.) Each SPE cartridge, loaded with a peak from the HPLC run, is then washed with solvent to remove any salts and the HPLC solvent; then a small amount of deuterated organic solvent is applied to each SPE cartridge to elute the bound compound into a 30 μL capillary tube. These capillaries, all filled offline, are then moved to the NMR spectrometer, where spectra can be recorded for the desired length of time.

The amount of material required to obtain useful spectra in each of these approaches is dependent upon the particular compound and its physical characteristics. UV–visible spectra can be collected with absorbance maxima of <0.01, and the sensitivity will depend upon the dimensions of the HPLC column and the detector cell. Subnanomolar amounts can be characterized in many cases, depending on the chromophore. Mass spectrometry has a similar detection limit, depending upon the ionization characteristics of the compound, the HPLC column bore, and the details of the specific mass spectrometer. Our own experience has been with ThermoFinnigan electrospray instruments, with the newer TSQ series having ~50-fold greater sensitivity than the older 7000 series.

NMR is usually the limiting factor in analyses of this type, depending upon the situation. If only a 1D ^1H NMR is needed, then, in principle, the UV–visible, mass, and NMR spectra can be collected with samples in the low microgram range.

One powerful approach to spectral analysis is the use of online systems, that is, liquid chromatography coupled to spectroscopy. An example from this laboratory is presented in Figure 40.26 and involves the characterization of an unknown oxidation product of testosterone by recombinant P450 3A4. Testosterone was incubated with 0.1 nmol of the P450 (plus NADPH–P450 reductase) for 5 min and the substrate and products were extracted and applied to an HPLC column. The effluent was split, with a minor fraction directed to a UV detector and a mass spectrometer. On the basis of the UV profile, fractions were collected (robotically) on small SPE cartridges. In this work, three HPLC runs were pooled before further processing. The SPE columns were switched and washed, to elute solvent and any salts, and then eluted with a small amount of deuterated solvent into small capillary tubes. All of this processing can be done offline from the NMR spectrometer. The capillary tube containing the peak of interest was then used for NMR analysis, in this case 1D (^1H), COSY (^1H–^1H), HSQC, and NOESY (^1H). In this case, most of the ^1H and ^{13}C resonances were already known from the literature on testosterone.

The UV spectrum (not shown) was very similar to that of testosterone, suggesting no change in the double bonds that constitute the chromophore (Figure 40.26). The mass spectrum confirmed the addition of one oxygen atom (+16). The chemical shift (δ) of the proton(s) associated with the new product (relative to the substrate testosterone) suggested that it was adjacent to either an allylic carbon or a carbon α to a carbonyl, that is, C-1 or C-7. Inspection of the COSY spectra indicates a lack of connection to C8, arguing against a hydroxyl at C-7 and thus in favor of C-1. The HSQC spectrum supports this view. The stereochemistry was easily established as 1β with the NOESY spectra, after considering the 3D possibilities for the molecule (Figure 40.28). If the H-7 proton were in the β-position, it would be expected to show a very strong cross peak with the (three) protons of the C-19 methyl group. It does not, and therefore the H-7 proton is α (and the hydroxyl group is β). The NOESY spectrum shows the expected coupling to H-2α,β, H-9, and H-11 (Figure 40.26). Thus, the structure was established as 1β-hydroxytestosterone with ~6 μg of the product.[184]

In some cases, the aforementioned information may not be sufficient to assign the structure. Other spectroscopy may be in order. Infrared (IR) spectra can be useful, particularly if the presence of certain diagnostic groups is suspected.[181] Circular dichroism (CD) spectra can be useful in assigning stereochemistry.[185,186] In the example shown here with 1β-hydroxytestosterone, an *exact* mass was obtained. As mentioned earlier, this is not always done as a first course to characterizing a molecule but can be very important in distinguishing among possibilities after obtaining a lower-resolution spectrum. High-resolution mass spectrometry is becoming much more important in such situations.[187] Another approach needed sometimes involves use of more NMR spectra, especially 2D spectra.[183,188]

A common next step in work of this type is development of an assay. One preferred approach is to synthesize

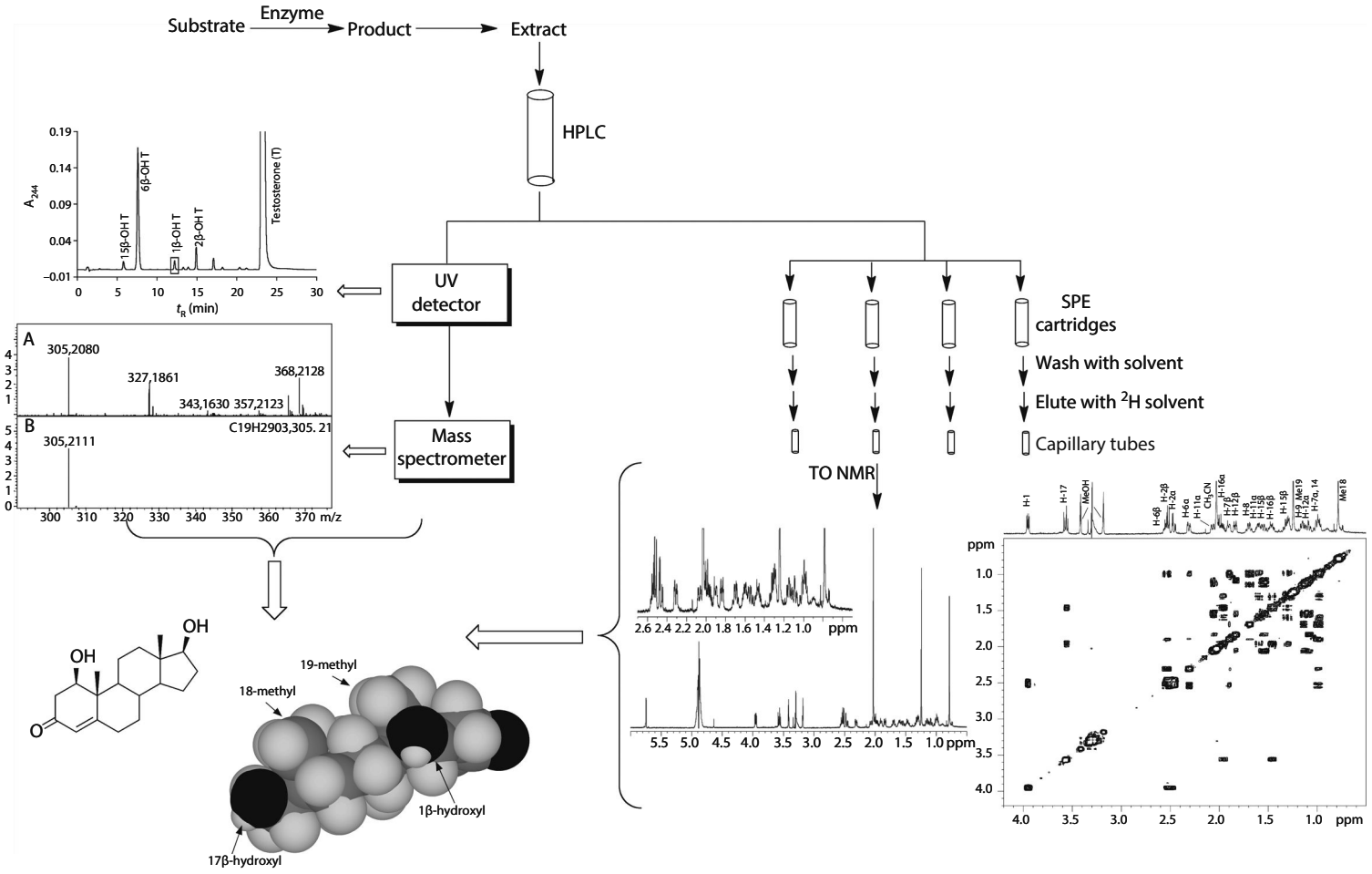


FIGURE 40.26 Determination of a structure of a reaction product by online LC–SPE–MS–NMR. The UV detector can also be used to obtain the UV spectrum of the peak of interest (indicated with box, spectrum not shown). The data are from the characterization of 1β-hydroxytestosterone as an oxidation product of testosterone by P450 3A4. (From Krauser, J. et al., *Eur. J. Biochem.*, 271, 3962, 2004.)

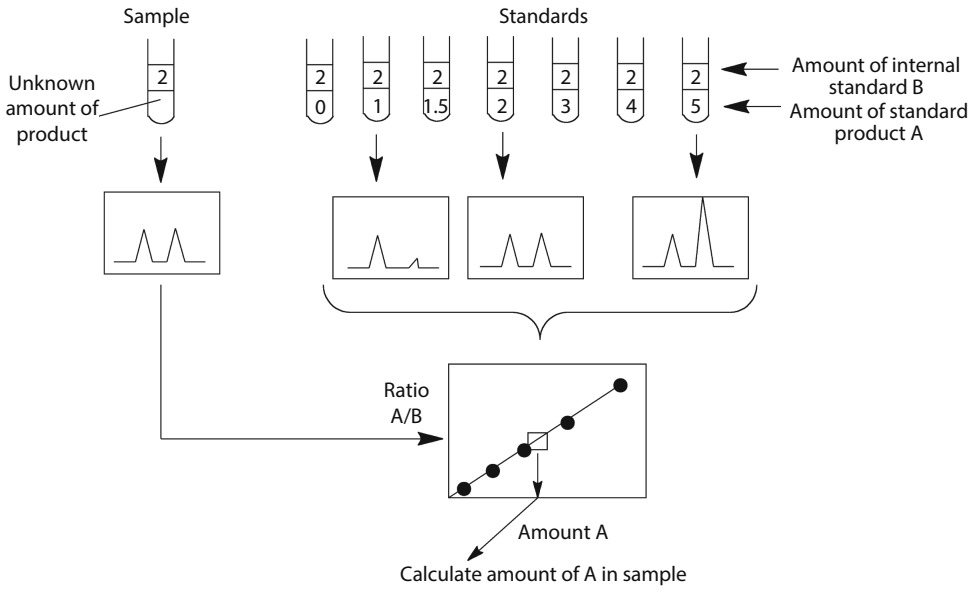


FIGURE 40.27 General approach to use of an internal standard (closely related compound) for quantitative HPLC analysis.

the reaction product and use it as a standard, preparing stocks by gravimetric methods. Typically, an assay would be set up using HPLC (in that in the case cited here [Figure 40.26], the product was first separated by this method). Detection could be by UV or fluorescence spectroscopy or mass spectrometry. A common approach is to use an internal standard, that is, a closely related compound that is still distinguished. This is added after the enzyme reaction, prior to the extraction step, in order to control for product recovery. If the analysis utilizes mass spectrometry, then ideally, the internal standard will be the same compound with heavy atoms (≥2 preferred) covalently attached (^{13}C, ^{18}O, and ^{15}N are preferred) to avoid isotopic (^{2}H) effects on HPLC t_R. In other cases, a compound differing slightly but having similar physical properties (extraction, ionization) is derived. The principal of internal standard curves is presented in Figure 40.27.

One problem that arises in many cases is that of quantifying a very small amount of a product that cannot be synthesized; also, the amount available may be too low for accurate gravimetric determination. One option is the use of a sensitive microbalance. Another, depending on the structure, is the use of the UV–visible extinction coefficient of a similar molecule with the same basic chromophore, in the absence of interference problems. Another method of quantifying small amounts of source materials is to use ^{1}H NMR spectra, which are absolute in their signal intensity. The choice depends upon the interfering signals; a choice used compound devoid of signals in the aliphatic region is CH_3CO_2H, which gives a sharp singlet (δ 1.91 in DMSO)[189,190] (Figure 40.28).

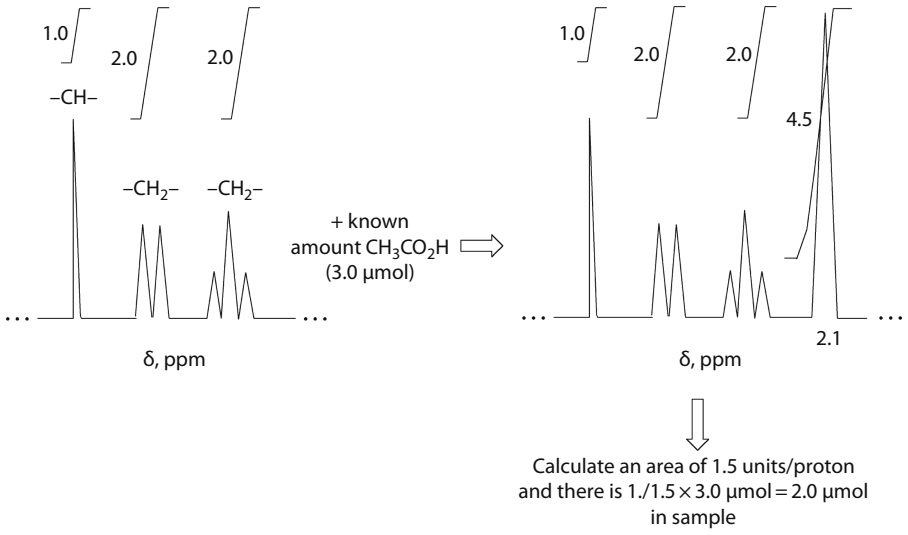


FIGURE 40.28 Use of NMR and an internal standard (CH_3CO_2H, δ 2.1) to establish the concentration of a small amount of a new product. (From Gottlieb, H.E. et al., *J. Org. Chem.*, 62, 7512, 1997; Wu, Z. et al., *Chem. Biodivers.*, 2, 51, 2005.)

An extensive discussion of all possible assays is beyond the scope of this article, and some representative assays are mentioned earlier. If interference is a problem (due to contaminating ions at the *m/z* of interest), a suitable assay may be set up by monitoring the loss of a specific fragment characteristic of the analyte of interest.

Heterologous Expression of Enzymes

The overall approach to handling enzymes has changed dramatically since the chapter published in the first edition of this book.[1] In today's world, the genomes of humans and many principal experimental animals are known. In such systems, the isolation of a new enzyme from a tissue or a microorganism is rare. Most enzymes are now prepared by heterologous expression methods. The advantages are simple: (1) The amount of enzyme that can be obtained is much less of an issue. (2) The expression system is usually cheaper to work with. (3) In principle, all human enzymes are accessible. (4) Individual amino acids can be replaced to study their roles. (5) Purification is easier, because (a) the starting concentration of the enzyme of interest is much higher, (b) the presence of interfering, closely related enzymes (e.g., other P450s) can be avoided, and (c) many proteins can be expressed with innocuous tags that will facilitate isolation.

A thorough treatment of heterologous expression is beyond the scope of this test, and the reader is referred to other work, including some compilations,[191–193] original work, and commercial literature. However, the point should be made that most practicing biomedical scientists (and certainly all enzymologists) must be able to utilize recombinant DNA methods and heterologous expression in today's scientific environment.

Considerations

Many methods for expression are available, and many scientists have personal favorites. What are some of the issues in deciding which system to use? Here are some issues to consider:

Amount Needed

Some scientific problems require large amounts of enzyme. For instance, x-ray crystallography and some physical techniques require considerable amounts, at least mg amounts of highly purified protein.

Posttranslational Modification

If the protein of interest is known to require posttranslational modification (other than incorporation of heme, flavin, metal ions), then eukaryotic expression systems are in order. The disadvantage is that these systems generally have lower yields. Another issue is that some posttranslational systems differ in lower and higher eukaryotes.

Difficulty of Reconstitution

Multiprotein systems can be difficult to reconstitute. However, if a protein has multiple subunits, coexpression of the components in a single system may facilitate the interaction, and the proteins may be isolated as a complex.

Needs for a Specific Cellular System

In some applications, the interest is not in using a purified enzyme but in doing studies within the context of a cellular environment because of the endpoints to be examined. For instance, in this laboratory, we have utilized bacteria to report genotoxicity, both in analysis of chemicals[176,194] and in doing random mutagenesis/molecular breeding.[195] Others have used mammalian cell systems to evaluate toxicity.[196] Thus, the complexity of the system depends upon the project.

An extension of this question is whether a transgenic animal will be used instead of a cellular system. Obviously, many mammalian responses can be detected only in such complex settings.

Use of Protein Tags

The purification of proteins is facilitated by the attachment of tags to either ends of the protein. In work in this laboratory, we have often employed a $(His)_5$ tag at the C-terminus.[197,198] N-terminal tags can be employed in some cases,[199] although that is the first end of the protein synthesized and it may be less tolerant to modification, particularly in bacteria.[200] In bacteria, the N-terminus can often be modified to improve protein expression, if there is no effect on the properties of the protein.

An alternate approach is to add larger peptide units to expressed proteins and utilize chromatography systems that will bind the tag. Some of the larger tags can be removed with selective proteases, if interference is a potential problem.

Heterologous Expression Systems Available for Use

As indicated earlier, these will be described only briefly, mainly in terms of the advantages and limitations of each, moving from the simplest to the more complex.

In Vitro Translation

A simple, low-cost instrument can be purchased (e.g., Roche) and this approach can be very useful if only small amounts of protein are required. The need to optimize organisms for expression efficiency is avoided. The reagents (aside from the cDNA) are commercially available and are expensive for large-scale work but reasonable for small projects.

Bacteria

Bacteria have many advantages and remain the preferred vehicle in this laboratory.[193] Most work is with *Escherichia coli*, although sometimes *Salmonella typhimurium*[201–203] and *Bacillus subtilis*[191] have applications. Bacterial expression is generally fast (1–2 days culture), relatively inexpensive, and gives high yields. Many vectors are available. The systems can be employed for various screens done in the cells.[193,201,202] Scale-up to high-volume production in fermentors is most easily accomplished with bacterial systems.

The limitations for bacterial expression are the need for posttranslational modification and problems in the proper

folding of source proteins. In addition, many proteins express better if the N-terminus is modified, for various reasons.[193,204] Thus, some are usually necessary with several constructs before proceeding with productive expression.

If expression of proteins is too rapid in bacteria, then misfolding can occur. Sometimes the misfolded proteins are accumulated in inclusion bodies, which are particles of damaged protein (devoid of prosthetic groups). Several approaches can be used to minimize this problem, including use of weaker promoters, lower incubation temperatures, and coexpression of chaperone proteins. For expression of human P450s in *E. coli*, the addition of chloramphenicol and ethanol has been helpful in some cases.[205] For the P450s that are more difficult to express, the addition of heme precursors has been helpful, probably by providing a prosthetic group for the protein to *wrap itself around*.

Yeast

Most work is done with *Saccharomyces cerevisiae* but other yeasts have some advantages.[206] Yeast expression of P450s has been done in this laboratory in the past[207] and is still done in other groups.[208,209] In addition to yeasts, some expression has been done in the *false yeast Pichia pastoris*, for example, with monoamine oxidase.[210,211]

Yeasts are eukaryotes and have advantages in terms of posttranslational processing capability and capacity for proper protein folding. Numerous vectors are available for expression, and generally, less manipulation of sequences is necessary for expression. *S. cerevisiae* does have an NADPH–P450 reductase (and cytochrome b_5), which couple with some mammalian P450s but not all.[212,213]

The expression levels achieved in yeast are generally lower than in bacteria. The recombinant DNA manipulations needed to prepare vectors are done in bacteria and then transferred to yeast. The expression times are longer than in bacteria. A major disadvantage of yeast expression is the difficulty in breaking yeast cells for enzyme purification.[207] Breaking cells with only (strong) mechanical methods is not very inefficient, and enzymes such as yeast lytic enzymes are required.

Insect Cell Systems

Systems based on baculovirus infections are useful.[214,215] The advantages and limitations of these systems are similar to yeast. Enzymes are not required to break the cells, but the costs of media are considerable. High levels of expression can be achieved, although the expression volume is usually a limitation because of the cost of media. As with yeast, posttranslational modification occurs; it may or may not be identical to what occurs in mammalian systems. Baculovirus-based expression of P450s and other heme proteins does require the addition of heme. As a general rule, baculovirus is a reliable means of expression proteins that are recalcitrant to expression in other systems.

These expression systems are usually used in *spinner* cultures, which do not require rigorous aseptic conditions. Insect cell culture is possible in shaking cultures (i.e., Fernbach flasks) although such methods have been used less. Large-scale cultures are possible although expensive.

Mammalian Cell Culture

COS-1 cells have a monkey kidney origin and have been used extensively for many proteins.[216] Other systems used for enzymes of relevance to toxicology include TKK lymphoblasts and V79 cells, which have been used in toxicity and mutation assays. Human embryonic kidney (HEK) cells have been used for higher level expression, usually in roller bottles. In some cases, these latter systems have been used to generate enough material for structural biology studies, although this is not a trivial or inexpensive process.

Another variation is vaccinia virus systems, which are used to produce proteins in mammalian cells.[217,218] These systems have been used for synthesis of drug-metabolizing enzymes and transporters, with higher levels of expression than some of the other mammalian systems.

The mammalian cell systems have the advantages of not requiring much optimization and rather faithfully doing post-translational modification. They also provide good models for cell-based assays. The disadvantages are the need for cell culture facilities, cost, and difficulties in large-scale work.

Transgenic Animals

A detailed discussion is far beyond the scope of this chapter.[219] Transgenic animals can be considered one type of heterologous expression, although they are usually not used to harvest the protein except in certain commercial settings with large animals and biopharmaceuticals (proteins used as drugs). However, transgenic mice are commonly used to address hypotheses in in vivo settings, both in terms of gain and loss of function (gene knock-in and knock-out animals, respectively). Transgenic rats are not very common, in that the technology is not as developed. Obviously, these are expensive experiments. The ease of developing transgenic mice has improved in recent years; the cost of maintaining the animals is still an issue. Transgenic animals do provide a reasonably appropriate setting for analyzing the functions of proteins in vivo.

Use of Purified P450 Enzymes

Many individual forms of P450 have now been purified and characterized from a number of species, including humans. A complete discussion of these preparations is beyond the scope of this article and the reader is referred to other references.[55,62,63,65,220,221] For a discussion of the current recommended nomenclature for the P450 families and subfamilies, see Ref. [222] and the website <http://drnelson.utmem.edu/CytochromeP450.html>. One of the reasons for purifying individual forms of P450 is to determine which individual forms are involved in particular reactions. This task has been made somewhat easier in the light of current knowledge available concerning cytochromes P450.

With rat liver systems, the first step is the development of an in vitro assay for the particular activity under consideration. This has to be developed with liver microsomes and the sensitivity should be optimized, along with conditions such as pH, time, and protein and substrate concentrations.

The next step involves comparison of rates of oxidation with microsomes isolated from (untreated) male and female rats and male rats treated with various inducing agents. A considerable body of knowledge now exists concerning the effects of gender and inducing agents on individual P450 forms and this information can be used to advantage.[63,65–67,223] For example, male rats (adult) contain P450 2C11 and P450 3A2 but not P450 2C12. Phenobarbital administration induces P450 2B1, P450 2B6, P450 2B2, and P450 3A1. Pregnenolone 16α-carbonitrile and dexamethasone induce P450 3A1. Levels of P450 2C11 are suppressed by administration of any of several of the typical inducers, particularly PAHs. From information obtained in such experiments, one can begin to suggest which forms are involved.

The next step involves examination of the relative abilities of individual forms of P450 to catalyze the reaction, if these are available. Reconstitution conditions are described elsewhere.

When specific inhibitors are known for individual forms of P450 (e.g., 7,8-benzoflavone [α-naphthoflavone] for rat P450 1A1 and P450 1A2), these can also be used to advantage (*vide infra*).[46,224]

Another step involves using specific antibodies with microsomal preparations to determine which will inhibit the reaction. This approach is discussed later in this chapter. These results should agree with and confirm those obtained in the aforementioned experiments.

The strategy is slightly different for studies with human enzymes, since induction and gender patterns are not involved. If some results are available on the catalytic selectivity of rat or other animal P450 enzymes, this information may be of some use in predicting human enzymes, although there are many cases of catalytic selectivity jumping subfamilies when making such comparisons, particularly in the P450 2C family.[56,225]

One way to proceed is to first use diagnostic chemical inhibitors[46,224] (Table 40.3). Because human samples are known to vary considerably in their P450 composition, it is risky to rely on a single human sample, particularly if there is little prior knowledge about its characteristics. Usually, several samples (≥3) should be used in initial screens. An alternative is to use a single experiment with microsomes pooled on the basis of protein content (≥10 samples). The same strategy applies to human hepatocytes (here, the number of samples available may be low).

Another useful strategy with microsomes is correlation analysis.[226,227] A set of microsomal samples is compared for the new activity under consideration and also marker activities diagnostic of individual P450 enzymes (Table 40.2 and Figure 40.29). In general, ≥10 samples should be used. Correlation analysis can be done with either linear or Spearman rank methods.[226,229] In principle, the parameter r^2 estimates the fraction of the variation accounted for by the relationship between two variables. Values of $r^2 \geq 0.5$ are generally judged to be significant (but the significance, p, is dictated by sample size).

An additional approach is to utilize purified or, more likely, recombinant enzymes to assay the activity. If this is done with a chemical, there should be some appreciation of the plasma or tissue concentrations relevant to the problem under consideration when a substrate concentration is selected. An improved approach involves measuring activity at several substrate concentrations and estimating V_{max} (or more appropriately k_{cat}, which is V_{max} divided by the enzyme concentration) and K_m.

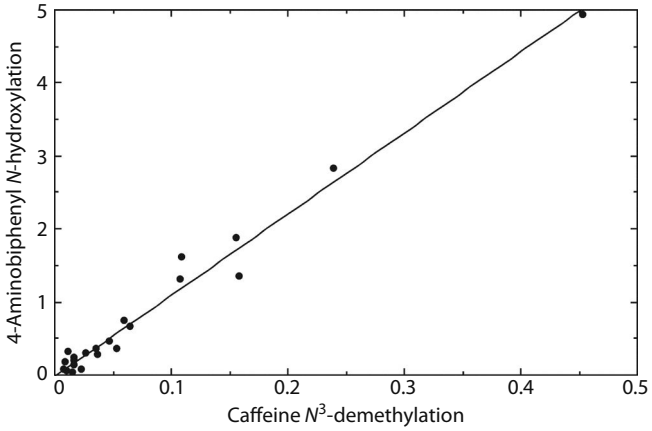


FIGURE 40.29 Correlation of caffeine N^3-demethylation and 4-aminobiphenyl *N*-hydroxylation in different human liver microsomal samples, indicative of P450 1A2 variability and involvement in both reactions.[228] All rates are expressed in nmol product formed/min/mg microsomal protein.

Inhibitory antibodies can also be useful. In principle, the fraction of inhibition one sees when one adds an antibody (to a particular P450) to microsomes is the fraction of that reaction catalyzed by the P450 to which the antibody was raised (Figure 40.30). There are several confounding problems. One is that antibodies generally do not distinguish between P450 subfamily members, unless extensive cross adsorption is done with the antibody. Despite early success in the area,[230,231] not all anti-P450 antibodies are inhibitory. Further, even antibodies raised against recombinant P450 proteins have sometimes showed reactions with similar epitopes in different P450 families.[232] This cross-reactivity can vary with individual rabbits. Although polyclonal antibodies raised against purified P450 enzymes have been highly useful in development of the field, there are two prospects for improvements. One is the use of monoclonal antibodies, which have already been exploited for some uses.[233–235] The other, more recent, is the use of antipeptide antibodies. Some of these have shown impressive specificity[236,237] although immunoinhibitory potency may still be a problem. A new development in this area is phage-display methods (*vide infra*).[238]

Two general points need to be made about use of such information (some points are also made later regarding heterologous expression systems). First, many of the parameters often cited for the in vitro systems used today are inappropriate, particularly with the recombinant systems. The expression of an activity of a recombinant system in terms of mg protein is not particularly helpful because no information is available about the level of expression. The concentration of the enzyme under consideration should be determined and used

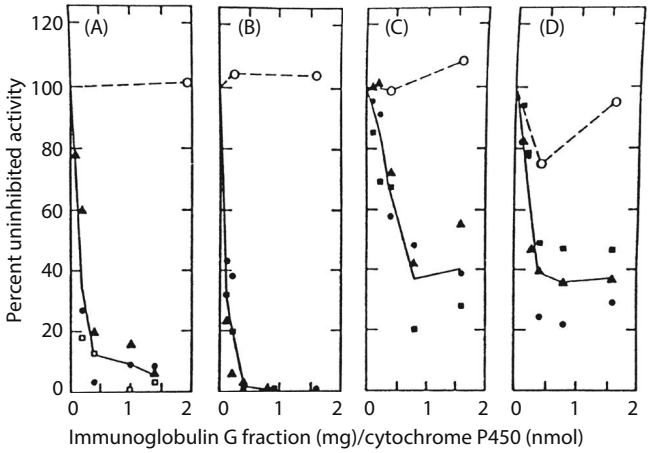


FIGURE 40.30 Inhibition of mixed-function oxidation of debrisoquine, sparteine, encainide, and propranolol in human liver microsomes by antirat P450 2D1. Incubations were carried out with microsomes prepared from liver samples 17 (△), 25 (□), 31 (●), 32 (▲), 34 (■), 72 (◊), and 86 (◆), which contained 0.32, 0.55, 0.77, 0.50, 0.55, 0.61, and 0.30 nmol P450 (mg protein)$^{-1}$, respectively. The microsomes were incubated with indicated amounts of anti-P450 2D1 (IgG fraction) for 30 min at 23°C in the appropriate buffer and other incubation components were then added. Results are expressed as percent of the control activity (obtained in the absence of antibody) for debrisoquine 4-hydroxylation (A), formation of Δ^5-dehydrosparteine from sparteine (B), encainide *O*-demethylation (C), and propranolol 4-hydroxylation (D). The solid lines are drawn connecting the means of the values obtained with the various samples, and the broken lines connect the means of the values (○) obtained with IgG prepared from preimmune antisera (individual values not shown). Control activities of debrisoquine 4-hydroxylation were 0.16, 0.060, and 0.19 pmol min^{-1} (nmol P450)$^{-1}$ for samples 25, 31, and 32, respectively. Control activities of Δ^5-dehydrosparteine formation were 26.6, 20.4, and 2.0 pmol min^{-1} (nmol P450)$^{-1}$ for samples 31, 32, and 34, respectively. Control activities of encainide *O*-demethylation were 0.044, 0.072, and 0.039 nmol min^{-1} (nmol P450)$^{-1}$ for samples 31, 32, and 34, respectively. Control activities of propranolol 4-hydroxylation were 0.17, 0.12, and 0.081 nmol min^{-1} (nmol P450)$^{-1}$ for samples 31, 32, and 34, respectively. (From Distlerath, L.M. and Guengerich, F.P., *Proc. Natl. Acad. Sci. USA*, 81, 7348, 1984.)

in the normalization. Such an analysis may be difficult if an immunochemical method is needed, since apoprotein or inactive enzyme will also be measured. Another common problem in P450 work (and with most of the other enzymes of interest here) is the attachment of too much significance to the K_m value itself. Contrary to what is often conveyed in toxicology and pharmacology texts, K_m does not generally note substrate affinity, unless specifically proven to be. The parameter K_m is an operational term, simply denoting the substrate concentration at which half-maximal velocity occurs.[239,240] It is a complex collection of microscopic rate constants, which are usually not known. In the case of P450 2E1, K_m can even be considered a function with k_{cat} appearing as a dependent variable.[154]

Second, the ratio of k_{cat}/K_m (or V_{max}/K_m) is considered the most appropriate estimate of enzyme efficiency by enzymologists.[240,241] In a plot of v versus S for an enzyme, this is the tangent to the plot at low substrate concentrations and has units of M^{-1} s^{-1} (or the equivalent). However, k_{cat}/K_m (or V_{max}/K_m) is *not* intrinsic clearance (nor *in vitro clearance*). Clearance is an in vivo parameter with a distinct meaning. If blood flow is not rate limiting and there are no complicating factors due to transport, then intrinsic clearance within a given organ might be a direct function of k_{cat}/K_m.[242] However, the more proper term for the ratio k_{cat}/K_m is simply *enzyme efficiency*. This is not to demean efforts at physiologically based pharmacokinetic modeling, which can be very useful.[241,243] However, these models must not make inappropriate assumptions about what they incorporate in the way of meanings of parameters, any more than they would incorporate the wrong pathways.

Purification of P450 from Bacterial Recombinant Expression Systems

Overview

A procedure is outlined that is used for purification of P450 3A4 from *E. coli* membranes in this laboratory. Details of construction of the expression plasmid (NF14) are presented elsewhere,[244,245] including the addition of the C-terminal pentahistidine ($(His)_5$) tag.[197,246] This chapter is an updated version of an earlier procedure published in this series.[247] The major change has been the incorporation of the $(His)_5$ tag and the use of metal-affinity chromatography (Figure 40.31).

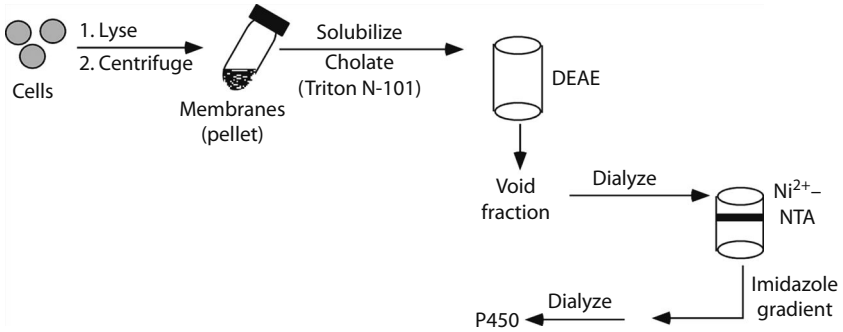


FIGURE 40.31 Purification of recombinant human P450 from *E. coli* membranes utilizing nickel affinity chromatography. (From Guengerich, F.P. and Martin, M.V., Purification of cytochrome P450: Products of bacterial recombinant expression systems, in: Phillips, I.R. and Shephard, E., eds., *Methods in Molecular Genetics, Cytochrome P450 Protocols*, Academic Press, Orlando, FL, pp. 31–37, 2006.)

Attachment of an oligo-His region, usually at the N- or C-terminus, has been used to facilitate protein purification.[248] The free His residues (usually $(His)_4$, $(His)_4$, or $(His)_6$) can chelate Ni^{2+} and similar metal ions; thus, a Ni^{2+}-chelate affinity column can be used for rapid purification.[249] Such approaches have been used with P450s, with His tags at either the C[250,251]- or N[199,252]-terminus. Detergent is needed to solubilize the membranes and keep the proteins disaggregated during chromatography, and the detergent must be removed in a subsequent step.

In our early work on the purification of *E. coli*-expressed human P450s, the procedures consisted mainly of ion-exchange chromatography methods.[244,245,247,253–256] The change to metal-affinity methods was made for two reasons: (1) the metal-affinity approaches can be used to reduce the need for nonionic detergents or to facilitate removal of these (nonionic detergents can yield artifacts and are even substrates[257]) and (2) some P450 mutants are relatively unstable and their purification requires more rapid methods.[258–260]

An example is provided in which an ion-exchange step is used prior to metal-affinity chromatography.[197] In some cases, the step can be omitted and solubilized P450 preparations can be used directly for metal-affinity chromatography (e.g., P450s 1A2 and 2D6[199,258,259,261,262]). In these cases, highly purified P450s can be prepared without the need for earlier steps.

Reagents:

1. 1.0 M Tris–acetate buffer (pH 7.6).
2. Sucrose.
3. Sodium EDTA, 0.10 mM in H_2O, adjusted to pH 7.5.
4. Lysozyme, 50 mg mL^{-1}.
5. 1.0 M potassium phosphate buffer (pH 7.4).
6. Magnesium acetate.
7. Glycerol.
8. 2-Mercaptoethanol.
9. PMSF, 0.1 M in *n*-propanol (stored at −20°C).
10. Leupeptin, 0.20 mM in H_2O.
11. Bestatin, 0.1 mM in H_2O.
12. Aprotinin, 4 U mL^{-1}.
13. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), Sol-Grade (Anatrace, Inc., Maumee, OH).
14. Sodium EDTA, 0.10 mM in H_2O, adjusted to pH 7.5.
15. Diethylaminoethyl (DEAE)-Sepharose, adjusted to pH 7.4 (GE Healthcare, Piscataway, NJ).
16. Nitrilotriacetate (Ni-NTA) agarose (QIAGEN, Valencia, CA). An alternative is a Co^{2+}-based matrix, for example, the Talon® system (Clontech, Mountain View, CA) although we have not used this in our own work.
17. DTT.

Preparation of Bacterial Membranes

Bacterial cells are recovered by centrifugation ($4 \times 10^3 \times g$, 15 min) and resuspended in 0.10 M Tris–acetate buffer (pH 7.6) containing 0.50 M sucrose and 0.5 mM EDTA at a concentration of ~70 mg wet cells mL^{-1}. The suspension is diluted twofold with H_2O and lysozyme is added to 0.1 mg mL^{-1}. The suspension is gently shaken and incubated on ice for 30 min in order to hydrolyze the outer membranes. The resulting spheroplasts are recovered by centrifugation at $4 \times 10^3 \times g$ for 15 min; the pellet is resuspended in buffer at a concentration of ~0.5 g mL^{-1}. This buffer for contains 0.10 M potassium phosphate (pH 7.4), 6 mM magnesium acetate, 20% glycerol (v/v), and 10 mM 2-mercaptoethanol. At this point, the preparation can be stored frozen at −70°C until further use.[245]

The frozen spheroplasts are thawed in a water bath at room temperature. During the thawing process, protease inhibitors are added to the spheroplasts to the following final concentrations: PMSF, 1.0 mM; leupeptin, 2.0 μM; bestatin, 1.0 μM; and aprotinin, 0.04 U mL^{-1}. Cells, in a Rosette cell packed in ice, are lysed for approximately 15 min (at ~70% full power) using a Branson sonicator or until the cell lysate is void of clumps. The lysate is subjected to centrifugation ($10^4 \times g$, 20 min) and the pellet is discarded. The supernatant is then centrifuged at $10^5 \times g$ for 90 min (e.g., 3.5×10^4 rpm in a Beckman 45 Ti rotor). The pelleted membranes are resuspended in a minimum volume of 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 6 mM magnesium acetate, and 10 mM 2-mercaptoethanol. The suspension is quickly frozen in liquid N_2 and stored at −70°C (unless further purification is started immediately).

The *E. coli* membranes prepared as earlier are diluted to a 20 mM potassium phosphate concentration by addition of 4 volumes of a solution of 20% glycerol (v/v), 1.25% CHAPS (w/v), and 10 mM 2-mercaptoethanol. Buffer, containing 20 mM potassium phosphate (pH 7.4), 10 mM 2-mercaptoethanol, 20% glycerol (v/v), and 1.0% CHAPS (w/v), is added, as necessary, to dilute to a protein concentration of ~2.0 mg mL^{-1}. Some P450s are not solubilized well with only an ionic detergent (CHAPS, cholate, etc.) and require the addition of a nonionic detergent (e.g., Triton N-101, Emulgen 911 or 913, Tergitol NP-10).[247] P450s in this category that we have encountered in this laboratory include P450 2A6 and 2D6. The mixture is stirred gently for 2–4 h and centrifuged at $10^5 \times g$ for 30 min; the pellet is discarded.

The resulting supernatant is applied to a 2.5 × 10 cm column of DEAE-Sepharose (suitable for ~1000 mL of solubilized membranes) that has been equilibrated with 20 mM potassium phosphate buffer (pH 7.4) containing 10 mM 2-mercaptoethanol, 20% glycerol (v/v), and 1.0% CHAPS (w/v) and 10 mL fractions are collected. After all of the sample has been applied, the column is washed with ~2 bed volumes of the equilibration buffer and the fractions containing red color (P450) are pooled. KCl (solid) is added to the pooled fractions to achieve a 0.5 M concentration. A spectral assay is done[114] to estimate recovery, and it may be useful to also monitor the progress of purification at this point by SDS–polyacrylamide gel electrophoresis.[263]

The pooled material is applied directly to a 1.5 × 5 cm column of Ni-NTA agarose that has been equilibrated with 20 mM potassium phosphate buffer (pH 7.4) containing 20%

glycerol (v/v), 0.5% CHAPS (w/v), 0.5 M KCl, and 10 mM 2-mercaptoethanol, at a flow rate of ~1 mL min^{-1}. Most of the brown color should be adsorbed to the column. The column is washed with 10 column volumes of the equilibration buffer. The column is then washed with 10 bed volumes of 20 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 0.5 M KCl, and 10 mM 2-mercaptoethanol. The protein (P450) is then eluted from the column with 20 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 0.5 M KCl, 10 mM 2-mercaptoethanol, and 400 mM imidazole, collecting 5 mL fractions. The pH of the elution buffer is rechecked after addition of the imidazole and is adjusted to 7.4 with 43% (or more dilute) H_3PO_4. The fractions are analyzed for A_{417} and by SDS–polyacrylamide gel electrophoresis, taking care to dilute samples to avoid potassium dodecyl sulfate precipitates. The P450 fractions (as judged by A_{417}) that are homogeneous (>95%) as judged by SDS–polyacrylamide gel electrophoresis are pooled and dialyzed extensively (4×, >6 h each time) with 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 0.1 mM EDTA, and 0.1 mM DTT, either before or after concentration with an Amicon ultrafiltration system and a PM-30 membrane. The concentration of P450 is estimated spectrally[114] (*vide supra*).

NADPH–P450 Reductase

Affinity procedures have been described by Yasukochi and Masters[264] and Strobel and Dignam[265] for purification of this enzyme from rat and hog livers using detergent extraction from microsomes, DEAE-cellulose chromatography, and 2′,5′-ADP- or $NADP^+$-agarose affinity chromatography. 2′,5′-ADP-agarose is commercially available from Sigma–Aldrich. Ion-exchange chromatography, or some other initial purification procedure, is often used to facilitate binding of the reductase to the affinity column.[264] Alternatively, the *n*-octylamino-Sepharose 4B reductase fraction from rabbit, rat, or human liver P450 purification procedures[135,266–271] can be directly applied to the affinity column. In our own laboratory, we now produce the rat or human reductase in *E. coli* and isolate it from the membranes, using the methods of Hanna et al.[272] These expression vector systems are freely available upon request.

The column is washed with 0.25 M potassium phosphate buffer (pH 7.7) containing 0.10 mM EDTA, 20% glycerol (v/v), and 0.20% Triton N-101 (w/v) (or other nonionic detergent) to remove other proteins. The detergent is then removed by washing the column with 30 mM potassium phosphate buffer containing 0.10 mM EDTA, 20% glycerol (v/v), and 0.10% sodium cholate (w/v). The reductase is eluted with the latter buffer containing 10 mM 2′-AMP or $NADP^+$ (and 0.10 mM PMSF) and dialyzed (48 h) versus 100 volumes of 10 mM Tris–acetate buffer (pH 7.4) containing 0.10 mM EDTA and 20% glycerol (v/v) to remove cholate and 2′-AMP (or $NADP^+$).[65,268,273]

This procedure has been used to obtain NADPH–P450 reductase in yields as high as 50%; specific activities for cytochrome *c* reduction range from 40 to 70 mmol min^{-1} (mg protein)$^{-1}$.[264,265,273] Spectra show the absence of nonflavin components; the A_{455}/A_{380} ratios are 1.10–1.15.[268,273] The apparent monomeric M_r is 74 kDa (the protein sequence has been deduced from cDNA and the actual mass is somewhat different[264]). Sometimes, proteolysis is a problem and results in cleavage of a peptide necessary for activity toward P450 (but not cytochrome *c*). This problem can be avoided by adding PMSF (from a stock ethanolic solution) to 0.10 mM to buffers immediately prior to use to inhibit serine-active proteases. Some preparations appear to lose some flavin mononucleotide (FMN), as evidenced by stimulation by 10 mM FMN. This problem can be minimized by including 1 mM concentrations of FMN in buffers and minimizing exposure to light during dialysis (use of amber glass).[269]

Methods for Use of Enzymes

Estimation of Protein Concentration

Estimates of protein concentrations are useful in a number of settings, ranging from standardization of crude assays, for the purpose of repeatability, to precisely establishing the amount of an enzyme being used in a reaction.

In the course of monitoring proteins during purifications, following the absorbance at 280 nm (due mainly to tryptophan) is a reasonable approach. However, even this can be a nontrivial issue if absorbing materials are present in buffers. For instance, alkyl phenyl ether detergents (Triton, Emulgen, etc.) are problems.

At least five methods are used to estimate protein concentrations in individual samples. The first three are colorimetric assays and are used widely.

The Lowry method is based on the use of Folin–Ciocalteu reagent[167] and yields a blue color, based on reactions with tryptophan and tyrosine (which still remains vague after all these years). A detailed protocol was published in this chapter in a previous edition.[4] The assay is reasonably rapid and sensitivity. A major limitation is the sensitivity to interference by contaminating materials (e.g., detergent, buffer salts).

The biuret assay is an older method, using complexation of Cu^{2+} ions with the amide bonds. Compared with the Lowry assay, this method has less interference by buffer anomalies but is also much less sensitive.

The Bradford method involves a color change of the dye Coomassie Brilliant Blue G upon binding to protein.[274] This assay is convenient and has sensitivity comparable to the Lowry method. However, as might be suspected, any extraneous hydrophobic material will interfere (esp. detergents).

Another method, the bicinchoninic acid, or *BCA* assay,[168] is even more sensitive than the Lowry method and generally has fewer interference problems. The reagents can be purchased (Thermo Scientific) and the manufacturer provides a detailed description of the method. It is technically simpler than the Lowry assay in that only a single reagent is used and the timing of additions is less critical; this makes the procedure more adaptable in microassay systems. The sensitivity

is increased at higher temperatures (e.g., 50°C), and detection of sub microgram amounts of protein is not unrealistic.

All of the aforementioned four systems are based on phenomena that are not well understood, and even if the basis were established, a real issue is the protein-to-protein variability of the colorimetric response. Thus, an assay used to establish the concentration of a protein by one of these methods can often show a twofold error. This inaccuracy may not be an issue in some settings but could be critical in others, such as establishing the stoichiometry of binding of a substrate or prosthetic group or the extent of a kinetic burst measurement.[275]

Two methods are accurate when the concentration of an individual protein must be known with great accuracy. One approach is to calculate the expected extinction coefficient (ε_{280}) from the content of tryptophan and aromatic amino acids in the sequence. The websites <http://www.biomol.net/en/tools/proteinextinction.htm> and <http://www.basic.nwu.edu/biotools/ProteinCalc.html> can be utilized. This approach can provide a reasonable value. Extinction coefficients at the lower UV wavelengths (i.e., 210, 215 nm) are based on the amide bonds and might be expected to be accurate. They are more sensitive[276]; however, the far UV absorbance is also very sensitive to interference by contaminants, including some buffer components.

The other accurate method is probably the best, although it is more laborious and expensive enough to restrict use to critical applications. The method is a throwback to a method called *quantitative amino analysis*, which has been used in protein chemistry. The protein is carefully hydrolyzed (usually 6 M HCl, 24 h at 110°C) to its component amino acids. These are derivatized, usually with phenyl isothiocyanate, and analyzed by HPLC. The amounts of each amino acid are determined by comparisons with external standards processed in the same way (using the integrals of the A_{254} peaks). Alternatively, the derivatization can be done with a fluorescent reagent to increase the sensitivity. The hydrolysis yields of tryptophan and cysteine are not ideal but the deviation due to these rarer amino acids is usually not significant enough to limit the application. The amount of the protein can be calculated from knowledge of the amounts of each amino acid and the known sequence of each protein.[275] In one application, three component DNA polymerase δ protein subunits were resolved by SDS–polyacrylamide gel electrophoresis and the amounts of each were determined by quantitative amino acid analysis.[277]

Methods for the Determination of Enzyme Purity

As in the case of other chemicals, no single technique can be used to establish purity; moreover, purity is always defined as a limit of given impurities in a given analytical system, and one can argue that nothing is really *pure*. However, some techniques are more useful than others in ruling out heterogeneity and will be discussed here. Some of the classical considerations about purity have been relaxed by the ability to express in recombinant systems, in that related enzyme family members are not expressed. However, for some purposes, proteins must still hold to strict criteria (e.g., antibody production, certain enzymology experiments):

1. An obvious criterion of homogeneity is the absence of suspected contaminants. For instance, P450 preparations should be devoid of NADPH–cytochrome *c* reduction activity and epoxide hydrolase preparations should be devoid of heme. Of course, such impurities must always be defined in terms of detectable limits of contamination. A point to be considered here is that an experimental situation may call for the absence of lipid or detergent contamination as well as protein contamination, and the limit of such impurity must be determined. Phospholipid can be determined by thoroughly dialyzing the enzyme versus Tris buffer and then H_2O to remove soluble phosphates; lipids are extracted as described by Bligh and Dyer[278] and phosphate content is determined according to Chen et al.[279] Ethylene oxide–based detergents (including Emulgens 911 and 913, Renex 690, Tergitol NP-10, Triton N-101, and Lubrol PX) can be extracted and assayed as described.[280,281] Alternatively, detergents can also be assayed by HPLC.[257]
2. Specific activity or specific content of the isolated enzyme is a guide to follow in purification. For instance, P450 preparations should contain *x* nmol of P450 (mg protein)$^{-1}$, where $x = 10^6$/subunit M_r (i.e., x = 16–22), NADPH–P450 reductase preparations should catalyze the reduction of 40–70 mmol cytochrome *c* min^{-1} (mg protein)$^{-1}$ under optimal conditions. However, such measurements are dependent on the accuracy of the protein estimation, which may be a problem. Specific activities are sometimes more variable than expected. In the case of P450, some forms may exist in vivo without a full complement of heme[282] (these are issues regarding prosthetic group loss that involve expressed proteins as well as those purified from tissues). These general guidelines about activity, and prosthetic group content are useful in evaluation of purity.
3. SDS–polyacrylamide gel electrophoresis[263] is routinely used as the main criterion for homogeneity. This technique has been quite useful in determination of homogeneity; moreover, the subunit M_r estimates appear to be reasonably valid for P450[127,135] and NADPH–P450 reductase.[79] The reader should remember, however, that even this powerful technique has its limitations. Evidence has been presented that different microsomal enzymes cannot always be distinguished by this technique.[65,283] Furthermore, the results obtained with this technique are rather dependent upon the exact procedure used, and the methods vary in resolving abilities.[65,135] Finally, apparent M_r values also vary depending upon the procedure and the standards used, and the reader is cautioned to compare results

from different laboratories carefully and allow for as much as 3–4 kDa differences. Further, it is well established that in some cases, such a change may result from a single amino acid replacement regardless of the effect on the true M_r.

Isoelectric focusing can provide resolution of enzymes and has been used in studying cytochromes P450. However, a number of artifacts can be encountered in the use of this methodology, at least when used without specialized procedures.[220,284–287]

Staining of electrophoretograms is usually done with protein stains. In the absence of SDS, NADPH–P450 reductase can be stained using tetrazolium dyes[288] and P450 can be stained using benzidine derivatives and H_2O_2.[289,290] The latter method has also been used to tentatively identify P450 in SDS–polyacrylamide gel electrophoresis. However, much of the heme leaves the enzyme, even in the absence of reducing agents. While others have claimed that heme does not bind to other proteins, Thomas et al.[290] found that heme was bound to albumin; thus, one must be cautious in interpreting data involving such a technique. Immunochemical techniques have been developed for the identification of P450 separated from microsomal membranes by SDS–polyacrylamide gel electrophoresis; these methods proved useful in answering a number of questions about P450 induction (*vide infra*).

Reconstitution of Enzyme Activity

Early work on reconstitution of mixed-function oxidase activity was reviewed by Lu and West.[125] The following general statements can be made. The optimum rate of enzyme activity, based upon P450, is obtained when NADPH–P450 reductase is present at an equimolar concentration or excess. Phospholipid enhances the rates of most activities; this phospholipid can be in the form of a microsomal extract or synthetic L-α-1,2-dilauroyl-*sn*-glyceryo-3-phosphocholine. Some nonionic detergents will partially replace phosphatidylcholine at low concentrations.[291,292] The activity toward some substrates can be further enhanced by small amounts of cholate or deoxycholate.[125] The role of phospholipid is not completely understood, but a dual role has been postulated[293]: L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine increased the affinity of a rabbit liver P450 for *both* organic substrate and NADPH–P450 reductase; all four components are complexed during catalysis. Several investigators have studied synthetic liposomal systems; however, no such system has been prepared to date that is more active in hydroxylation than a system reconstituted in the presence of subcritical micelle concentration levels of phospholipid. Neither cytochrome b_5 nor NADH is required for activity in many reactions, although some are definitely enhanced considerably by cytochrome b_5. The effect of the phospholipid appears to be kinetic and can, at least in some cases, be overcome with high protein concentrations and extended preincubation conditions.[59] However, see also Halvorson et al.[60] and Imaoka et al.[61]

A basic procedure for reconstituting mixed-function oxidase activity is outlined in the succeeding text. Equimolar concentrations of P450 and NADPH–P450 reductase (both of which have been stripped of excess detergent by treatment with polystyrene beads and/or calcium phosphate gel or hydroxylapatite)[107] are first mixed in the presence of 40 mM sonicated L-α-1,2-dilauroyl-*sn*-glyceryo-3-phosphocholine, and after 5 min, an appropriate buffer (i.e., 50–100 mM potassium phosphate or other buffer, pH 7.0–7.7) is added plus a sufficient volume of H_2O. The substrate is then added, preferably in H_2O if possible. If not, the substrate should be dissolved in $(CH_3)_2CO$, $(CH_3)_2SO$, or CH_3OH such that the final concentration of organic solvent is ≤1% (v/v) for the enzymes. Some forms of P450 oxidize these compounds or are inhibited by them (e.g., P450 2E1) and caution must be exercised.[148,149] The system is preequilibrated at 37°C for 3–5 min and the reaction is initiated by the addition of NADPH (0.15–0.5 mM) or, preferably, an NADPH-generating system. (The organic substrate should not be the last addition, as prior addition of NADPH will result in generation of H_2O_2, which can destroy P450.)[273] The length of the time for which the reaction is linear depends upon the substrate; in general, rapidly metabolized substrates do not give long periods of linearity and some substrates are converted to metabolites that destroy P450 rapidly. The addition of catalase (~800 U mL^{-1}, dialyzed before use to remove the preservative/inhibitor thymol) and superoxide dismutase (40 μg mL^{-1}) may be useful in avoiding heme destruction due to the generation of reduced oxygen species.[273,294]

FMO activity requires only the single protein, of course, but the enzyme is dramatically less heat stable than others.[295–297] The enzyme is stabilized by pyridine nucleotides, so NADPH should be added to the enzyme before incubation at 37°C, and then the organic substrate should be added. NADPH–P450 reductase has other enzyme activities in the absence of P450; many of these require no special conditions but may be stimulated, as is the case for lipid peroxidation, by high salt concentrations.

Use of Selective P450 Inhibitors

Chemical inhibitors offer considerable potential in the discrimination of enzymes involved in reactions. Unlike antibodies, they can readily be obtained by many laboratories by chemical synthesis or, in many cases, purchased directly. Further, they have the advantage that they can be used in vivo in many cases, even in humans. The approach of using chemical inhibitors with crude enzyme preparations to discern catalytic specificity has been most highly developed for the P450 enzymes (Table 40.3). P450 inhibitors have been known for some time but many of the early compounds (e.g., SKF-525A [*N*,*N*-DEAE-2,2-diphenylvaleric acid] and metyrapone) are only partially selective. Newer and more specific compounds are now available. A list of those used for human P450 enzymes is presented in Table 40.3.[46,72]

A few comments are in order. First, the specificity tends to carry over within each enzyme family (1A, 2A, 2B, 2C,…) between species, although some differences can be noted. For instance, quinine is a better inhibitor of rat P450 2D enzymes than its diastereomer quinidine, while the opposite

is true in humans. Some inhibitors are highly effective with more than one enzyme (e.g., diethyldithiocarbamate). Some inhibitors are irreversible and act by modifying the protein and/or prosthetic group. Ketoconazole is able to inhibit several P450 enzymes but many of its in vivo effects can probably be attributed to P450 3A4. The same is probably true of cimetidine.[298]

Immunochemical Techniques

Antibodies have been raised to P450, NADPH–P450 reductase, epoxide hydrolase, and many of the other enzymes considered here. For instance, these antibodies have been used to show the involvement of P450[229–231,299–302] and its reductase[288,303] in a number of reactions. Antibodies have also been used to examine the homogeneity of isolated enzyme fractions, the multiplicity of enzymes in microsomes, and the amounts of individual enzyme forms in microsomal preparations.[64,65,283,304–308] The topical location of the enzymes in microsomal membranes has also been studied with immunological techniques[230,309] as have several aspects of enzyme biosynthesis. Antibodies have also been used to study the localization of the enzymes in various sections of individual organs.[310,311]

Preparation of Antibodies

All three of the aforementioned enzymes are rather antigenic, and antibodies have been raised in rabbits using <50 μg of protein. Sheep, goats, and guinea pigs have also been used for antibody production. A number of different immunization schedules can be used, depending upon the animal and the dose. Antisera can be used in some procedures, but immunoglobulin G (IgG) fractions are needed in some applications.[231,299] To prepare these fractions, antisera are heated 20 min at 56°C and centrifuged at $10^4 \times g$ for 10 min. The supernatants are mixed with equal volumes of 50% $(NH_4)_2SO_4$ (w/v) and recentrifuged. The pellets are washed with 25% $(NH_4)_2SO_4$ (w/v) to remove most of the color and then dissolved in 10 mM potassium phosphate buffer (pH 8.0) and dialyzed against the same buffer (at 4°C). The dialysates are passed through columns of DEAE-cellulose equilibrated with the same buffer. The void volume fractions (measured by absorbance at 280 nm) are pooled, retreated with $(NH_4)_2SO_4$ as earlier to remove color if necessary, concentrated by ultrafiltration (up to 50 mg mL^{-1}), and stored at –20°C. There are a number of alternative procedures available using commercial Protein A or Protein G affinity columns.

An alternative approach to using the purified protein of interest is to utilize peptides to generate antibodies. This approach has become more popular with P450s, as well as many other enzymes, for several reasons. First, the intact protein does not have to be expressed do isolated, although it may be necessary for use as a standard in either defining specificity or quantifying the protein. Second, the ability to use only part of the protein provides an opportunity to choose sequences that are specific for a particular protein compared to closely related forms. Some caution (and experimental work) is necessary because antibodies can show reactivity with less closely related proteins, for example, some antibodies recognize P450 proteins from multiple gene families.[232] Finally, the purification of peptides is generally more straightforward than proteins, in that they are smaller and can be readily subjected to methods such as reversed-phase HPLC with organic solvents and acidic conditions. Thus, the prospects of including contaminating antigens are less. Many commercial vendors supply peptides already purified. The quality of these peptides is important, and the purity and identity can be readily confirmed by methods such as capillary electrophoresis and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. Peptides are generally conjugated to a carrier protein (e.g., keyhole limpet hemocyanin) before injection into animals.

Another approach involves the production and use of monoclonal antibodies. This methodology, originally developed by Milstein,[312] involves initial injection of animals and utilization of spleen cells, either in in vitro or intact animals for the hybridoma production. An advantage of using a monoclonal antibody is that it is only one of a complex mixture of antibodies that normally is generated in the polyclonal response. Therefore, no problems should result from the variability one often sees with polyclonal antibodies, that is, with variation in the individual antibodies produced among different animals and at different times.[232]

Another approach avoids the need to use animals and has other advantages. Large phage-display libraries exist (e.g., 10^9 antibody molecules). These systems are bacteria based and can be readily screened. For instance, blots containing the protein of interest (e.g., a particular P450 and other proteins that one wishes to avoid recognition of) can be used with such a library. The phage that selectively binds can be recovered and cultured in bacteria. Recovery can be expedited using epitope tags. Further, such approaches can be used to select antibodies using unpurified systems, for example, one can screen an existing phage-display library for antibodies (or, more correctly, F_c chains) with a set of cells that is hypothesized to contain a specific protein and another set is not. If a difference is observed, in terms of which F_c chains are selected, then that F_c chain can be recovered and used as a reagent to either monitor purification of the protein or to immunopurify the protein directly.

The list of immunochemical techniques that can be used with such antibodies is quite lengthy and the reader is referred to texts on the general subject[313]; the procedures include double-diffusion analysis, inhibition of enzyme activity, radioimmunoassays (RIAs) and enzyme-linked immunoassays, immunoprecipitation, immunoaffinity column chromatography, and immunohistochemical localization. These techniques continue to be useful in studies of the roles of individual forms of these microsomal enzymes in various processes.

Immunoinhibition of Catalytic Activity

In this approach, one adds an antibody to an enzyme preparation and determines if that antibody preparation can block

catalytic activity (Figure 40.30). This approach is most useful with a crude enzyme system, such as a subcellular organelle preparation. Thus, one can ask what fraction of total activity is the result of the enzyme specifically recognized by the antibody (if the antibody completely inhibits the activity of the antigen itself). Such an approach has been useful in a number of cases in our own laboratory[220,229,314,315] as well as many others.

Antibodies to some proteins tend to be more inhibitory than others. In general, polyclonal antibodies raised against cytochromes P450 are often, but not always, inhibitory. However, inhibitory antibodies have not been reported for microsomal FMO and only occasionally has inhibitory anti-epoxide hydrolase been prepared.[316] Apparently, antibodies raised against GSTs are not inhibitory. Some of the difference may be due to the size of the substrate or accessory protein that interacts with the antigen in catalysis. Thus, one would expect binding of an antibody to block binding of very large substrates (viz., other proteins) more readily than smaller compounds. In support of this view, anti-NADPH–P450 reductase blocks reduction of cytochrome *c* but not ferricyanide or neotetrazolium blue.[288] Another general trend is that only a limited fraction of monoclonal antibodies are inhibitory.[317,318]

Analyzing for antibody inhibition is a relatively straightforward process. In general, the enzyme preparation of interest is mixed with the antibody and incubated for 20 min at room temperature. Other components are then added and catalytic activity is measured in the usual manner.

A general way to properly assess enzyme inhibition is to run several incubations, varying the amount of antibody and holding the amount of enzyme constant. Parallel assays should be done in which a nonimmune antibody preparation prepared in the same way is added at the same levels to the enzyme preparation of interest (Figure 40.30). (Alternatively, one can mix varying ratios of immune and nonimmune antibodies with each aliquot of enzyme, maintaining a constant *total* amount of antibody added.)

Most assays of this type are done with IgG antibody fractions. Serum and ascites fluid contain other materials that can cause nonspecific inhibition. However, if the antibody titer is very high (with regard to inhibition) or if the catalytic assay is so sensitive that little antibody is needed for inhibition, then such crude materials may be used.

In general, little can be said about inhibition <15% of the total unless enough careful replicates are done, and the difference between immune and nonimmune serum incubates is reproducible, concentration dependent, and statistically significant. To a first approximation, the percentage of inhibition is a reflection of the fraction of the total catalytic activity in the preparation that is due to the protein that reacts with the antibody. The antibody should completely inhibit the purified enzyme itself, however, for this analysis to be valid, for the possibility exists that noninhibitory antibodies may hinder the binding of inhibitory antibodies and total inhibition might never be achieved.

Quantitation of Proteins by Immunoblotting

In many cases, the absolute concentration of a particular protein in a sample is desired, apart from its catalytic activity. A direct way to obtain such measurements is with the use of specific antibodies. A variety of immunochemical techniques are available for use, including various types of RIAs and enzyme-linked immunosorbent assays (ELISAs).[319] However, knowledge concerning the specificity of the antigen–antibody reaction must be available. Probably the single most reliable technique for evaluating specificity is coupled SDS–polyacrylamide gel electrophoresis/immunoperoxidase staining, or immunoblotting (which often goes by the slang term *Western blotting*), where a crude mixture of protein is separated by electrophoresis and the resolved proteins are transferred to a thin sheet of nitrocellulose paper where they can be detected after binding antibodies and antibodies coupled to enzymes with chromogenic substrates. In our early studies with this system, we found that the intensity of the staining of protein bands was proportional to the amount of antigen applied and that such a procedure could be utilized in making quantitative measurements (Figure 40.32). We still continue to use such a system to quantify many proteins,[320] for several reasons. Under appropriate conditions,

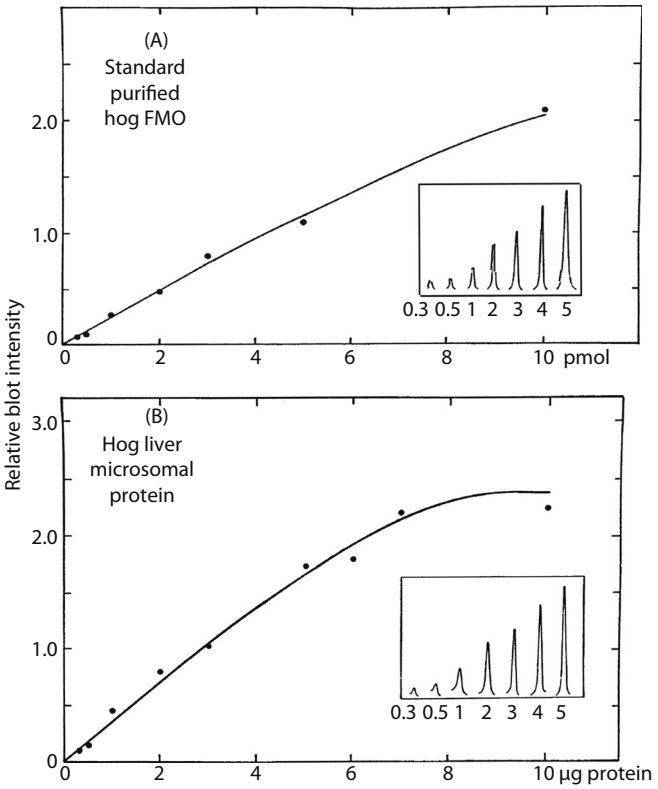


FIGURE 40.32 Immunoelectrophoresis and densitometry of FMO in purified samples and porcine liver microsomes.[283] (A) Area under the densitometric peak as a function of the amount of purified porcine liver FMO used for electrophoresis. The *inset* shows the actual densitometric traces. (B) Area under the densitometric peak as a function of the amount of porcine liver microsomal protein used for electrophoresis. The *inset* shows the actual densitometric traces.

the method is accurate and quite sensitive. It provides a check on the specificity of antigen–antibody interaction in each individual antigen sample and provides data even when cross-reactive materials are present (if they can be resolved in a single electrophoretic dimension). The method is relatively rapid and straightforward, even when new systems are explored little optimization is required.

Samples of roughly 5 μg of microsomes or cellular homogenate protein are solubilized by heating with SDS and 2-mercaptoethanol. The samples are electrophoresed in a typical system based on the procedure of Laemmli[263]—a slab gel is used with up to 25 samples. Five or six lanes are used to prepare a standard curve for each gel: the lanes contain, for example, 0.5, 1, 2, 3, 5, and 10 pmol of the purified antigen. Crude protein samples to be analyzed are loaded into the wells for the other lanes. Typically, 1–50 μg of microsomal protein might be loaded per well for analysis of P450 proteins. Protein samples are dissolved in a mixture of 63 mM Tris–HCl buffer (pH 6.8) containing 10% glycerol (v/v), 1.0% SDS (w/v), 0.001% pyronin Y (w/v), and 5.0% 2-mercaptoethanol (v/v) and heated for 60 s at 95°C. Aliquots are loaded into the wells of a 1.5 mm × 16 cm × 20 cm gel (e.g., Bio-Rad, Richmond, CA). The separating gel is poured from a mixture of 0.375 M Tris–HCl buffer (pH 8.8) containing 7.5% acrylamide (w/v), 0.03% tetramethylethylenediamine (TEMED) (w/v), 0.10% SDS (w/v), and 0.0425% $(NH_4)_2S_2O_8$ (w/v) and the stacking gel, in which the wells are formed, is poured from a mixture of 0.14 M Tris–HCl buffer (pH 6.8) containing 3.5% acrylamide (w/v), 0.057% TEMED (v/v), 0.65% sucrose (w/v), 0.11% SDS (w/v), and 0.045% $(NH_4)_2S_2O_8$ (w/v). The electrode buffer (pH 8.3) contains 190 mM glycine, 25 mM Tris, and 0.10% SDS (w/v). Power is applied to the system at a constant current setting of 25 mA per gel to move the samples through the separating gel. The electrophoresis takes ~4 h. When the pink dye front has moved to within about one cm of the edge of the gel, the power is turned off and the system is separated. One of the glass plates on the gel is removed and water is sprinkled on the surface. A wetted piece of nitrocellulose paper (0.45 mm, Whatman) is laid over the wet gel. Care should be taken (and enough water used) to avoid trapping air bubbles. Two sheets of Whatman #3 paper, prewetted with water, are laid over the nitrocellulose. The glass plate is removed from the other side of the gel and replaced by a wet sheet of Whatman #3 paper. The entire *sandwich* is placed between two wet sponges and then enclosed between the two electrode baffles of an electrotransfer apparatus, with the nitrocellulose closer to the anode than the cathode. The apparatus, with the gel and nitrocellulose between the baffles, is filled with 25 mM Tris–HCl buffer (pH 8.2) containing 190 mM glycine and 20% CH_3OH (v/v). Constant current (400 mA for 1 h or 200 mA for 2 h) is applied to the system.

After the blotting operation, the polyacrylamide gel and the filter papers are discarded and the nitrocellulose sheet is placed in 25 mL of a solution of PBS (phosphate-buffered saline: 10 mM potassium phosphate buffer [pH 7.4] plus 0.9% NaCl [w/v]) containing 0.50% Tween 80 (w/v). The sheet is conveniently placed in a plastic box of only slightly larger dimensions (13 × 18 × 3 cm) with a lid. The gel is shaken in a 37°C water bath for 30 min to block reactive sites on the nitrocellulose sheet with serum proteins so that antibodies will not be bound in subsequent steps. After the blocking step, the nitrocellulose sheet is washed twice with 60 mL of PBS at room temperature. In practice, the box is rocked on a platform rocker (Bellco Glass, Vineland, NJ) for 5 min, the buffer is decanted, and 60 mL of fresh PBS is added each time.

In the next step, 25 mL of PBS containing 0.50% Tween 80 (v/v), and an appropriate dilution of the antiserum of choice, is poured into the box over the nitrocellulose sheet. The system is shaken (or rocked) at 37°C for 30 min and then overnight at 4°C; alternatively, 37°C for 2 h is usually satisfactory. Typical antisera dilutions range from 1/100 to 1/2000 (some monoclonal antibodies have been successfully used at $1/10^6$ dilutions of ascites fluid). Then the nitrocellulose sheet is washed six times (5 min each, room temperature) with 60 mL of PBS. The next addition is 25 mL of PBS containing 0.50% Tween 80 (w/v) and 0.20% (v/v) goat antirabbit IgG antiserum (if the primary antiserum was made in rabbits). This solution is rocked or shaken with the nitrocellulose sheet at room temperature for 30 min, and the sheet is then washed again six times with PBS as before. The next addition to the sheet is 25 mL of a solution of PBS containing 0.50% Tween 80 (w/v), and 0.20% (v/v) horseradish peroxidase/rabbit anti-horseradish peroxidase complex (Promega, Madison, WI). The sheet is rocked in this solution at room temperature for 30 min and washed six times with PBS as before.

Development of the stain is done in the following manner. 4-Chloro-1-naphthol (32 mg) is dissolved in 12 mL of CH_3OH and diluted with 60 mL of PBS. H_2O_2 (120 mL of a 30% solution) is added and the solution is poured over the nitrocellulose sheet; bands usually appear within a few minutes. The solution is removed; the nitrocellulose sheet is washed three times with PBS and twice with H_2O. Sheets can be dried between two layers of Whatman #3 filter paper, with a uniform weight applied.

When the gel is dry (within 1–2 h), the bands can be scanned using a densitometer. The integrals are used to construct standard curves and estimate the amount of antigen in each sample (Figure 40.31).

In practice, a standard curve is constructed on each nitrocellulose sheet. An additional way to reduce error is to include an internal standard in each protein sample. Equine alcohol liver dehydrogenase can be used for this purpose, adding 0.2 mg to each sample prior to electrophoresis. The buffer containing the primary antisera is fortified with a 1/500 dilution of rabbit antisera raised against equine alcohol dehydrogenase. When the nitrocellulose sheets are visualized, the P450 band in the 50–60 kDa region is accompanied by a second band migrating with apparent M_r of 43 kDa. The integrals of both bands are obtained from the densitometer. The ratio of the areas of the two bands can be compared to the ratios found with the standard antigen samples.

Even if the antigen–antibody system is not specific enough to visualize only a single electrophoretic band, useful

information can be obtained, if the different antigens are electrophoretically separable. For instance, rat P450s 1A1 and 1A2 usually cross-react but can be separated and quantified.[64,321] The same situation exists with human P450 2C enzymes.[315]

Since the original immunoblotting work was done,[322] a number of variations of the procedure have been reported. Many of these are cited in subsequent reviews.[323,324] For instance, different additives can be used in the buffers for blocking the sheets. Nylon membranes, such as Zeta-Probe (Bio-Rad, Hercules, CA), have increased capacity and can be used to increase the sensitivity of the methods. *Staphylococcus aureus* protein A conjugates can be bound to the primary antibody. Other enzymes such as alkaline phosphate can replace peroxidase; alternatively, ^{125}I-labeled antibodies can be used with autoradiography, as described in the original Towbin et al.'s paper.[322] Another alternative is the use of IR fluorescent reagents (e.g., LI-COR system), which works very well for quantitation (LI-COR, Lincoln, NE).[320] If monoclonal antibodies are used, the methods must be adapted by including a step with rabbit antimouse immunoglobulin G[317,318]; many monoclonal antibodies give poor responses in this system because the individual epitopes do not have sufficient affinity constants.

In our own laboratory, we have applied this approach to rat and human microsomal epoxide hydrolase, rat NADPH–P450 reductase, several different forms of rat and human P450, and FMO. The method can be utilized with cells[325] or tissue homogenates[283] as well as with subcellular organelles.

One related new alternative to determining concentration of proteins in tissues and other crude samples is the use of proteomics.[326,327] No antibodies are needed, and the method makes use of peptides within the proteins of interest. A crude sample is cleaved with trypsin (or another protease), and the crude mixture of peptides is analyzed by LC–mass spectrometry, quantifying an individual peptide or set of peptides. The method is sensitive and we have used it with studies of DNA polymerases in *Sulfolobus solfataricus*. The sensitivity is comparable to that of immunoblotting and the time, and the cost of raising antibodies is eliminated.

Recombinant DNA Techniques

mRNA Isolation

Analysis of mRNA levels provides insight into regulatory mechanisms. In many cases, regulation of enzymatic activity occurs primarily at the level of transcription and mRNA levels are well correlated with protein levels. However, this is not always the case, and notable exceptions have been documented with some P450 enzymes.[328,329] Techniques of mRNA isolation and handling have become routine and may be mastered without considerable difficulty. Moreover, the generation of highly specific probes can be easily achieved by synthesis of oligonucleotides or long probes developed by polymerase chain reaction (PCR) technology, in contrast to the production of antigens and antibodies for analysis of protein levels (*vide supra*). The most widely used procedure for RNA isolation is that of Chomczynski and Sacchi.[330] It is rapid, reliable, and useful with large numbers of samples.

First and foremost, one of the critical aspects of working with RNA is to avoid RNase. Any traces of this protein on glassware, dust, or fingertips will be devastating. Disposable gloves must be worn during all steps and all glassware and plasticware should be treated as described in the succeeding text and stored specifically for RNA procedures. Several commercial kits for total RNA isolation are currently available based on the guanidine method. These kits do offer the ease of ready-made reagents or a single monosolution combining all the initial reagents but at much higher costs.

Reagents for RNA Isolation:

1. Stock guanidine thiocyanate: 250 g of guanidine thiocyanate (Sigma–Aldrich) is dissolved (at 65°C) in a mixture of 293 mL H_2O, 18 mL of 0.75 sodium citrate buffer (pH 7.0), and 26 mL of 10% sarkosyl (w/v). This solution can be stored ≥3 months at room temperature.
2. Denaturing solution: add 0.36 mL of β-mercaptoethanol per 50 mL of the solution just described (add before use—can be stored 1 month at room temperature if necessary).
3. Water-saturated phenol: nucleic acid grade phenol, saturate with H_2O. Once made, the solution may be stored for <1 month at 4°C. This may be purchased ready-made from a variety of sources.
4. 2 M sodium acetate (pH 4.0).
5. $CHCl_3$/isoamyl alcohol (49:1, v/v) or bromochloropropane.[331]
6. 100% isopropanol.
7. 75% C_2H_5OH.
8. All glassware and plasticware to be utilized should be previously treated with a solution of diethylpyrocarbonate (DEPC) and autoclaved, preferably in individually self-sealed pouches. Autoclaving does not inactivate all RNases. Alternatively, glassware can be baked at 300°C for 4 h. Some types of plasticware may be washed with $CHCl_3$ as an alternative to DEPC.[332]
9. If possible, solutions should be shaken with 0.1% DEPC (w/v) and then autoclaved to destroy excess DEPC, which can react with nucleic acids. For chemicals that react with DEPC (e.g., Tris buffers) or that cannot be autoclaved, sterile filtration through 0.2 μm Nalge filters may reduce potential RNase contamination. Presterilized disposable pipettes, pipette tips, filter units, and such may be used directly if individually wrapped as supplied.
10. Whenever possible, all chemicals to be used in RNA work should be reserved for this purpose only and weighed out only with the use of DEPC-treated spatulas.

This procedure may be used for 100 mg of tissue or an equivalent amount of cells, 10^7 cells.[332] The tissue is removed

from the animal and minced on ice. If frozen material is utilized, the tissue should be ground with a mortar and pestle in liquid nitrogen. It is important to note that substantial RNA degradation may occur during this step. Homogenize the material in 10 mL of denaturing solution per g of tissue with either a glass Teflon homogenizer or a Tissue-mizer (Cole Palmer, Vernon Hills, IL). Transfer the homogenate to either a 25 mL glass Corex tube or a smaller polypropylene centrifuge tube. To this are added, sequentially, per 1.0 g of tissues 1.0 mL of 2 M sodium acetate (pH 4.0) and mix thoroughly by inversion, 10 mL of H_2O-saturated phenol and repeat mixing, and 2 mL of the $CHCl_3$-isoamyl alcohol or bromochloropropane. The final mixture is mixed vigorously for 10 s and incubated on ice for 15 min. Centrifuge the suspension at $10^4 \times g$ for 20 min at 4°C and transfer the aqueous (upper) RNA-containing phase to a new tube. Precipitate the RNA by the addition of 1 volume of 100% isopropanol. Incubate for 30 min at –20°C and then centrifuge at $10^4 \times g$ for 20 min at 4°C. Discard the supernatant. Redissolve the RNA pellet in 0.3 mL of the denaturing solution and transfer to a microcentrifuge tube. Reprecipitate the RNA with 1 volume of 100% isopropanol for 30 min at –20°C. Centrifuge for 10 min at $10^3 \times g$ and discard the supernatant. Resuspend this RNA pellet in 75% C_2H_5OH (v/v) and collect the pellet as before. Dry the RNA pellet under vacuum for 5 min. Redissolve the RNA pellet in 100–200 µL of DEPC-treated H_2O and incubate 10–15 min at 55°C–60°C. Store the final RNA at –70°C. The total RNA concentration may be estimated by measuring the absorbance at 260 nm (A_{260} = 1.0 for a solution of 40 µg mL^{-1}, with a 1 cm pathlength).

Reagents for mRNA Preparation:

1. Oligo(dT)-cellulose is available from a number of supplies.
2. Binding buffer: 10 mM Tris–HCl (pH 7.5) containing 0.5 M NaCl, 1 mM EDTA, and 0.5% SDS (v/v). Prepare a 2× binding buffer as well.
3. Wash buffer: 10 mM Tris–HCl (pH 7.5) containing 0.1 M NaCl and 1 mM EDTA.
4. Elution buffer: 10 mM Tris–HCl (pH 7.5) containing 1 mM EDTA.
5. 3 M sodium acetate.
6. Microfuge spin columns ultrafree-MC 0.45 µm filter units (Waters, Bedford, MA).
7. 100% C_2H_5OH.
8. As described in the previous section, all reagents and glassware must be meticulously treated and subsequently handled to avoid contamination with RNases.

The basic procedure can be varied depending upon the scale.[333] Oligo(dT)-cellulose (0.1 g [mg total RNA]$^{-1}$) is suspended in 1–5 mL of binding buffer and equilibrated for 60 min at room temperature with gentle agitation. The slurry is then pelleted and resuspended in fresh binding buffer, rewashed, and finally resuspended in binding buffer (50 mg mL^{-1}). The RNA is suspended at 1 mg mL^{-1} in elution buffer and heated at 65°C for 5 min. Chill on ice and dilute the sample with an equal volume of 2× binding buffer. The RNA sample can be added to equilibrated oligo(dT)-cellulose (from which excess binding buffer has been removed by a brief spin) immediately prior to loading. RNA is incubated with the oligo(dT)-cellulose for 15 min at 23°C with gentle rocking. The sample can then be loaded into a DEPC-treated microfuge spin column. The flow-through (void) fraction should be collected by a brief spin and reapplied to the column, repeat. The column is washed with 5–10 volumes of binding buffer followed by 5 volumes of wash buffer. The bound RNA is eluted with 2–3 column volumes of elution buffer adjusted to 0.5 M NaCl with 2× binding buffer; the entire procedure is repeated (i.e., binding, washing, elution). RNA in the final sample is recovered via ethanol precipitation by adding 0.1 volumes of ethanol and centrifuging. The reuse of the oligo(dT) resin is not recommended.

Northern and Southern Blotting

Specific DNA and RNA sequences can be detected by blotting and hybridization, referred to as Southern and *Northern* blotting.[334] Northern blotting differs from Southern blotting primarily in the initial gel fractionation step (*Northern* and *Western* are slang based on the first use of a method developed by Southern for DNA).[335] RNA molecules are single stranded and thus can form secondary structures. They must be electrophoresed under denaturing conditions for good separations to occur. Denaturation is achieved by the addition of formaldehyde to the gel and buffers or by treating the RNA with glyoxal and dimethyl sulfoxide (DMSO). Previous precautions mentioned for dealing with RNA apply here. All materials must be handled meticulously and all glassware should be DEPC treated and baked. A gel tank should be set aside for RNA work. You should not utilize a DNA tank for RNA work.

Reagents for Electrophoresis and Blotting[336,337]:

1. 5× MOPS [3-(*N*-morpholino)propanesulfonic acid] running buffer and 1×: 0.2 M MOPS buffer pH 7.0 with 50 mM sodium acetate and 5.0 mM EDTA.
2. 37% formaldehyde (12.3 M), pH > 4.
3. Deionized formamide: if formamide has a yellowish color, it can be deionized by adding 5 g of mixed-bed ion-exchange resin (e.g., Bio-Rad AG 501-X8) per 100 mL, stir 1 h at room temperature, and filter through Whatman #1 filter paper. Formamide is a teratogen, so handle with care.[335]
4. Loading buffer: 1 mM EDTA, pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, and 50% (v/v) glycerol. This can be stored up to 3 months at room temperature.
5. 1× SSC buffer: 15 mM sodium citrate (pH 7.0) containing 150 mM NaCl. Prepare 20×, 10×, and 0.25× as well.
6. Ethidium bromide, 5 mg mL^{-1}.
7. Prehybridization buffer: mix 60 mL formamide, 3 mL of sonicated solution of 10 mg mL^{-1} salmon

sperm DNA, 12 mL of 100× Denhardt's solution (2.0% Ficoll [w/v], 2.0% polyvinylpyrrolidone [w/v], and 2.0% bovine serum albumin [w/v]), 6 mL of a solution of 1 mg/mL polyA, 1.2 mL of 10% SDS (w/v), 30 mL of 20× SSC buffer (*vide supra*), and 7–8 mL DEPC-treated H_2O.

8. SDS, 10% (w/v).

To pour a 20 × 20 cm gel, boil 3 g of agarose in 186 mL of DEPC-treated H_2O until dissolved and then cool to 60°C. Add 60 mL of 5× MOPS buffer and 54 mL of 37% formaldehyde. Pour the gel into the gel box with the comb such that the slots are 1 mm above the horizontal surface. Once the gel has set, remove the comb and add enough 1× MOPS buffer to completely cover the gel.

Each sample should contain 2 µL of 5× MOPS buffer, 3.5 µL of 37% formaldehyde, and ≤20 µg RNA in 5 µL. Mix using a vortex device, centrifuge in a microfuge for 5–10 s, and incubate for 15 min at 55°C or 5 min at 65°C. Add 2 µL of loading buffer to every sample and spin again for 5–10 s in a microfuge. Load samples onto the gel and run at a constant voltage of 5 V cm^{-1} until the bromophenol blue band has migrated halfway to two-thirds down the gel. It can be advantageous to run duplicate sets of gels, in that one gel may be stained with ethidium bromide and the other used for transfer. For staining, place the gel in pan, cover with 20× MOPS, and add a few drops of the stock ethidium bromide solution. Incubate for 40 min, then examine the gel on a UV transilluminator to visualize bands, and photograph. Two sharp bands, the 18S and 28S rRNA, should appear if total RNA was used in the electrophoresis.

The gel to be transferred should be rinsed several times with H_2O. It is necessary to remove the formaldehyde as it can hinder retention of RNA to nitrocellulose and nylon membranes.[335] Replace the H_2O with 500 mL of 20× SSC. A piece of nitrocellulose is cut 3 mm smaller on each side than the gel. Wet this for 1 min in H_2O followed by 5–10 min in 20× SSC buffer. Cut five sheets of Whatman 3MM paper 3 mm smaller on each side than the nitrocellulose. In order to prepare a wick, cut a piece of chromatography paper 2 cm larger than the gel with four tabs extending out 10 cm on each side. A glass plate, slightly larger than the gel, is placed on top of a large buffer reservoir. One wick is place on every side of the glass plate hanging down into the 20× SSC buffer reservoir. Remove any air bubbles trapped between the wick and the glass plate with a glass rod. At this point, the gel is carefully placed on top of the wicks, taking care to remove any air bubbles. Cover the four edges of the gel with either plastic wrap or parafilm to prevent wicking at the edges as the gel contracts. The nitrocellulose sheet is placed on top of the gel. This is in turn covered with the Whatman papers one at a time, taking care to remove air bubbles after the addition of each new sheet. Cover the stack with 5 cm of paper towels cut to the same size followed by another glass plate. Add a weight to hold everything in place. Transfer will occur overnight.

Remove all the materials to expose the nitrocellulose sheet. Mark the wells and front to back. It is best to use pencil, as ink will wash off in subsequent washes. If nitrocellulose was used, place it between two sheets of Whatman paper and bake for 2 h at 80°C. Nylon membranes may be baked as described or wrap the dry membrane in UV-transparent plastic wrap, such as Saran wrap, and place the RNA side down on a UV transilluminator (254 nm) and irradiate for the appropriate length of time. For hybridization, the blot is placed in a sealable bag with 1 mL of formamide prehybridization solution per 10 cm^2 of membrane. The bag is rotated and incubated at 37°C–42°C for 2 h to overnight. An appropriate probe might be either (1) nick translation of a cDNA fragment, (2) random labeling, (3) PCR, or (4) oligonucleotide synthesis followed by ^{32}P-end labeling. For additional information, consult current literature in the field. Longer probes with higher levels of incorporation are more sensitive, while shorter probes can be more specific when optimized for differences. The probe is mixed with hybridization solution and heated at 95°C for 10 min. Hybridization will occur overnight at the same temperature used for prehybridization. On the next day, the bag or box is opened and the filter is removed. The filter is washed twice, 1–2 min each, with 2× SSC buffer at room temperature. Two similar washes follow 45 min each with 1× SSC containing 0.10% SDS (w/v). The hybridization can be changed by varying the temperature of the hybridization or the wash and the salt concentration of the wash buffer, in order to increase the *stringency* of the wash and the specificity of hybridization.[338] The filter is exposed to film. Time needed for an appropriate exposure varies with the specific activity of the probe, stringency of the hybridization, and the concentration of the RNA and DNA. Most work has been done with ^{32}P-labeled probes. Alternative procedures utilize ^{33}P, avidin/biotin, immunochemical, and luminescence procedures (consult commercial vendors). These procedures may increase in use due to decreased cost and increased restrictions on the disposal of radioactive waste.

Reagents for Southern Gels:

1. 0.25 M HCl
2. Denaturation solution: 1.5 M NaCl plus 0.5 M NaOH
3. Neutralization solution: 1.5 M NaCl plus 0.5 M NaOH (pH 7.0)
4. 20× and 2× SSC

Once the DNA samples have been digested with the restriction enzymes of choice, run an agarose gel with the appropriate markers and stain with ethidium bromide. Photograph the gel with a ruler alongside so that band positions may be compared to the membrane after hybridization. Rinse the gel in distilled water and place it in a dish containing 10 gel volumes of 0.25 M HCl. Shake slowly for 30 min at room temperature. Remove the HCl and rinse with distilled H_2O. Add 10 gel volumes of denaturation solution and shake for 20 min at room temperature. Repeat this step one time. After the second incubation, rinse the gel and incubate 2 times, 20 min shaking with 10 gel volumes of neutralization solution.[339] The setup for a Southern transfer is exactly

the same as for the Northern transfer. Refer to the Northern section (*vide supra*) on how to assemble the gel and membrane for transfer. After the DNA has been transferred to the membrane, it must be immobilized. The remainder of the protocol is the same as for a Northern blot. Place the nitrocellulose membrane between two sheets of Whatman 3MM paper and bake for 2 h at 80°C. The nylon membrane may be UV irradiated. Hybridization of probes can proceed as described under Northern blot.

Plasmid DNA Isolation (Minipreps)

Alkaline lysis is the most common procedure used for minipreps.[340,341] This procedure is simple and multiple samples can be run at the same time. Many commercial vendors offer kits based on this procedure. Plasmid DNA is isolated from small amounts of plasmid-containing bacteria. The bacteria are lysed using SDS and NaOH. The SDS denatures the bacterial proteins and the NaOH denatures the chromosomal and plasmid DNA. This solution is neutralized with potassium acetate. The covalently closed plasmid DNA reanneals rapidly. The chromosomal DNA and proteins precipitate and are removed by centrifuging the sample. The plasmid DNA is recovered from the supernatant by ethanol precipitation.[342]

Reagents for DNA Isolation:

1. Luria–Bertani (LB) media with an appropriate antibiotic (i.e., selective for your plasmid).
2. Cell resuspension buffer: 25 mM Tris–Cl (pH 8.0) buffer containing 10 mM EDTA and 50 mM glucose. Sterilize and store at 4°C.
3. Cell lysis buffer: 0.2 M NaOH, 1% (w/v) SDS (prepare immediately prior to use).
4. Cell neutralization buffer: 5 M potassium acetate (pH 4.8): start with 29.5 mL glacial CH_3CO_2H and add KOH pellets to pH 4.8. Add H_2O to 100 mL. Do not autoclave. Store at room temperature.
5. 95% and 70% C_2H_5OH (v/v).

Inoculate a single colony in 5 mL of LB^+ antibiotic medium and grow overnight. Spin down 1.5 mL of the cells in a microcentrifuge for 20 s at maximum speed. Remove the supernatant and add 100 µL of the cell resuspension buffer. Incubate for 5 min. (It is important that the cells are completely resuspended.) Add 200 µL of the cell lysis buffer and mix by tapping the tubes (by finger). Place tubes on ice for 5 min. Next, add 150 µL of cell neutralization buffer and mix. Incubate on ice for 5 min. Precipitate the chromosomal DNA and cellular debris by spinning in microfuge for 3 min. Transfer the supernatant to a new tube and add 0.8 mL of 95% C_2H_5OH (v/v). Incubate for 2 min at room temperature. The DNA is precipitated by microcentrifuging for 1 min. Carefully remove the supernatant and add 1 mL of 70% C_2H_5OH to wash the pellet. Dry the pellet under vacuum. The DNA pellet may be resuspended in 30 µL of sterile H_2O and stored at –20°C or –70°C. Some procedures call for storing the DNA in TE buffer (10 mM Tris–HCl [pH 8.0] containing 1.0 mM EDTA); however, TE can interfere with sequencing reactions. The DNA concentration can be determined by measuring A_{260}: a solution with an A_{260} of 1.0 contains 50 µg mL^{-1} of DNA (1 cm pathlength).[343] Plasmids can be maintained for a short amount of time (2–4 weeks) on selective media plates by storing them at 4°C. Permanent storage should involve storage of the isolated plasmid in addition to storage of the bacterial strain. Bacterial strains can be stored be growing the cells to saturation in the presence of the selective antibiotic. An equal volume of bacteria is added to sterile 100% glycerol and quick frozen in liquid nitrogen (*glycerol stocks*). Cells may then be stored at –70°C. For recovery, simply streak out a sample on selective media and grow.

Polymerase Chain Reaction

The basic principles of the method were developed in 1986 and are relatively straightforward.[344] The process provides the opportunity for considerable innovation in the application to a wide variety of research problems. The reader is advised to consult the current literature, in that only the basic points are mentioned here. The double-stranded DNA is heat denatured, and the two primers complementary to the ends of the target segment are annealed at a temperature close to the temperature of the oligomers and then extended at a temperature optimal for the polymerase used. One set of these three consecutive steps is referred to as a cycle. Thus, the amount of the original sequence of interest is expanded in a geometric progression. The process is greatly facilitated by the use of heat-stable polymerase isolated from a thermophilic bacterium; such polymerases function most effectively at high temperatures, for example, 72°C–78°C. Many cycles can be carried out with the same enzyme. The cycle times are relatively short and commercial instruments can be programmed to cycle through steps automatically. DNA segments can be amplified 10^5- to 10^9-fold. Heat-stable polymerases of very high fidelity are available and reduce errors.

A typical PCR would be performed on a 100 µL scale in 0.5 mL sterile thin-walled microcentrifuge tubes. There is considerable potential to generate false positives and it is imperative to minimize laboratory errors. Reagent purity is important, and avoiding contamination at every step is crucial. Carryover of amplified sequences contributes to the majority of false positives; thus, appropriate precautions include physical separation of pre- and post-PCR and aliquoting reagents to minimize the number of repeated samplings. Positive placement pipettes are the best choice for PCR work. Some other general precautions include changing gloves frequently, careful uncapping of samples to prevent aerosol formation, addition of nonsample components (buffer, nucleotides, primers) to the reaction mixture prior to DNA addition, and (absolutely) the use of new pipette tips for every addition.

The PCR sample may be single- or double-stranded DNA or RNA. If the starting sample is RNA, reverse transcriptase is used to first prepare cDNA prior to conventional PCR, that is, RT-PCR. PCR primers are 20–30 base long oligonucleotides that are complementary to sequences defining the 3′ ends of the complementary template strands. Several computer

programs can assist in primer design. Bear in mind that computer design is not foolproof. Utilizing these programs will aid in detecting primer pairs with intra- or intermolecular complementarities (primer dimers). Ideally, 40%–60% G+C content is recommended, without long stretches of either base. Many computer programs will analyze the primer secondary structures and calculate the T_m values. The T_m values for both primers should be well matched. Internal secondary structure (e.g., hairpin loops) should be avoided in primers. Nontemplate-complementary 5′ extensions may be added to primers to allow a variety of useful operations on the PCR product, for example, the addition of restriction enzyme sites, without significant perturbation to the amplification.

Optimal annealing temperature must be determined empirically. A good annealing temperature from which to begin optimizing is 5°C–10°C below the T_m of the primers. Another critical parameter for PCR is $MgCl_2$ concentration needed in the reaction to generate maximum product. Titration of the Mg^{2+} concentration over a range of 1.5–4 mM is recommended to find the concentration producing the highest yield of product (the fidelity of *Taq* polymerase decreases in the presence of high Mg^{2+}). *Pfu* polymerase has a higher fidelity rate compared to *Taq* (Stratagene). This multifunctional, thermostable enzyme possesses both 5′–3′ DNA polymerase activity and 3′–5′ exonuclease activity. This latter activity results in a 12-fold increase in fidelity over *Taq* polymerase. A typical PCR (performed in 100 µL) would contain dATP, dCTP, dGTP, dTTP (200 µM each), primer (0.25 µM), template (0.1–500 ng), and 10 µL of a stock buffer containing 0.20 M Tris–HCl (pH 8.75), 0.10 M KCl, 0.10 M $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 1.0% Triton-X 100 (w/v), and 1.0 mg nuclease-free bovine serum albumin mL^{-1} (w/v). The tubes are mixed gently and centrifuged briefly. It is important to note that some protocols call for the addition of mineral oil. If the thermocycler being utilized has a heated lid, then mineral oil is not needed in the reaction; add 50 µL of mineral oil if the thermocycler does not have a heated lid. Heat samples to 95°C for 5 min and then cool to the desired annealing temperature to allow the primers to anneal to the template DNA. *Pfu* polymerase (2.5 units) is added. Primer extension proceeds at 75°C. Cycles are then repeated to achieve adequate amplification. PCR products are checked by electrophoresis, with detection by ethidium bromide staining or hybridization to labeled probes as appropriate. An alternative to ethidium bromide staining is SYBR Gold nucleic acid gel stain from Molecular Probes/Life Technologies (Grand Island, NY), which is 25–100 times more sensitive than ethidium bromide, is easier to use, and permits optimization of 10- to 100-fold lower starting template copy number.[345]

Site-Directed Mutagenesis

Site-directed mutagenesis has been defined as "any of various techniques by which defined mutations can be made in vitro in a cloned DNA."[346] The Kunkel method of site-directed mutagenesis is a classic method for introducing mutations into a DNA sequence. This method of site-directed mutagenesis by deoxyuridine incorporation depends on the host strain to degrade template DNA that contains uracil in place of thymidine.[347,348] A number of dUTPs are incorporated into the template strand in place of dTTP in a host that lacks dUTPase (i.e., it is *dut*$^-$) and uracil *N*-glycosylase (i.e., *ung*$^-$) activities. Uracil itself is not mutagenic and base pairs with adenine. Under normal cellular conditions, dUTPase degrades deoxyuridine and uracil *N*-glycosylase removes any incorporated uracil. Postmutation replication in a *dut*$^+$*ung*$^+$ *E. coli* strain is used to then degrade the nontarget strand DNA. This approach requires single-stranded DNA be utilized so that only one strand contains the uracils that are susceptible to degradation.

A more convenient method for site-directed mutagenesis is based on PCR using the selection method of DAM methylation. This is commonly sold in a commercial kit available from Stratagene known as QuickChange™. However, the procedure can be accomplished without the purchase of a kit, and everything needed is available individually. There is no need to isolate single-stranded DNA. The entire process is PCR based, utilizing *Pfu* polymerase. After the PCR has finished, the products are digested with the restriction enzyme *Dpn*I. This restriction enzyme is specific for DAM-methylated $G^{Me6}ATC$ sequences.[349] The wild-type template will be digested by *Dpn*I and the PCR-generated mutant DNA will not be cleaved by this enzyme. Therefore, the reaction is enriched for the mutant DNA population. Multiple mutations can be introduced using the *Dpn*I method. A selection feature one can incorporate through the primer design is the addition of other unique restriction enzyme sites (e.g., unique to the DNA sequence of interest). This method works well with plasmids of moderate size (<8 kb) and eliminates the need for subcloning.

Another type of mutagenesis is cassette mutagenesis. In the basic form of cassette mutagenesis, a small section of DNA is removed from the wild-type gene and is replaced with a synthetic segment that carries one or more desired mutations.[350]

Cassette mutagenesis can also be utilized to produce multiple mutations within a target zone. One efficient way to generate the pool of oligonucleotides is to synthesize one strand of the cassette with equal mixtures of all four nucleotides in the first two positions and an equal mixture of G and C in the third.[351] This composition will result in all 20 amino acids.[352] The second strand for the cassette is synthesized with inosine at each target position. Inosine will base pair with all four bases.[353] The two strands are annealed and the population of cassettes is ligated into a vector.

GENOTOXICITY ASSAYS LINKED TO METABOLISM

One use of bacterial P450 systems is the development of convenient genotoxicity assays. This area has been reviewed elsewhere, including the expression of enzymes in *S. typhimurium*.[193,202] More recently, *E. coli* systems have

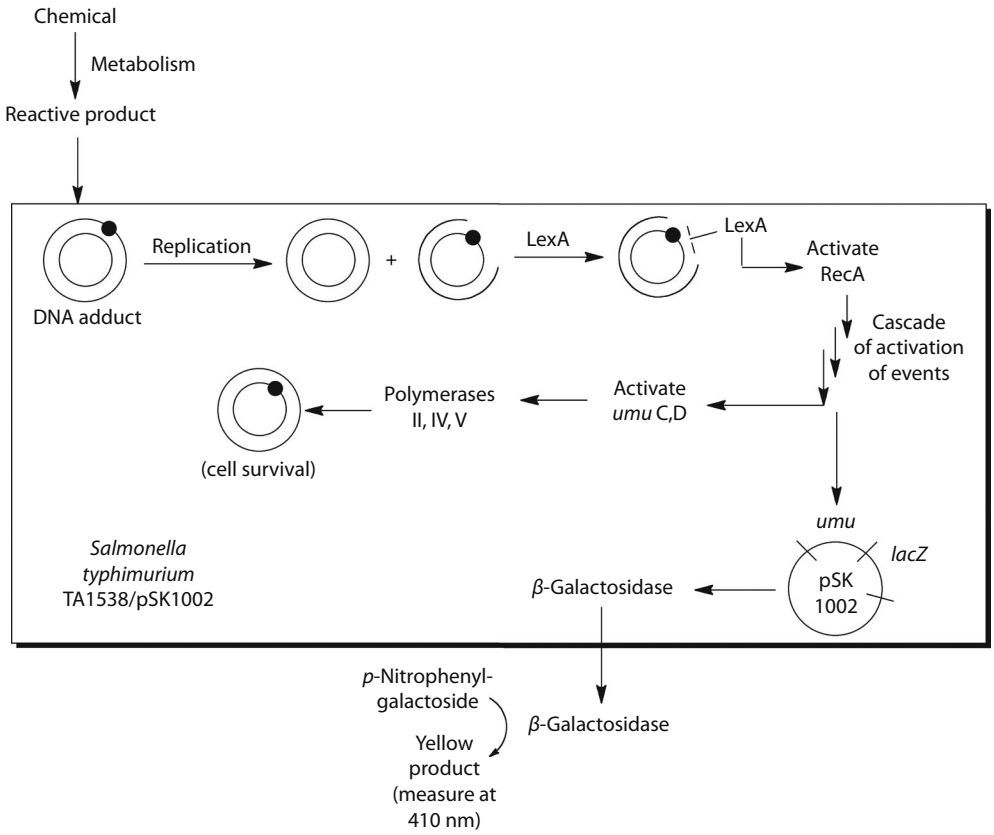


FIGURE 40.33 Use of bacterial SOS response to identify genotoxins with the *umu* response. A chemical is incubated with a metabolic system capable of converting it to a reactive product, in the presence of *S. typhimurium* cells containing the plasmid pSK1002. Formation of DNA adducts blocks replication of the modified strand and leaves regions of single-stranded DNA, to which the protein LexA binds. The protease LexA is then activated and cleaves RecA, leading to more events in a cascade and activating >30 genes. One of these is *umuC*, which codes for a translesion polymerase that the bacteria use to bypass DNA adducts and replicate. A reporter plasmid in the cell (pSK1002) contains the regulatory region of the *umu* gene attached to *lac*Z. Thus, generation of DNA adducts has the overall effect of activating this *umu*-based plasmid and producing the products β-galactosidase. The production of β-galactosidase can be quantified by lysing cells and using a colorimetric assay.

been developed where the endpoint is *lac* protrophy instead of the usual *his* in the Ames test. A bacterial *N*-acetyltransferase can be concurrently expressed to increased sensitivity to aryl and heterocyclic amines. Such systems have been shown to respond to concentrations of some aryl amines as low as the classical *S. typhimurium* systems.[176] These systems offer several advantages. In other work, it has been possible to express rat and human GSH transferases along with *N*-acetyltransferase in *S. typhimurium* for use in genotoxicity assays.[201,354,355] More recently, several human P450s have been coexpressed with NADPH–P450 reductase in *S. typhimurium*.[356] These systems have considerable potential, in that bacterial systems will continue to be the mainstay of high-throughput, first-check genotoxicity screens. In these systems, reactive products are generated with the cells and more closely approximate the normal cellular situation.

Some examples are the *S. typhimurium umu* test (or the similar *E. coli* chromotest) in which the reporter gene is linked to a promoter that responds to a cascade emanating from damage to bacterial DNA.[175,357] These systems often have faster readout than colony-counting systems (e.g., conventional Ames test) with their colorimetric endpoints, which are often seen within a few hours. The Ames test relies on the reversion of a variety of different *S. typhimurium* mutants with histidine dependent growth requirements to prototrophy by a chemical carcinogen. All of the strains contain two additional mutations that disrupt excision repair and cause loss of the lipopolysaccharide barrier.[358] In the *S. typhimurium umu* test, DNA damage as a result of chemical carcinogenesis invokes the SOS response (Figure 40.33). The SOS response initiates proteolytic cleavage of the LexA protein by the activated RecA protease and ultimately results in β-galactosidase activity that can be colorimetrically quantified.[175] Briefly, a chemical is incubated with a metabolic system capable of converting it to a reactive product, in the presence of *S. typhimurium* cells containing the plasmid pSK1002. This can be liver microsomes or a recombinant P450 system expressed within the cells. Formation of DNA adducts blocks replication of the modified strand and leaves regions of single-stranded DNA, to which the protein LexA binds. The protease LexA is then activated and cleaves RecA, leading to more events in a cascade and activating >30 genes. One of these is *umu*C, which codes for a translesion polymerase that the bacteria use to bypass DNA adducts and replicate. A reporter plasmid

in the cell (pSK1002) contains the regulatory region of the *umu* gene attached to *lac*Z. Thus, generation of DNA adducts has the overall effect of activating this *umu*-based plasmid and producing the products β-galactosidase. Production of β-galactosidase can be quantified by lysing cells and using a colorimetric assay (Figure 40.33). The assay can be done in microtiter plates.

The *E. coli* chromotest is very similar although unlike the previous examples, this system utilizes only one strain and also relies on the cell SOS response system. In this case, cell division is tied into the assay; thus, survival of the host is not important.[359] The *E. coli* strain has the same mutation that renders the strain lipopolysaccharide deficient, allowing for better diffusion of chemicals through the outer membrane.[360] Several steps occur between the reaction of a carcinogen with DNA and a resulting mutation.

In this laboratory, we have recently utilized two different systems to study DNA repair. One system is the human p53-driven *Ade* reporter system in the yeast *S. cerevisiae*. Cells with the wild-type p53 human tumor suppressor gene express *Can1* and these cells are able to take up canavanine, which is cytotoxic and limits growth on selective media. Cells with mutant p53 are unable to express *Ade2* accumulate an adenine metabolite intermediate and the colonies are red in color.[361]

The second system utilizes resistance to the antibiotic rifampicin in *E. coli* through mutations in the *rpoB* gene as a result of carcinogen exposure.[362,363] Utilization of this system enabled the identification of the direct inactivation of O^6-alkyguanine DNA alkyltransferase (AGT) and formation of AGT-Cys^{145}-CH_2-DNA adducts by which methylene dibromide (dibromomethane) can cause genotoxic damage.[363] Additional work resulted in identification of another ethylene dibromide DNA adduct, guanine N^7-alkylation, and a potential second site that was not identified.[362]

CONCLUSIONS

Enzymes have important roles in the metabolism and toxicology of many chemicals. Important examples are known with drugs and with *environmental* chemicals, and the principles are the same. Some background has been presented here, along with some detailed procedures for working with these systems. Obviously, the examples are not comprehensive, and they can be modified to make them more useful for particular needs. The need for better understanding of the biochemical and molecular events involved in toxicology (Figure 40.4) will continue for some time, and we hope that this information will facilitate some of the studies in the area.

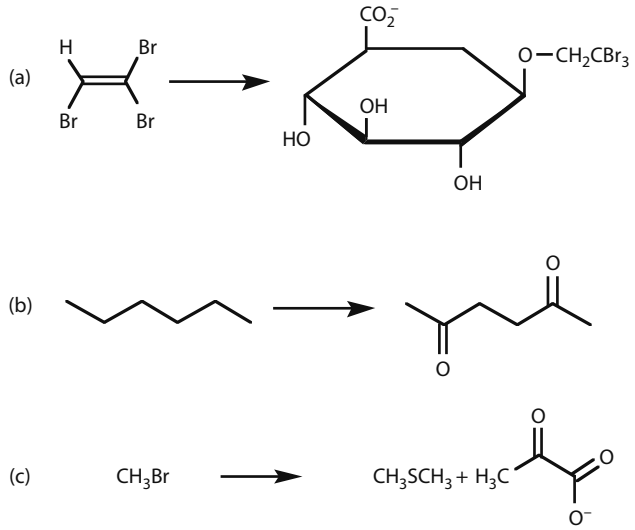
QUESTIONS

40.1 Most assays for competitive inhibition of cytochrome P450 enzymes involve direct studies with assays of substrate oxidation. How could you develop a high-throughput screening strategy for identifying high-affinity inhibitors of a particular cytochrome P450 (e.g., 2D6) without the need to do assays of catalytic activity?

40.2 A new chemical of interest induces total hepatic cytochrome P450 (as measured spectrally) and is hepatotoxic (in rats). Are the two phenomena necessarily related? How would you establish the relationship?

40.3 An investigator tells you that he has found that a particular recombinant human cytochrome P450 enzyme catalyzes a reaction of interest to you. What other pieces of evidence are needed to put this information into the perspective of how much this enzyme contributes to the process in human liver?

40.4 The pathways shown in the following have been extended to demonstrate an example of situations often encountered in practical situations. Provide reasonable stepwise pathways to the products indicated. The number of steps is not specified. For each step, provide the necessary cosubstrate(s) and name of enzyme (if enzymatic, only group name, no need for individual form).



40.5 You have encountered an interesting enzyme in your research and wish to further study its function. This enzyme requires glycosylation for activity. How does this influence your choice of expression systems?

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41 Modern Instrumental Methods for Studying Mechanisms of Toxicology

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CONTENTS

INTRODUCTION

The mechanisms underlying an organism's response to a toxic insult are usually complex, involving the toxicant itself, metabolites derived from it, and numerous tissue-derived endogenous compounds. The ability to monitor the chemical changes that result from intoxication is critical to understanding these mechanisms. Our ability to monitor chemical changes associated with intoxication has often limited our mechanistic understanding of toxicity. The development of gas chromatography (GC) in the 1950s and 1960s extended the lower range for detecting chemical changes in organisms and in the environment. The realization that exposure to chemicals such as DDT was widespread and that these chemicals could concentrate in the food chain fueled the development of modern toxicology. This early breakthrough in instrumental analysis led to the development of a host of powerful instruments and techniques that have profoundly increased our ability to define mechanisms of toxicity. Because each instrumental method has both strengths and limitations, it is important for toxicologists to be aware of available analytical methods and to understand the types of studies for which each is suited.

This chapter is not intended to be a comprehensive survey of modern instrumental methods; rather, it focuses on those techniques that are likely to be most useful to toxicologists. Some of these techniques, such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, are widely used in toxicology. Others, such as near-infrared (IR) spectroscopy, are not as commonly used but have proven extremely useful for toxicological studies. Recent advances in the important areas of in vivo toxicology and metabolomics further underscore the importance of these analytical methods in toxicological research. Emphasis has been placed on practical applications, not in-depth theoretical discussion. This chapter serves as a starting point for further study. The interested reader is encouraged to consult the cited references to gain a more in-depth understanding of modern instrumental methods.

MASS SPECTROMETRY

MS is an analytical technique that determines the mass-to-charge (m/z) ratio of ionized molecules and their fragments and adducts. It is perhaps the most generally applicable tool in chemical analysis and the method of choice for many specific analytical procedures. In addition to its application as a universal detector in qualitative analysis, MS can be fine-tuned to quantify trace amounts of specific components in complex mixtures. Mass spectrometers are often part of a hyphenated analytical system where chromatography (usually gas or liquid) precedes mass spectrometric detection. MS has impacted biomedical, environmental, and toxicological research, including the study of toxic substances and their fate in the body. Considerable progress has been made in toxicology using MS as an analytical tool. This section looks at the basic principles of MS, new trends in the field, and some examples where MS has been applied to toxicology studies such as elucidation of detoxification mechanisms and exposure assessment. This is not a comprehensive review of recent breakthroughs in the area as much as it is a starting point for the curious student of the art. A good text covering routine techniques in MS with consideration of biochemical applications has been written by Boyd et al. [21], and Richardson has recently reviewed applications of MS specifically related to environmental toxicology [163].

Mass Spectrometers and Mass Spectra

There are many different types of mass spectrometers [49], but certain features are common to them all. Ions, unlike neutral compounds, can be manipulated by electromagnetic forces such that a separation by m/z ratio is possible. A mass spectrometer is a device that creates ions from sample molecules, separates them by m/z ratio, and determines the number of ions at each particular ratio. The mass spectrum is a plot of m/z ratio on the x-axis vs. relative ion abundances (RA) on the y-axis. The mass spectrum can give molecular weight and structural information about the compound that was ionized. Ions are normally produced with single charges ($z = 1$), so the m/z ratio gives the mass in daltons (Da) of intact molecules and fragments directly. A simple model of a molecule AB undergoing electron ionization (EI) is shown in the following:

$$AB \rightarrow \text{Ionization process} \rightarrow AB^{+\bullet} + A^{+} + B^{+}$$

The ionization process in this case produces a molecular ion $AB^{+\bullet}$ (usually designated as $M^{+\bullet}$) that is the sample molecule with an electron removed. Often, enough energy is imparted in the process so that a portion of the molecular ions decompose to form various stable fragment ions as shown by A^{+} and B^{+} earlier. The spectrum is highly characteristic of a particular compound and has been likened to a fingerprint that can be

used for identification purposes. Mass spectra of compounds with known structures are continuously reported and compiled, so patterns of fragmentation are established following EI. This knowledge is useful in interpreting spectra of unknown compounds. In addition, computerized searches of mass spectral databases can facilitate compound identification. The sensitivity of conventional mass spectrometers permits good spectra to be obtained on less than 10 ng of material, so, although the ionization process destroys the sample, the mass spectrometer requires very small amounts of material for analysis.

Instrument Design

Basic Configuration

The many different types of mass spectrometers vary in size and complexity from small benchtop units to large multiple-sector analyzers. Nevertheless, mass spectrometers can all be broken down into a few basic components and their functions: sample introduction, ion production, mass analysis, and ion detection. The ion source configuration is dependent on the physical state of the sample matrix entering the mass spectrometer. Ionization sources have been developed for gas, liquid, or supercritical fluid inlets. Electron ionization (EI) sources have been used for gas and liquid inlets and must be under vacuum. Atmospheric pressure ionization (API) techniques are available for liquid and supercritical fluid inlets. These components are shown schematically in Figure 41.1.

Inlet Systems

Samples are introduced into the mass spectrometer housing via some type of inlet. The inlet selected depends on the sample, the nature of the sample matrix, and the type of ionization desired. Many mass spectrometers are designed with multiple inlets to accommodate a wide variety of samples with minimal time required for instrument reconfiguration. For solid materials, the simplest means of introduction is to place the sample onto a probe or surface that is inserted directly into the ion source or atmospheric pressure source such as electrospray. For samples that are thermally stable, a probe containing the sample can be placed directly into the EI source. Analytes that occur in mixtures may require some separation before direct probe analysis. Although some separation of components in time is achieved by heating the probe slowly, it is a crude means of separation and works best on reasonably pure samples. Headspace sampling is often used as a means of introducing volatile analytes onto a GC column and offers the advantage of leaving nonvolatile components behind. This technique also allows for portable sampling and analysis in the field or in the laboratory [78,226]. Probes are also used in conjunction with other ionization techniques where they serve merely to place the sample in a position to be ionized. Examples of this would be *laser desorption* (LD), desorption electrospray ionization (DESI), and direct analysis in real time (DART), which are described later in this chapter.

Chromatographic separation techniques coupled to the source add another dimension of analysis, and most mass spectrometer systems used in biological sciences are configured in this manner. GC-MS has worked very well in this regard, making GC-MS perhaps the most common hyphenated method for performing organic analysis [22]. Although applications with packed GC columns are still reported with various types of interfaces, most GC-MS is performed with fused silica capillary columns. These columns use gas flows of 1–2 mL/min, and the ends can be placed directly into the ionization source. High-resolution chromatography of reasonably volatile and thermally stable samples is possible with these columns.

Many compounds will not pass through a gas chromatograph, and *liquid chromatography–MS* (LC-MS) has been

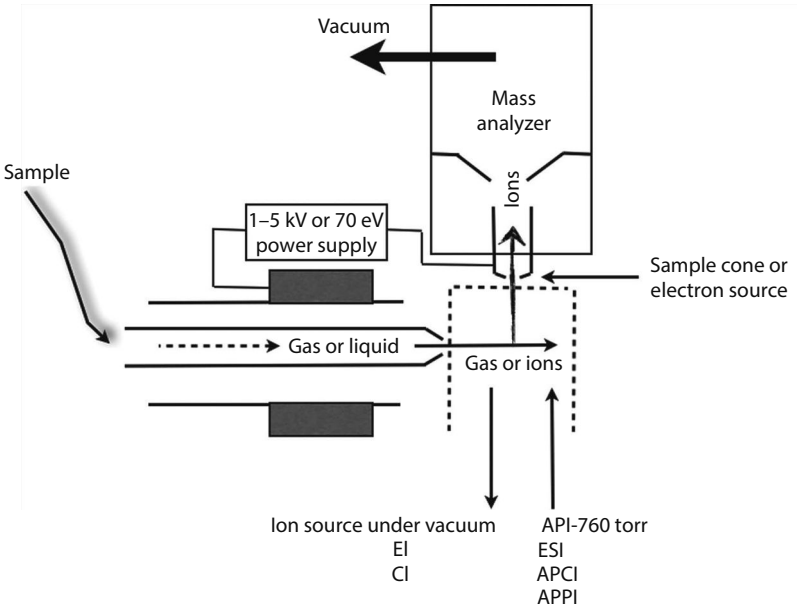

FIGURE 41.1 Schematic of a basic mass spectrometer system interfaced to an inlet. API and EI are both represented here.

developed as an alternative method for introducing analytes dissolved in solution into the mass spectrometer. In fact, because biological samples are usually aqueous based and include thermally labile and polar substances, LC-MS now rivals GC-MS in popularity. This is due to the development and refinement of many reliable interfaces in the last two decades. *High-performance liquid chromatography* (HPLC) methods using a variety of columns and flow rates have used MS as a detector. General procedures are well documented for LC-MS techniques [217].

Another chromatographic technique used with MS is *capillary electrophoresis* (CE) [196]. A high electric field in a small-diameter capillary tube filled with aqueous solution is used to separate charged compounds. CE offers the advantages of high resolution, low sample consumption, and short analysis times. It is well suited to the analysis of ions in solution. Interfacing to the mass spectrometer is often accomplished through electrospray ionization (ESI) or continuous-flow fast atom bombardment, which are described in the succeeding text. Due to the high salt concentration required for effective CE, coupling CE to MS remains a challenge and these two techniques are rarely used together.

Supercritical fluid chromatography has been coupled to MS and proven to be a powerful technique for resolution of closely related isomers, including enantiomers [32,188].

Ionization Sources for Volatile Compounds

This discussion of ionization sources is divided into two parts based on the volatility of the compound of interest. It might well be divided into the same parts based on the two popular chromatography methods interfaced to mass spectrometers: GC and LC. Because MS was originally limited to volatile samples, these types of ionization sources are considered conventional. Volatile samples are introduced directly into the ion source using a leak valve for gases, a heated direct insertion probe described earlier for solids, or a GC. This section describes methods used in these types of applications.

The most established means of ionization for volatile samples is EI, in which a sample is volatilized into the gas phase and passes through a beam of energetic (70 eV) electrons boiled from a filament. The process may be written as

$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

The high-energy electron displaces an electron from molecule M, which may remain intact as the molecular ion $M^{+\bullet}$ and thus provide direct molecular weight information. The energetics of the process can also cause fragmentation of the molecule. Fragmentation patterns are related to the structure of the molecule and thus can be interpreted as representative of that compound. As an example, Figure 41.2 shows the EI mass spectrum of nicotine identified in an extract of urine from a smoker. The highest mass ion in the spectrum is *m/z* 162, which is the molecular ion. The most abundant peak in the spectrum (referred to as the base peak) is *m/z* 84 and

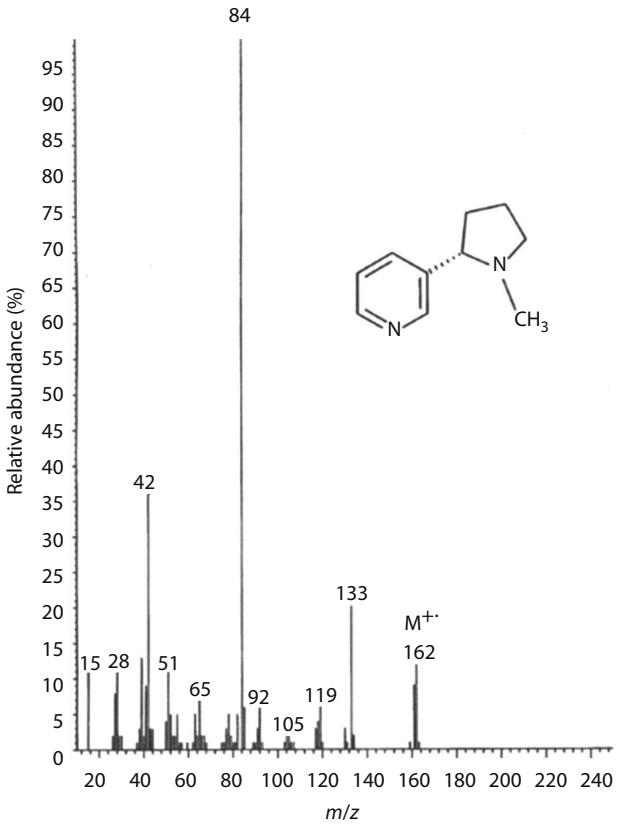

FIGURE 41.2 EI mass spectrum of nicotine. Data acquired on a quadrupole GC-MS system.

results from cleavage of the bond between the two rings with the charge remaining with the pyrrolidine ring.

EI has several advantages. Both fragment and molecular ions are produced in most EI spectra. The mass spectra are fairly reproducible from one instrument to the next, which makes it possible to match sample spectra to reference EI mass spectra with some certainty. Several large mass spectral databases of compounds are available that can be readily searched against an EI spectrum produced on most types of mass spectrometers. Some disadvantages of EI include the occasional lack of a molecular ion for some compounds, the difficulty of distinguishing between mass spectra of isomers, and limited application to samples with sufficient gas-phase volatility and thermal stability.

An alternative ionization method called *chemical ionization* (CI) can be used in cases where the molecular ion is weak or not present in the EI mass spectrum. The process derives its name from the use of gas-phase chemical reactions to produce ions from the sample. The two types of CI are *positive-ion chemical ionization* (PICI or PCI) and *negative-ion chemical ionization* (NICI or NCI). In both cases, the ionization is softer than EI, resulting in less fragmentation. PICI is most often accomplished through a gas-phase proton transfer reaction. The ion source is flooded with a reagent gas, usually methane, at a relatively high pressure (1 torr). During electron bombardment under these conditions, a series of gas-phase reactions occur

as depicted in the following for reagent gas methane and sample molecule M:

$$CH_4 \rightarrow CH_4^+$$

$$CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3$$

$$CH_5^+ + M \rightarrow CH_4 + (M+H)^+$$

Methane molecular ions formed in the high-pressure source collide with neutral reagent molecules (CH_4) and produce protonated methane. CH_5^+ acts as a strong Brönsted acid and transfers a proton to the sample molecule to produce the protonated molecular adduct $(M + H)^+$. PICI works best for samples with a relatively high proton affinity such as those containing a heteroatom. Fewer fragment ions and more abundant molecular ions characterize PICI mass spectra. In addition, sensitivity is enhanced relative to EI. The higher source pressure used, however, contaminates the instrument more rapidly and results in more frequent maintenance. Also, some modifications to the source are usually required when switching from EI to CI operation:

$$M + CH_4 + e^{-\bullet} \rightarrow M^{-\bullet} + CH_4$$

This process is rather selective, as not every molecule will readily form a stable $M^{-\bullet}$. Another process is adduct formation where a background anion such as Cl^- will attach to a molecule to form $(M + Cl)^-$. Numerous ion–molecule reactions are possible, and some can be rather complex. Proton abstraction is common and works well for samples with an acidic proton. Negative-ion formation can be enhanced using derivatization to form an analog with a high electron affinity. The sensitivity and selectivity of such assays can be very high; for example, one group reports conversion of nicotine using heptafluorobutyric anhydride to a stable electron scavenger derivative that can be detected at the femtogram (10^{-15} g) level on column [37].

Ionization Sources for Nonvolatile Compounds

This section describes ionization sources for compounds that cannot be volatilized sufficiently for EI, particularly those that decompose upon heating. These include very polar or very large molecules such as those encountered in biological samples. For these types of samples, LC is preferred over GC for chromatographic separation. A number of interfaces deal with samples in solution such as those that emerge from an LC column. Before introduction into the high vacuum of the mass spectrometer, most of the solvent molecules must be removed and the sample molecules ionized. Heat, nebulization with gas, and differential pumping are techniques used to remove the solvent. Ionization of the sample molecules can occur by a variety of different methods as described in this section. Some methods, such as particle beam, have distinct ionization steps, while others, such as electrospray, have ion formation inherent in the process. With the exception of particle beam, LC-MS ionization methods are soft like CI and produce mostly molecular adduct ions with gain or loss of a proton being the most common means of ionization.

Early development of these LC-MS interfaces was designed for low flow rates (i.e., 0.05–10 μL/min) because vacuum system was not capable of efficiently removing solvent when higher flow rates were employed. The early interfaces included thermospray [18], particle beam [12,13], and continuous-flow fast atom bombardment [224]. These earlier techniques required frequent maintenance and suffered from poor sensitivity. These techniques are only mentioned briefly here to provide the reader with a historical perspective. The exponential growth in MS as a routine detection methodology was made possible by the development and commercialization of API techniques: ESI, atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). A discussion of these ionization interfaces follows.

A widely used method of API, ESI, is simply an existing ion from solution into the gas phase by using a high electric field [213]. For Figure 41.1, the ESI interface is under high vacuum starting at the sample cone. The LC effluent passes through a capillary needle that is maintained at a high voltage (1–5 kV). At low flows (1–10 μL/min), the high electric field at the tip produces a mist of charged droplets at atmospheric pressure. For higher flow rates (i.e., 50–1000 μL/min), a nebulizing gas delivered coaxially to the needle assists production of the mist. As evaporation decreases droplet size, ions are ejected as depicted for sample molecules M and solvent molecules S in Figure 41.3. Of course, the analyte must already

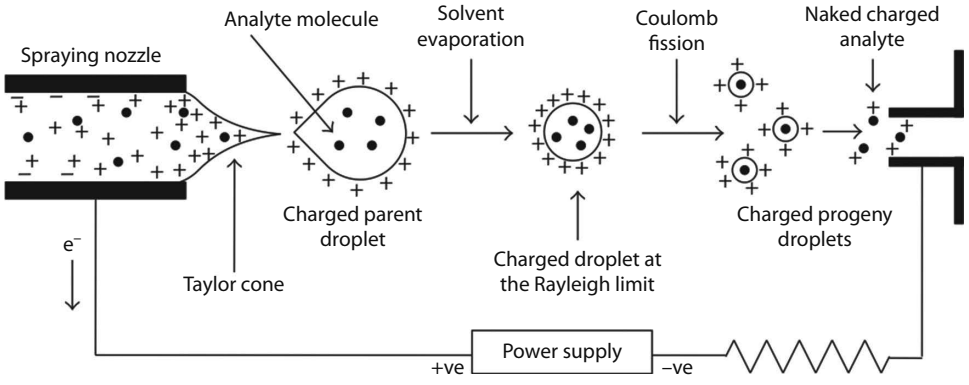

FIGURE 41.3 The ESI process.

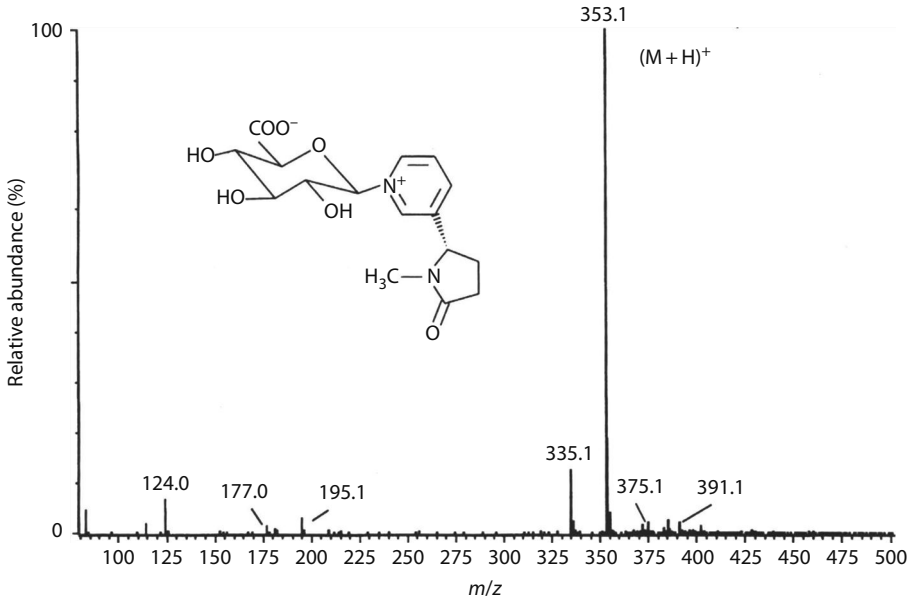

FIGURE 41.4 ES mass spectrum of cotinine-*N*-glucuronide taken with a quadrupole mass analyzer.

exist as an ion in solution, but this is easily accomplished by adjusting the pH. Positive ions are shown in the figure, but the process works equally well for negative ions. The ions are swept through a sampling cone and passed through a differential pumping system to remove solvent and nebulizing gas molecules before the ions enter the mass analyzer through a tiny aperture. ESI is a very gentle ionization method that can be used with either small or large molecules. Figure 41.4 shows an ESI spectrum of the glucuronide conjugate of cotinine, a phase II metabolite of nicotine. This thermally labile compound produces $(M + H)^+$ as the base peak at *m/z* 353. An interesting feature of ESI is the ability to produce ions with multiple charges. Because mass spectra are plotted with *m/z*, this permits the available mass range of the analyzer to extend to thousands of daltons; for example, a compound with $(M + H)^+$ at *m/z* 5000 would have $(M + 5H)^{+5}$ at nominal *m/z* 1001. This feature is less useful for small molecules such as common pharmaceuticals and their metabolites but is very useful for macromolecules such as proteins. ESI works well with aqueous mobile phases and some organic solvents.

APCI shares the same atmospheric pressure interface as ESI but uses a different probe for producing ions [81]. Instead of the capillary needle shown in Figure 41.3, APCI introduces the LC effluent to a heated tube (400°C–550°C), where the solvent and samples are volatilized at atmospheric pressure. A nebulizing gas (nitrogen) is used to assist with volatilization. A discharge needle after the heated tube creates plasma where reagent ions and electrons are produced. CI occurs in the gas phase to produce protonated solvent ions and this proton is transferred to the analyte. Therefore, analyte ionization is preferred when the solvent is a stronger acid than the analyte. APCI is rugged, reliable, and very sensitive due to efficient ionization, especially for molecules with heteroatoms. The rapid heating does produce some thermal degradation of labile species. APCI can accommodate analytes that are less polar than those suited for ESI and HPLC flows of 2 mL/min and works well with most types of solvents.

Direct APPI or dopant-assisted APPI has gained popularity due in large part to favorable ionization of less polar analytes, when compared with that typically achieved with ESI or APCI. It was first described by Jorgenson and coworkers with an interface to open tubular LC [46] and later adapted for use with traditional LC by Robb et al. [164]. A proposed mechanism for ionization with APPI is illustrated as follows:

$$\text{Dopant} + \text{hv} \rightarrow \text{Dopant}^{+\bullet} + e^-$$

$$\text{Dopant}^{+\bullet} + n[\text{solvent}] \rightarrow [\text{dopant} - \text{H}] + [(\text{solvent})_n + \text{H}]^+$$

$$[(\text{solvent})_n + \text{H}]^+ + \text{M} \rightarrow n[\text{solvent}] + [\text{M} + \text{H}]^+$$

The dopant is a compound with an ionization energy (I_1) less than $h\nu$ (10.6 eV), while the solvent or mobile phase has ionization energy greater than $h\nu$. Toluene (I_1 = 8.8 eV) is typically used as the dopant due to its relative safety and compatibility with material components of the MS system. Methanol and acetonitrile have favorable proton affinities and have been found to work well with APPI interfaces as well as begin versatile organic modifiers for reversed-phase LC [93].

DESI is an exciting ionization technique that eliminates the need for a bulk solvent but uses a traditional ESI probe [36,112]. The DESI experiment is performed by spraying a stream of charged solvent particles across an analyte surface while collecting the deflected droplets in a portable, charged wand, which serves as an atmospheric pressure ion transfer inlet into the MS. In this way, analytes may be collected from the surface of an object, such as a briefcase, a floor tile, or an article of clothing, which is suspected of being contaminated

with toxins, drugs, or other materials of interest. The portability and ease of sample handling make DESI a particularly promising technique for remote environmental testing, field forensics, and counterterrorism. Growing interest in these toxicological disciplines continues to foster the development of portable field MS units for remote sensing.

For all of these interfaces, an important consideration in coupling an HPLC method to a mass spectrometer is mobile-phase compatibility. Popular LC-MS mobile-phase solvents are water, methanol, and acetonitrile. They are good solvents for a wide range of samples, and their low molecular weights reduce background ions in the mass spectrometer. Organic modifiers such as triethylamine, which are often used to improve chromatography, should be avoided as they can suppress ionization of sample molecules. Although many of the interface/ionization sources listed here work best with a buffer at low concentrations (<20 mM), it must be a volatile organic buffer such as ammonium formate or ammonium acetate, or simply formic acid, which will not leave deposits that block the apertures leading to the mass analyzer. When a basic mobile is warranted, ammonium bicarbonate (5–10 mM) is very effective.

Other Ionization Sources

The bombardment of solid or liquid surfaces with intense light from a laser offers a soft means of ionizing nonvolatile, thermally labile compounds. This technique is called LD. In *matrix-assisted laser desorption ionization* (MALDI), the sample is suspended in a matrix material that absorbs light near the wavelength of the laser. Absorption of the energy desorbs and ionizes the analytes in the matrix, resulting in molecular adducts such as $(M + H)^+$ or $(M + Na)^+$ with little fragmentation. MALDI is used with isolated samples rather than online chromatography and has been used to successfully distinguish various bacteria when combined with high-resolution MS [97].

In addition to organic and bioanalytical analyses, MS used for inorganic analysis figures prominently in toxicology [8]. Unlike organic MS, inorganic MS is mostly concerned with atomic ions instead of molecular ions; thus, methods have been developed for producing atomic ions of various elements from samples. A common ionization method used in inorganic MS is *thermal ionization* that takes place when an atom or molecule interacts with a heated surface. Samples are deposited directly on a filament and heated. Another method is the use of *inductively coupled plasma* (ICP), where the sample is volatilized, atomized, and ionized in a few milliseconds at plasma temperatures of 7000–8000 K.

An interesting field of expanding applications is *accelerator mass spectrometry* (AMS). Unlike the ion sources described previously, AMS is a high-energy (MeV) nuclear physics technique that uses a Van de Graaff accelerator to measure very small amounts of rare and long-lived isotopes [175]. For ^{14}C analysis, the sample is converted to graphite powder by oxidation followed by reduction. A cesium ion gun bombards the sample, and the negative carbon ions produced are selected from the source and accelerated at 3–10 MeV. They pass through a thin foil or gas where electrons are stripped from C^- to create positive ions. These ions are then accelerated to several MeV, selected by a mass analyzer (quadrupole or magnet), and detected. AMS was first applied to geochemical and archaeological samples but has seen growing biomedical applications, particularly in the monitoring of ^{14}C-labeled drugs. The high sensitivity of this method (10^{-18} mol) permits low doses of labeled materials to be monitored in humans. Given its high sensitivity and specificity, AMS has been particularly useful for in vivo studies involving microdosing of xenobiotics [106]. Because the sample is prepared in a manner that is unique to any other MS-based analysis and the LC is offline from the mass spectrometer, a unique validation procedure has been developed [74].

Mass Analyzers

Mass analysis is the process by which a mixture of ions is separated according to their *m/z* ratios. Several types of mass analyzers are commonly used, and these form the primary differences between various mass spectrometer systems. Each analyzer uses electric or magnetic fields or both for separating ions. Although many experimental combinations and variations are available, this section briefly describes the popular commercial models and the basis of their operation.

The basic modes of analysis for mass spectrometers are *repetitive scanning* and *selected ion monitoring* (SIM). In repetitive scanning, a mass range is covered in a fixed interval and constantly repeated; for example, a GC-MS system might be set to scan repetitively *m/z* 40–400 in 1 s. This would detect any compound producing ions in this mass range every second, which is necessary for a high-resolution separation technique such as capillary GC. The resulting plot of all responses vs. time is a *total ion chromatogram* (TIC). Wide-range repetitive scanning is very useful for screening samples such as body fluid extracts for all types of compounds present. The mass range may be adjusted to detect only signals of interest or to exclude interferences such as low-molecular-weight solvents. This procedure offers less sensitivity than SIM but provides much more information. In addition, ion chromatograms can be produced from the stored data file. Ion chromatograms are reconstructed responses for a particular ion and are useful in quantitation to exclude interferences in complex chromatograms. For quantifying specific analytes, it is useful to restrict the ions detected to those of interest. Operating in the SIM mode, where the analyzer scans discontinuously, rapidly switching between only a few ions does this. Because more dwell time is spent on these ions, sensitivity is increased on the order of 10–100 times. This technique ignores ions from background materials and is useful in quantifying analytes in fairly complex matrices. Multiple-stage analyzers are capable of several more modes of scanning, and these are described later in a separate section.

The most popular analyzer system used today is the quadrupole mass analyzer. These compact systems are relatively inexpensive and durable and can scan very rapidly. The quadrupole assembly consists of four parallel rods arranged

equidistant around a central axis. Direct-current (dc) and radio-frequency (RF) voltages are applied to opposite pairs of rods to form a fluctuating electric field. Ions formed in the source are directed and focused into this field by electrostatic lenses or a series of parallel plates that generate a square wave. Only ions with a particular *m/z* ratio as determined by the dc and RF field strength can pass through to the detector; thus, the analyzer scans for particular ions by ramping these parameters. Typically, a full mass spectrum of *m/z* 40–400 can be taken in less than 1 s with good sensitivity. After ions are converted to electrons, a detector is located at the end of the quadrupole assembly.

By using quadrupole analyzers in series (*multiple-stage quadrupoles*), tandem MS is possible. Although sector instruments with magnetic and electrostatic fields can be used as stages, quadrupoles are more often used for tandem MS. These systems operate by selecting a particular ion from the first-stage analyzer and sending it into a second stage where the ion collides with an inert gas (such as argon) to produce further fragmentation. A third stage analyzes the resulting fragments, thus producing a mass spectrum from an ion in a mass spectrum. This technique is referred to as *MS/MS* or *collision-induced dissociation* (CID) and is often used with soft ionization such as CI, ESI, APCI, or APPI to produce fragments from molecular adducts. An example is given in the following for a molecular adduct produced by ESI:

Source	Analyzer 1	Collision Cell	Analyzer 2
$[M + H]^+ \rightarrow$	$(M + H)^+ \rightarrow$	Argon (eV)$\rightarrow$	A^+, B^+, C
	(Precursor)		(Products)

The selected ion is usually referred to as the *precursor* or *parent*, and its fragments as *products* or *daughters*. Precursors and products can be linked to establish fragmentation pathways, thereby producing additional structural information. MS/MS (MS^2) systems can also be set to monitor specific fragmentation pathways, a process known as *multiple reaction monitoring* (MRM). Setting the mass analyzer for a specific transition creates a very specific detector that can monitor an analyte in very complex samples. In this regard, it is analogous to SIM. Although the cost of an MS/MS system is more than for a single-stage mass spectrometer system, the increased structural information and selectivity of these instruments are very attractive. They have become workhorse systems for characterizing and quantitating metabolites or pharmacokinetic samples, particularly when interfaced to HPLC. Further structural information about an analyte can be gained by MS/MS/MS and can be accomplished using triple quadrupole instruments, if one applies sufficient energy for fragmentation to occur in the source.

Mass analyzers that are also found in analytical laboratories are the *ion trap* [196,217]. These mass analyzers are a type of 3D quadrupole composed of a doughnut-shaped ring and two end caps. Ions are contained in the circular electromagnet by an RF field until swept into a detector by an applied RF voltage–amplitude ramp. Ions are ejected according to their resonance energy, which is related to their mass, and a spectrum is thus produced. A time sequence of events produces ions in the trap and then ramps the RF field to detect specific masses. Both GC-MS and LC-MS ion trap systems are commercially available. When coupled directly to a GC, ions are formed by EI just as for a quadrupole system, and switching between EI and CI modes is simple. For LC-MS, ions are usually injected into the trap from an external source such as ESI. Ion traps are similar to quadrupoles in sensitivity, size, and cost, but they are also capable of MS/MS analysis. MS/MS in the ion trap is performed in a timed series of events by colliding the ions while trapped either with themselves or a collision gas. The product ions are then detected. In addition, MS/MS on the product ions may be performed such that MS/MS/MS/… (MS^n) is possible but not practical unless the sample concentration is sufficiently high. Ions are ejected from the ion trap each time an MS experiment is sampled. This limits the usefulness of ion traps to roughly three (e.g., MS/MS/MS) transitions. For LC-MS interfaces that produce molecular adducts, the ion trap is a less expensive option over the triple-stage systems described previously to perform CID analyses.

Somewhat related to the ion trap but longer in use is Fourier transform MS (FTMS) [90]. Ions are produced by EI in a cubic cell consisting of opposing pairs of trapping plates, transmitting plates, and receiving plates. Ions may also be injected into the source from an external ionization source such as ESI. A high constant magnetic field and electrostatic trapping plates trap the ions formed. Each ion undergoes cyclotron motion at a frequency determined by its *m/z* ratio. All the ions are excited (resulting in increased radius of motion) simultaneously by applying a burst of RF energy over a range of frequencies corresponding to the cyclotron frequencies of the ions. The ions are detected simultaneously by measuring the image current induced on the detection plates of the cell. The frequencies form a beat pattern, and by Fourier transformation, the individual cyclotron frequencies of the ions are determined and the *m/z* values produced. Many operational aspects of FTMS are similar to FT-NMR. High mass resolution (discussed in the following text) is possible with these systems, as is MS^n described for the ion trap previously.

Time-of-flight (TOF) analyzers work on the simple principle that ion velocity is mass dependent [220]. A high voltage sent down a tube accelerates ionized sample ions, and the different *m/z* ratios are separated in time. The arrival time at the detector is based on *m/z*, and a mass spectrum is produced. Naturally, scanning speed is very fast and mass range is large. Coupled with MALDI ionization sources described earlier, they have been applied to a variety of studies in biomedical research that require the mass analysis of very large molecules. TOF mass spectrometer systems have shown a growing popularity recently due to techniques that have improved their performance, including mass resolution. TOF has been interfaced with LC and GC systems, and these systems are often complement each other [72,124,143,151].

High-resolution mass analyzers can measure masses to within a thousandth of a mass unit (0.001 Da) or better.

The utility of this feature is based on the fact that atomic masses, although close to integral values, are not truly integral. Although ^{12}C is assigned 12.000000 Da, ^{16}O is actually 15.994915 Da, ^{1}H is 1.007825 Da, ^{14}N is 14.003074 Da, and so on. Thus, high-resolution mass measurements can lead to the determination of elemental composition; for example, the exact mass of citrulline + H ($C_6H_{13}N_3O_3$) is 176.1030. Using a high-resolution mass spectrometer, its molecular ion would be distinguishable from that of another compound with a nominal *m/z* 176 but different molecular formula such as *3,3-dinitroazetidine-1-carbaldehyde* ($C_4H_6N_3O_5$ = 176.0308). The slightly higher mass for citrulline is due to fewer number of oxygen atoms relative to the latter compound. As detectors, high-resolution mass spectrometers offer more selectivity than low-resolution mass analyzers by monitoring very narrow mass ranges and thus eliminating potential interferences. Quadrupole analyzers are not capable of resolving power much in excess of 0.1 mass units and cannot produce high-resolution spectra. The most commonly used high-resolution system is a double-focusing instrument with an electrostatic sector to first select ions of a specific kinetic energy before analysis by magnetic sector. As mentioned earlier, FTMS and most TOF systems are capable of high-resolution mass spectra.

Ion Monitoring

Conventional mass spectrometer systems (quadrupoles and magnetic sector instruments) focus the resolved beams of ions from the mass analyzer onto a detector. The most common detector is an electron multiplier, which is a series of electrodes. When an ion impinges on the first electrode, it releases a shower of electrons that impact a second electrode and so on. The cascading effect produces gains on the order of 10^6. Such high gain produces sensitivity, so a complete mass spectrum can be produced from a few nanograms or less of material.

Interpretation of Mass Spectra

Interpretation of mass spectra involves correlating the plots of ion abundance vs. *m/z* ratio with structure. For unknowns, it is best performed with all auxiliary information possible regarding a compound such as its origin and preparation, chromatographic behavior, and spectra from ultraviolet (UV), IR, NMR, etc., to assign structure with confidence. In this section, basic interpretation of EI mass spectra is reviewed followed by a discussion of mass spectra produced by softer ionization techniques.

EI Mass Spectra

Fragmentation in EI MS can be complex but rich in information if correctly interpreted. It remains difficult to interpret all features in a mass spectrum, but a few simple rules can be used to glean useful information and there have been recent descriptions of automatic algorithms for interpreting data [174]. The most significant datum in identifying an unknown compound is its molecular weight; thus, assignment of the molecular ion in the spectrum is the most important step in its interpretation. The molecular ion must be the highest mass ion in the spectrum and fragment ions must be logical neutral losses from this ion. Keep in mind that not all compounds will give a molecular ion. Fragmentation peaks in the spectrum result from one or more cleavages that may or may not involve rearrangements. The particular fragments produced are related to the strength and chemical nature of the bonds that held the fragment to the rest of the molecule; thus, an understanding of organic chemistry is useful in assigning fragment ion structures and the associated neutral species lost. EI mass spectra are very reproducible on different instruments, and assistance is available in the form of libraries of mass spectra that are searchable by computer. These are included with most data systems and are very straightforward to use. The data system suggests the best matches for your compound and provides some number indicating its confidence in the assignment. These libraries are useful for quickly identifying known compounds and suggesting identities for others. The ability to create your own library is an option in most data packages.

Soft Ionization Mass Spectra

These mass spectra are those produced by less energetic ionization methods such as CI, MALDI, and LC-MS interfaces such as ESI, APCI, and APPI. They are all marked by the appearance of molecular adducts such as $(M + H)^+$. Other common adducts depend on the sample matrix and may include $(M + Na)^+$ and $(M + K)^+$. When using ammonium buffers in LC-MS, $(M + NH_4)^+$ is common along with adducts formed with solvent molecules such as $(M + H_2O + H)^+$ or $(M + CH_3CN + H)^+$. Also, clusters such as $(2M + H)^+$ may occur, especially when higher concentrations of analyte are present. These spectra serve primarily to verify the molecular weight of the compound.

CID Mass Spectra

Mass spectra produced by MS/MS are greatly influenced by different parameters such as collision energy and collision cell pressure [141]. These conditions can vary from one compound to the next and also from one instrument to the next for the same compound; thus, CID spectra are less quantitatively reproducible than EI mass spectra, yet CID spectra remain extremely useful for qualitative structure elucidation. Common neutral losses such as H_2O for hydroxyl groups $[M + H - 18]^+$, loss of glucuronic acid from a glucuronide conjugate $[M + H - 176]^+$, or loss of sulfur trioxide from a sulfate conjugate $[M + H - 80]^+$ are very informative and complement the soft ionization techniques. Fragmentation mechanisms for CID involve the movement of $2e^-$, which is different from what occurs with EI where $1e^-$ movements cause bond breakage. A recent review by Holčapek et al. offers the toxicologist excellent background material and examples of interpretation of these CID spectra [75].

Experimental Design

With such a variety of instruments and methods available, some thought must be given to designing a mass

spectrometric analysis. This section provides some basic guidance for acquiring qualitative and quantitative information for a sample.

Method Selection

The nature of the sample determines the best approach; for example, if the sample is a large protein, it is unlikely to ionize by EI or pass through a GC. Another consideration is the complexity of the sample. Purified samples may not require chromatography but mixtures do. GC-MS and LC-MS are established techniques that perform separation and mass analysis and can rapidly identify unknowns and assess the relative composition of a sample. Samples that are thermally labile or nonvolatile must be ionized with a soft ionization method such as ESI or APCI. MS/MS techniques are useful for providing structural information and a dramatic improvement in signal to noise ratio (S/N). If high mass resolution is required, the mass analyzer must be a double-focusing sector instrument, an FTMS, or a TOF system.

Qualitative Analysis

Qualitative analysis refers to correctly identifying an unknown substance. Although interpretation of mass spectra has already been discussed, other considerations must be mentioned. Many compounds have similar mass spectra, and a single mass spectrum cannot give certain identification in every case. For example, at least three polycyclic aromatic hydrocarbons have a molecular weight of 252 (benzo(*a*) pyrene, benzo(*e*)pyrene, and perylene), and all have similar EI mass spectra. In these situations, using a separation technique such as GC or LC prior to mass analysis can assist identification. When an unknown gives a matching spectrum and coelutes with an authentic standard, then confidence in its identification is high. When no authentic standard is available, the use of multiple chromatographic techniques and ionization methods can enhance confidence in the identity of an unknown. It should be emphasized that, even though MS is a powerful tool in determining the identity of an unknown, auxiliary techniques outside of MS should be used whenever possible to provide increased confidence in the identification of a compound.

Quantitative Analysis

The number of ions detected in the mass spectrometer is proportional to the amount of material introduced to the source, making quantitative analysis possible. Using standard analytical calibration principles, mass spectrometers are capable of very precise, accurate, and sensitive determinations of analytes. The response of the mass spectrometer is calibrated using a series of standards that contain known amounts of the analyte or analytes of interest. External standard calibration plots are possible but are prone to errors due to instrument response variation through the course of analyzing several standards and samples.

A preferred calibration system is the use of internal standards where a known amount of a reference compound is added to the samples and also to the standards. A response ratio of the analyte to an internal standard is produced, which can partially offset variations in sample preparation and instrument sensitivity. The internal standard should be added to the sample prior to any mixing, extraction, derivatization, enzyme treatment, or chromatography to account for any sample losses during these processes. The instrument is usually calibrated with a series of standards that contain a fixed amount of the internal standard and varying amounts of the analyte of interest. The range of analyte concentrations in the standards must cover the values expected for the samples. A calibration plot can be produced from the response ratio vs. analyte concentration. Analyte concentration can be obtained from this plot by interpolation. Most analysts prefer linear curves because the data are easier to process; however, many analytical software packages are available that will fit curves to nonlinear plots. Good texts are available that provide rigorous coverage of calibration plots, calculation of concentration, and limits of detection and quantitation [21,199].

A special case in quantitative MS is the use of a labeled analog of the analyte as an internal standard in a procedure known as *isotope dilution*. Isotope dilution provides a more accurate and precise means of calibration than either external standards or conventional internal standard methods. The isotopically labeled analog has physical, chromatographic, and mass spectral properties that are nearly identical to those of the analyte and thus compensates in like manner for any losses due to sample preparation or instrument variability [34,56]. Stable isotopes (such as ^{2}H, ^{15}N, and ^{13}C) are preferred to radioactive isotopes because of reduced hazards of handling the samples.

Sample Preparation

Investigations in toxicology involve the analysis of biological samples from in vivo and in vitro sources. Such samples include urine, blood, saliva, tissue extracts, and cell suspensions. The use of specific detection methods such as SIM or MRM coupled with chromatography permits these very complex samples to be analyzed directly with LC-MS; sample preparation is minimal (e.g., filter and inject). As trace analysis becomes important, however, many samples require a preconcentration step prior to analysis so adequate limits of detection can be obtained. Isolation and purification techniques commonly employed are solid- or liquid-phase extraction, preparatory HPLC, thin-layer chromatography (TLC), or a combination of these. GC-MS analyses require the sample to be volatile and thermally stable. If the analyte of interest does not meet these requirements, derivatization of an analyte can assist in its analysis. Derivatization can enhance sensitivity, selectivity, and chromatographic performance in some cases. Thus, it should be considered as an alternative when difficulties with an analyte are discovered. Ideally, the mass spectrum of the derivative will provide an intense molecular ion or unique fragment ion. A common derivatization choice is trimethylsilylation of hydroxyl or amine groups to increase volatility and thermal stability.

Several commercial trimethylsilylating reagents are available such as bis-trimethylsilylacetamide (BSA).

Applications

MS analysis is extensively employed in general toxicological disciplines, such as clinical toxicology, forensics, and metabolomics. This versatile technique is also highly specific, as it has been used for the study of specific toxins, including heavy metals, aromatic amines, natural products, and DNA adduct formers. In the emergency clinical setting, the presentation of a highly intoxicated patient requires prompt blood and body fluid analysis, as one or a number of common intoxicating agents may be involved. Van Hee et al. [200] developed a method for rapidly screening body fluids for a series of intoxicating agents and their characteristic metabolites. A 20 μL sample of blood, plasma, or urine is derivatized with a trimethylsilyl agent, mixed with a 1,3-propylene glycol internal standard, and analyzed by GC/EI MS. γ-Hydroxybutyric acid, a common "date-rape" drug, and several other potential intoxicants and metabolites were readily separated and quantified using this method. These compounds were detectable at body fluid concentrations of less than 1 mg/L, and the entire process, from derivatization to analysis, took less than 30 min.

Metabolomics is the exciting discipline of studying changes in the production and relative concentration of low-molecular-weight endogenous biochemicals and metabolites in living organisms before and after exposure to xenobiotics or pathological processes [4,5,92]. Metabolomic fingerprinting enables the toxicologist to identify likely xenobiotic exposure, monitor environmental stressors, and predict chemical toxicity. Dynamic metabolomic profiles consist of complex mixtures of very similar compounds that are constantly changing in terms of composition and relative amount. Accordingly, the precision and extreme sensitivity of MS are very useful in metabolomic studies, and examples of this application are numerous. Using a targeted metabolomics approach, Wang and coworkers demonstrated a positive correlation between the development of future diabetes and five branched-chain and aromatic amino acids [207]. Targeted metabolomics begins with some knowledge of the disease process so that authentic standards and appropriate stable isotope standards can be used. Analytical methodologies, even LC-MS, lack the selectivity necessary for an untargeted approach. Identification of metabolites with authentic standards should always be used to validate a metabolomic study.

Determination of Toxic Metals Using Stable Isotope Dilution

Cadmium is another toxic heavy metal derived from a variety of industrial and environmental sources. Aggarwal et al. [1] have developed a stable isotope dilution GC-MS method for determining cadmium exposure. Their method determined cadmium in urine by using the chelating agent lithium bis(trifluoroethyl)dithiocarbamate (Li[FDEDTC]), to form the chelate $Cd(FDEDTC)_2$, which was analyzed by GC-MS with EI. $M^{+\bullet}$ was monitored for quantitation. The internal standard used was ^{106}CdO. Cd determination in urine was possible with this method at the 10 μg/L level with good precision and accuracy. The researchers note that the isotope dilution technique provides freedom from matrix effects so that precision and accuracy are not affected by incomplete recovery.

Determination of Aromatic Amines in Human Milk Using GC-MS

Aromatic amines come from several environmental pollution sources and have been associated with breast cancer in humans. The presence of these compounds in human breast milk was demonstrated in a straightforward manner by DeBruin et al. [44] using GC-MS. The method used *solid-phase microextraction* (SPME) to sample the headspace over a heated milk sample. The SPME fiber was then inserted into the injector port of a GC-MS and desorbed onto the column. Aromatic amines identified were aniline, *o*-toluidine, and *N*-methylaniline. Two internal standards, aniline-d_5 and *o*-toluidine-d_9, were added to the milk samples prior to preparation. For quantitation, a quadrupole mass spectrometer was operated in the SIM mode with a base ion for each analyte selected. A qualifying ion was also used to confirm the identity. Using a calibration curve constructed from standards made in bovine milk, the authors quantified the three analytes in milk samples at the sub-ppb level.

Serum Cotinine by LC-MS/MS Using APCI

Serum cotinine is often used as a biomarker of exposure to nicotine. If methods have sufficient limits of detection, exposure to extremely low amounts of nicotine as in environmental tobacco smoke can be assessed. The analysis of low concentrations of analytes in complex matrices may be approached in different ways. One approach seeks to perform extensive sample cleanup to present a relatively clean sample to the instrument. Although this is labor intensive and requires meticulous attention to handling of the sample, it permits the analytical system to operate longer and more reproducibly between servicing. A good example of this approach is the determination of cotinine in serum by the method of Jain and Bernert [86]. They desired to measure exposure to nicotine for large numbers of samples from both smokers and nonsmokers. A rapid, sensitive LC-MS/MS method with APCI was developed for serum cotinine that utilized sample extraction and concentration. Although sample preparation has many steps, the results of this method are impressive. The HPLC method had a retention time of less than 1 min for the analyte and internal standard, which permitted samples to be injected every 2 min. Under routine operation, 100 samples a day were analyzed. A detection limit of 0.05 ng/mL was achieved that was sufficient to monitor serum cotinine in nonsmokers exposed to environmental tobacco smoke.

Sample preparation began with the addition of the internal standard, methyl-D_3-cotinine, to 1 mL serum. The sample was acidified and centrifuged to remove protein. The supernatant was basified and extracted with methylene chloride,

dried, reconstituted for transferal to a microvial, evaporated to dryness again, and finally reconstituted in 20 µL of toluene. For LC-MS/MS analysis, 10 µL was injected onto a 4.6 mm × 3 cm C_{18} column for an isocratic separation with a flow rate of 1 mL/min of 80% methanol and 20% 2 mM ammonium acetate. The effluent was introduced to an APCI source on a triple-stage quadrupole mass spectrometer set to operate in the MRM mode. Ions monitored were *m/z* 177 → *m/z* 80 for cotinine and *m/z* 180 → *m/z* 80 for the internal standard. For a confirming transition for cotinine, *m/z* 177 → *m/z* 98 was also monitored.

The key features of this method were sample extraction and concentration followed by very specific detection. Recoveries were 60%–70%, and most background materials were removed. Reducing the final volume to 0.02 mL resulted in a >10-fold concentration of the sample. Highly specific MRM analysis and focusing on only one analyte reduced chromatography requirements so retention time could be minimal, and a simple isocratic method that did not require equilibration could be used. Thousands of serum samples were analyzed by this method but only for cotinine. The mass spectral parameters of this method could be modified to include nicotine as it could be extracted by this same procedure. Extension to other more polar metabolites of nicotine such as *trans*-3′-hydroxycotinine would be problematic as the method now exists, because they are more difficult than cotinine to efficiently extract into an organic phase.

Determination of DNA Adducts

LC with tandem MS (LC-ESI-MS/MS) is a powerful tool in the analysis of DNA adducts [82,83]. Modified bases can be determined in tissue with excellent sensitivity. The use of stable isotope-labeled standards is necessary to compensate for the loss of analyte during sample preparation and is a critical step in eliminating the matrix effects on the analysis of modified bases by ESI-MS. Ishii et al. have recently described methodology for the determination of lucidin-3-*O*-primeveroside (LuP) adducts of guanosine and adenosine in rats. The animals were fed a diet containing LuP. The adducts were detected in the liver and kidney of the exposed animals [82].

RATIONALE FOR CHOOSING MS FOR AN ANALYSIS

Contemporary MS combines specificity and sensitivity and gives highly reliable analytical results. Femtomolar determinations are commonplace. The unique fingerprint identification provided by mass spectra gives a great deal of confidence that an analysis has been performed correctly. Improvements in inlet systems, ionization sources such as ES and APCI, and mass analyzers such as multiple-stage quadrupoles for tandem MS have greatly expanded the applicability and portability of MS. LC-MS is the workhorse in modern bioanalytical laboratories. It is the method of choice for toxicokinetic and pharmacokinetic studies and is commonly used for metabolite characterization, quantification, and metabolomic profiling. Its high sensitivity makes it ideal for quantifying exposure to environmental toxins. Nevertheless, the analyst must keep in mind that MS is a technique that destroys the sample. This is particularly important in cases where sample sizes are very small and result from time-consuming and labor-intensive isolation and purification methods. Despite these limitations, MS is critical to studying mechanisms of toxicity.

NMR Spectroscopy

In 1946, Purcell, Torrey, and Pound of Harvard University and Bloch, Hansen, and Packard of Stanford University independently detected NMR effects of the hydrogen nucleus in paraffin wax (Purcell) and water (Bloch). These first observations of NMR won the Nobel Prize in Physics for Bloch and Purcell in 1952. In 1951, Packard reported that the NMR spectrum of ethanol consisted of three distinct resonances and that these resonances arose from the three different chemical environments for the hydrogen nuclei in the molecule (CH_3, CH_2, and OH). This discovery quickly caught the attention of organic chemists who realized that NMR spectroscopy could be used as a tool for determining chemical structure [94,154]. NMR spectroscopy has since emerged as the most powerful technique for structural characterization of organic compounds. NMR is the method of choice for determining the identity of xenobiotic metabolites if they can be isolated in microgram to milligram quantities. In this regard, it is often used in conjunction with chromatographic and mass spectral methods. It can also be used to probe the structures of xenobiotic adducts to biological macromolecules such as DNA and proteins. NMR is used to quantify xenobiotic exposure and changes in endogenous compounds in response to such exposure, and it is widely employed in metabolomic studies.

Due to its selectivity and the wealth of information that is available from NMR spectroscopy, it has found increasing application in pharmacology, toxicology, and biomedical research. Recent advances in NMR technology have enabled researchers and clinicians to study chemical and physiological changes within cell suspensions, isolated organs, and living organisms in a noninvasive manner. In vivo spectroscopic techniques have been used to measure cellular metabolism, intracellular pH, cytosolic sodium, magnesium, and calcium levels, organ damage in response to toxicants, and a host of other toxicologically relevant phenomena.

NMR spectroscopy is a very powerful tool for studying mechanisms of toxicology. Toxicologists should be familiar with the strengths and limitations of NMR spectroscopy and the types of studies for which it is suited. The following sections include an overview of the theory of NMR and numerous examples of its application in toxicology and related disciplines. Although a detailed mathematical treatment of magnetic resonance is beyond the scope of this chapter, mathematical formulae will be used to describe and clarify the phenomena. It is assumed that the reader has encountered traditional proton and carbon NMR spectroscopy in an introductory organic chemistry course, and very little discussion of the interpretation of NMR spectra appears. A number of

extremely good texts on the theory, application, and interpretation of NMR are available. The interested reader might refer to them for more detail [45,47,154,159,166].

Basic Theory of NMR Spectroscopy

Nuclear Spin

An understanding of the NMR phenomenon begins with a look at the atomic nucleus. Nuclei are composed of protons and (except for ^{1}H) neutrons; therefore, all atomic nuclei carry a positive charge. Many nuclei also rotate about the nuclear axis, and the angular momentum of this spinning charge is described in terms of the spin quantum number, *I*. Spin numbers have values of 0, 1/2, 1, 3/2, and so forth. For our purposes, the most important spin-active nuclei are ^{1}H, ^{13}C, ^{31}P, and ^{19}F, all of which have $I = 1/2$.

Behavior of Nuclei in an External Magnetic Field

Because a spinning nucleus ($I > 0$) is a charge in motion, it generates a magnetic field and behaves like a small bar magnet with a magnetic moment (μ). Just as a bar magnet will align itself with an external magnetic field, so too will any nucleus with $I > 0$. For $I = 1/2$, the nuclei can adopt one of two orientations relative to the external magnetic field. They may be aligned with the field (low-energy state) or against the field (high-energy state).

A nucleus with $I > 0$ exhibits another important property when placed in an external magnetic field. That property is precessional motion. We have all observed the behavior of a spinning top. As it spins, the axis of the top slowly revolves around the vertical. The top is said to be precessing around the vertical axis, and this type of behavior is called *precessional motion*. A nucleus spinning in an external magnetic field also exhibits precessional motion, with the magnetic moment (μ) precessing around the axis of the applied magnetic field B_o (Figure 41.5). The frequency at which the magnetic moment precesses about B_o is called the precessional frequency, ν. While the spinning frequency of any given nucleus is constant, ν varies directly with the strength of the external magnetic field B_o:

$$\nu \propto \mathbf{B}_o$$

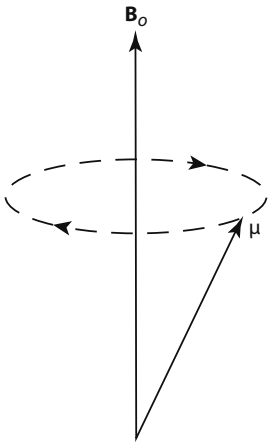

FIGURE 41.5 Magnetic moment, μ, of a spinning nucleus precessing about the applied magnetic field, $\mathbf{B}_o$, with precessional frequency ν.

This proportionality is the most fundamental relationship of NMR spectroscopy and leads to the fundamental NMR equation:

$$\nu = \frac{\gamma \mathbf{B}_o}{2\pi} \tag{41.2}$$

The proportionality constant (γ) is the magnetogyric ratio and is a fundamental nuclear constant. It is related to the nuclear magnetic moment (μ) and the spin quantum number (*I*):

$$\gamma = \frac{2\pi\mu}{hI} \tag{41.3}$$

where *h* is Planck's constant.

A proton (hydrogen nucleus) has a magnetogyric ratio of 2.6752×10^8 rad/s/T; therefore, in a magnetic field of 7.1 T, its precessional frequency is approximately 300 MHz. A proton in a magnetic field of 14.1 T will have $\nu \approx 600$ MHz. The magnetogyric ratio of ^{13}C is 6.727×10^7 rad/s/T, so in a field of 7.1 T, $\nu \approx 75$ MHz. Table 41.1 lists the precessional frequencies at selected field strengths for several common magnetic nuclei. NMR spectrometers are most frequently

TABLE 41.1
Precessional Frequencies of Several Common Nuclei at Selected Field Strengths

	Precessional Frequency (MHz) at Field Strength (T)									
Nucleus	**1.4**	**2.3**	**4.7**	**7.1**	**9.4**	**11.7**	**14.1**	**17.6**	**18.8**	**21.1**
^{1}H	60.0	100.0	200.0	300.0	400.0	500.0	600.00	750.0	800.0	900.0
^{2}H	9.2	15.4	30.7	46.1	61.4	76.8	92.1	115.1	122.8	138.2
^{13}C	15.1	25.1	50.3	75.4	100.6	125.7	150.9	188.6	201.2	226.3
^{14}N	4.3	7.2	14.4	21.7	28.9	36.1	43.3	54.1	57.8	65.0
^{15}N	6.1	10.1	20.3	30.4	40.5	50.7	60.8	76.0	81.0	91.2
^{17}O	8.1	13.6	27.1	40.7	54.2	67.8	81.3	101.6	108.4	122.0
^{19}F	42.4	94.1	188.2	282.2	376.3	470.4	564.5	705.6	752.6	846.7
^{23}Na	15.9	26.5	52.9	79.4	105.8	132.3	158.7	198.4	211.6	238.1
^{31}P	24.3	40.5	81.0	121.4	161.9	202.4	242.9	303.6	323.8	364.4

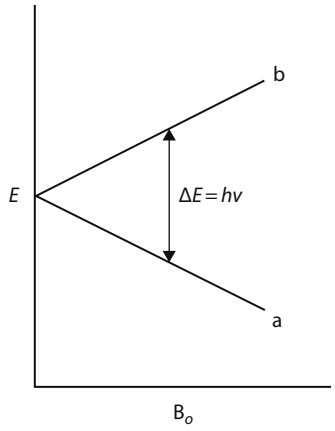

FIGURE 41.6 Energy level diagram for a spin 1/2 nucleus in a magnetic field $\mathbf{B}_o$.

classified by the precessional frequency of the ^{1}H nucleus at the field strength of the magnet; for example, a spectrometer with a field strength of 7.1 T is commonly referred to as a 300 MHz NMR.

A spin 1/2 nucleus in a magnetic field will have a precessional frequency (ν) and will be able to adopt one of two orientations, or spin states, relative to B_o. It will be aligned with (parallel) or against (antiparallel) the external field. The parallel orientation (α) is a low-energy state for the system, and the antiparallel orientation (β) is a high-energy state (Figure 41.6). A nucleus precessing in the parallel orientation can absorb energy (electromagnetic radiation) and be excited to the high-energy, antiparallel orientation. The precessing nucleus will only absorb electromagnetic radiation with a frequency equal to ν. Because precessional frequencies are on the order of MHz, the electromagnetic radiation that will excite a precessing nucleus is in the RF range. When the applied RF equals ν, the precessing nucleus and the RF are said to be in resonance, hence the name NMR. The frequency at which resonance occurs for a given nucleus is called its *resonance frequency.*

Relaxation Times

When a nucleus has absorbed energy and is excited to the high-energy spin state, it will tend to lose energy and return to the low-energy state. In addition to direct reemission of RF, there are two radiationless processes by which nuclei can exchange energy with their environment. These relaxation processes are a direct result of interaction of the nucleus with some electromagnetic vector in the local environment. The nucleus is surrounded by solvent molecules, and energy can be transferred to the solvent or other nearby atoms in a process called *spin-lattice* relaxation. The spin-lattice relaxation time (T_1) depends on such factors as temperature and solvent viscosity, with higher temperature and lower solvent viscosity slowing T_1 relaxation.

The nucleus can also transfer energy to nearby nuclei in a process called *spin–spin* relaxation. In spin–spin relaxation, one nucleus loses energy and the other one gains energy so there is no net change in the populations of the two spin states. The spin–spin relaxation time (T_2) depends on molecular mobility, and nuclei in large molecules that have highly constrained molecular motions have very efficient spin–spin relaxation (short T_2). The magnitude of T_1 and T_2 determines the line widths of NMR spectral lines; short relaxation times lead to broad lines, and long relaxation times lead to sharp lines. This means that NMR spectra obtained in viscous solvents (short T_1) will have broader lines than those obtained in nonviscous solvents. Also, NMR spectra of biological matrices such as plasma or cell suspensions will show many very broad resonances from proteins, nucleic acids, and other macromolecules that have very short T_2 relaxation times. These broad resonances can obscure the signals of small molecules of interest such as xenobiotic metabolites. A variety of NMR techniques have been developed that enable the observation of low-molecular-weight compounds in the presence of macromolecules [160].

Chemical Shifts

In the simplest NMR experiment, *continuous-wave NMR* (CW-NMR), a sample is placed in a strong, uniform magnetic field and the nuclei in the sample precess with their characteristic frequency ν. The sample is irradiated with radiowaves of increasing frequency, and when the RF equals ν, the nuclei are excited to the high-energy state and RF is absorbed. This absorption is recorded as the NMR spectrum. If all nuclei of a given type (e.g., ^{1}H) precessed with exactly the same frequency, an NMR spectrum would convey very little structural information. It would consist of a single line. Fortunately, the exact value of ν for a given nucleus depends on the chemical environment of that nucleus.

So far, we have discussed the magnetic properties of spinning nuclei, but atoms and molecules also contain electrons. Under the influence of an external magnetic field, electrons will circulate and generate their own magnetic field that will tend to oppose $\mathbf{B}_o$. This results in a magnetic shielding of nearby nuclei, which slightly shifts their precessional frequencies. The density of the circulating electron cloud depends greatly on its chemical environment, so different nuclei in a molecule will experience different degrees of shielding and hence will have different values of ν. The shift in ν depends on the chemical environment of the nucleus, and for this reason, it was given the name *chemical shift.*

Relative to the resonance frequency, chemical shifts are quite small. For protons in a field of 7.1 T ($\nu \approx 300$ MHz), the chemical shifts cover a range of about 4000 Hz. The absolute value of chemical shifts in frequency units depends on the strength of the applied magnetic field. The greater the value of B_o, the greater the chemical shift range. This explains, in part, the desire to move to higher and higher field strengths for NMR spectroscopy. The higher the field, the greater the resolution of chemical shifts.

Chemical shifts are rarely (if ever) expressed in absolute frequency units; instead, they are measured relative to a standard reference compound. For ^{1}H and ^{13}C NMR, the universally accepted reference is tetramethylsilane (TMS). It is commonly added to samples as an internal standard

at concentrations <1%. TMS has several important properties that make it a near ideal reference standard. It contains 12 magnetically equivalent protons and 4 magnetically equivalent carbons, so it gives a single intense peak in the ^{1}H and ^{13}C NMR spectra. It is chemically inert, soluble in most organic solvents, and volatile (b.p. = 27°C), so it can be removed easily from the sample after analysis. The protons and carbons of TMS absorb at a lower frequency (are more shielded) than those of almost all other organic compounds, so their chemical shifts can be arbitrarily set to 0 Hz, and most other chemical shifts measured relative to them will be positive. TMS is not soluble in water, so for aqueous solutions, a suitable water-soluble internal standard must be chosen. The most widely used internal standard for aqueous samples is the sodium salt of 3-(trimethylsilyl)-propanoic acid (TSP).

Because chemical shifts vary with the strength of the applied magnetic field, their values (in Hz) vary from one NMR spectrometer to the next. Protons that absorb at 300 Hz relative to TMS in a field of 7.1 T (300 MHz instrument) will absorb at 100 Hz on a 2.3 T (100 MHz) instrument. To avoid confusion and enable comparisons of chemical shifts from instrument to instrument, chemical shifts are expressed in dimensionless units designated δ. The chemical shift in δ units is defined according to the following relationship:

$$\delta = \frac{\vartheta_s - \vartheta_{\text{std}}}{\text{Operating frequency}} \times 10^6 \text{ ppm} \qquad (41.4)$$

where ϑ_s and ϑ_{std} are the resonance frequencies of the sample and standard, respectively.

Spin–Spin Coupling

Whereas a great deal of structural information may be obtained from the chemical shifts of nuclei in a molecule, even more information is available from an NMR spectrum. Nuclei that are closely connected through bonding electrons are influenced by the spin state of their neighbors. Consider two closely connected protons, H_a and H_b:

$$\begin{array}{ccc} H_a & & H_b \\ | & & | \\ C & - & C \end{array}$$

The bonding electrons in the H_a–C bond tend to align their spins with the spin of H_a, the C–C bonding electrons tend to align with the spin of the H_a–C bonding electrons, and the H_b–C bonding electrons tend to align with the spin of the C–C bonding electrons. Finally, H_b tends to align its spin with the spin of the H_b–C bonding electrons. In this way, the spin state of H_a directly influences the spin state of H_b. Because H_a can have two spin states, parallel or antiparallel, the resonance line of H_b is split into two closely spaced lines, a so-called *doublet*. H_b influences the spin state of H_a in exactly the same way, so H_a also appears as a doublet. The spacing between the lines in the H_a doublet is the same as that in the H_b doublet. Figure 41.7 illustrates the appearance of the H_a and H_b NMR signals. This phenomenon, called *spin–spin coupling* (or simply *coupling*), results in characteristic splitting of NMR signals, which is dependent on the number of neighboring spin-active nuclei, their geometrical arrangement, and the number of bonds between them. Coupling is usually not important beyond four bonds, and magnetically equivalent nuclei do not show coupling. Unlike chemical shifts, coupling is independent of $\mathbf{B}_o$.

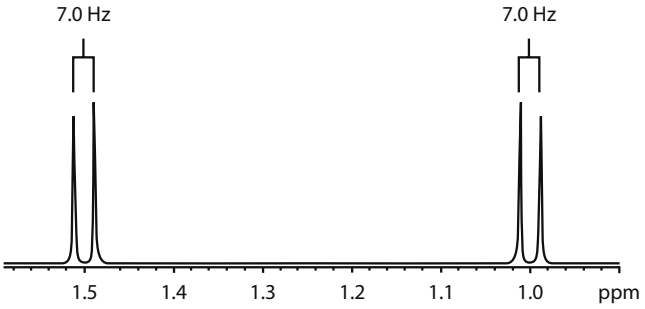

FIGURE 41.7 Proton NMR spectrum of the H_a/H_b spin system. Note the coupling constant, *J*, is 7.0 Hz.

The number of lines into which an NMR signal is split depends on the number of adjacent nuclei. The general rule is that *n* adjacent spin-active nuclei will split a signal into $2nI + 1$ lines. For spin 1/2 nuclei such as ^{1}H, two adjacent nuclei will split a signal into three lines (a triplet), three adjacent nuclei will split the signal into four lines (a quartet), and so forth. The relative line intensities within a multiplet are determined by the coefficients in the binomial expansion. A triplet will have intensities of 1:2:1, a quartet will have intensities of 1:3:3:1, and so on. The separation between spectral lines due to coupling is the coupling constant (*J*) (Figure 41.7). The magnitude of a coupling constant depends on the number of intervening bonds and the bond geometry. The coupling constants for protons that are *trans* to one another in a rigid molecule are greater than those for protons that are *cis*. Spin–spin coupling is most important in proton NMR spectroscopy, as most organic molecules have adjacent protons and show extensive coupling. In ^{13}C NMR spectroscopy, proton coupling to the carbons is often undesirable because it leads to very complex spectra and reduced S/N ratio. For these reasons, it is usually suppressed by irradiating over the entire proton resonance frequency range to saturate the proton transitions. This so-called decoupling produces carbon spectra made up of only singlets.

Integral Areas

The area under a peak in an NMR spectrum is proportional to the total number of nuclei giving rise to that signal; in other words, the area of a peak can be used to gain information about the concentrations of the spin-active nuclei. All modern NMR spectrometers are capable of integrating peak areas, and the resulting integrals can be plotted on the spectrum. In the case of a multiplet, the total area of the signal is integrated. By comparing integrals within a proton spectrum, it is possible to determine which signals are due to

methyl (3 H), methylene (2 H), or methine (1 H) groups. This is a powerful aid in interpretation of proton NMR spectra.

Because peak areas are proportional to the concentrations of the spin-active nuclei, NMR can be used for quantitative analysis of molecules in solution. This is particularly useful for quantification of xenobiotic metabolites in biological matrices. If the analytes are present in high enough concentration (see succeeding text for a discussion of NMR sensitivity) and signals due to the presence of the analytes of interest can be distinguished in the spectrum, then very little sample preparation is required. For quantitative analysis, analyte integrals are compared to integrals of an internal standard that is added to the sample at a known concentration. Quantitative analysis by NMR requires very careful calibration and attention to spectral acquisition parameters, so it should be carried out cautiously. Excellent discussions of quantitative analysis by NMR are available [154,197].

For heteronuclear NMR (such as carbon) where broadband proton decoupling is used, the peak areas do not always give an accurate estimate of the concentration of spin-active nuclei. Decoupling gives rise to nuclear Overhauser effects that complicate the interpretation of integral areas. For this reason, carbon spectra are rarely integrated; however, spectral acquisition parameters can be adjusted to correct for these effects if accurate integrals are required from a carbon (or other heteronuclear) NMR spectrum.

Sensitivity

For nuclei in an external magnetic field, the energy difference between spin states is quite small. This means that the two spin states are almost equally populated at room temperature, with the population of the low-energy state exceeding that of the high-energy state by only about 0.001%. The intensity of absorption (and hence the sensitivity of NMR) is proportional to the number of nuclei absorbing RF energy. As the population difference between spin states increases, there will be more nuclei to absorb RF energy, and the sensitivity will increase. From Equation 41.3, we see that the energy difference between spin states ($\Delta E = h\nu$) is proportional to the magnetogyric ratio (γ). This means that the sensitivity of NMR depends on the magnitude of γ. Detection of nuclei with relatively large magnetogyric ratios (such as ^{1}H and ^{19}F) will be fairly sensitive. The sensitivity of NMR also depends on the natural abundance of the spin-active nucleus under observation. Nuclei with a high natural abundance will be detected with greater sensitivity. Table 41.2 lists natural abundance, spin quantum number, magnetogyric ratio, and sensitivity for some selected nuclei.

The energy difference between spin states ($\Delta E = h\nu$) is also proportional to the magnetic field strength B_o. One way to increase the sensitivity of NMR is to increase the field strength. This approach has been somewhat successful; however, even at a field strength of 14.1 T, the energy difference between spin states is only on the order of 10^{-4} kJ/mol. NMR is (and is likely to remain) less sensitive than optical techniques such as electronic absorption spectroscopy, where ΔE is considerably larger. Even with the most sensitive high-field instruments, tens to thousands of micrograms of sample are required.

FT-NMR

Because the energy difference between spin states is small, NMR signals are invariably weak. In fact, they are often only slightly more intense than the background noise caused by the electronics of the NMR spectrometer. To improve the S/N ratio, several spectra can be obtained and the resulting collection of data averaged. The NMR signals in these spectra would always occur at the same frequencies; however, the random noise would not. The desired signals would build up over time relative to the noise and the S/N would increase. It can be shown that, for n repetitions, the signal increases by a factor of n and the noise increases by a factor of $\sqrt{2}$. As a result, signal averaging increases the S/N by a factor of $\sqrt{2}$.

If signal averaging were applied to the CW-NMR experiment described previously, the spectrum would be scanned repetitively over the frequency range of interest and the resulting spectra averaged. This would result in an increase in S/N, but the time required for the experiment would be quite long. Consider a proton spectrum covering 10 ppm at a field strength of 2.3 T. The sweep width (frequency range) of this spectrum is 1000 Hz. A sweep rate of 1 Hz/s is required to achieve a spectral resolution of 1 Hz, so it would take 1000 s

TABLE 41.2
Selected NMR Properties of Several Common Nuclei

Nucleus	Natural Abundance (%)	Spin Quantum Number *I*	Magnetogyric Ratio ($\times 10^{-7}$ rad/s/T)	Relative Sensitivity at Constant Field
^{1}H	99.985	1/2	26.752	1.000
^{2}H	0.015	1	4.107	9.65×10^{-3}
^{13}C	1.108	1/2	6.727	0.016
^{14}N	99.635	1	1.933	1.01×10^{-3}
^{15}N	0.365	1/2	2.711	1.04×10^{-3}
^{17}O	0.037	5/2	3.627	0.029
^{19}F	100	1/2	25.167	0.834
^{23}Na	100	3/2	7.076	0.093
^{31}P	100	1/2	10.829	0.067

to acquire one spectrum. Increasing the S/N by a factor of 4 requires 16 repetitions ($\sqrt{2}$), so this experiment would take almost 4.5 h. You can see that signal averaging in CW-NMR is not very practical. How, then, do we achieve a significant increase in the S/N in a reasonable amount of time?

The solution to this problem is found in a technique called *pulse NMR*. In pulse NMR, the sample is irradiated with a pulse of RF energy containing all the frequencies required to excite the nuclei of interest. This pulse is applied for a very short period of time (on the order of μs), and the nuclei are allowed to relax to their equilibrium state with the emission of all the frequencies previously absorbed by the nuclei. This signal decays over time and is called *free induction decay* (FID). The FID contains all the spectral information including chemical shifts, coupling constants, and intensity. To convert this information from the time domain to the frequency domain, which is the normal mode for an NMR spectrum, the FID is subjected to a mathematical operation called *Fourier transformation*. To achieve signal averaging, a series of FIDs are acquired and averaged prior to Fourier transformation. The resulting FT-NMR spectrum is equivalent to a CW spectrum and is obtained in considerably less time. Figure 41.8 shows the FID and resulting proton FT-NMR spectrum of a 0.1% solution of ethylbenzene. For this spectrum, 64 repetitions, or transients, were acquired, requiring a total time of 4.7 min. Note the large TMS peak at 0 ppm and the presence of an impurity peak at about 1.55 ppm. Figure 41.9 shows the effect on the S/N of increasing the number of transients. These proton spectra were obtained on the same ethylbenzene sample used to generate Figure 41.8, and only the quartet is plotted.

All modern NMR spectrometers are of the FT type. FT-NMR has allowed insensitive nuclei such as ^{13}C to be studied routinely and sensitive nuclei such as ^{1}H and ^{19}F to be studied at much lower concentrations than previously possible. It has also opened up a multitude of powerful new multipulse and 2D NMR experiments, some of which will be discussed in a later section.

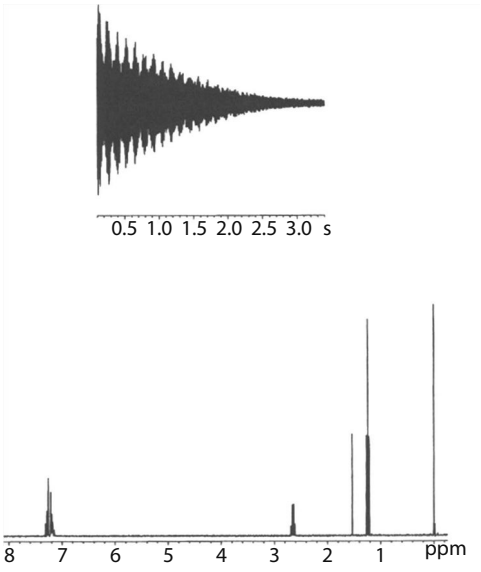

FIGURE 41.8 FID and proton NMR spectrum of a 0.1% solution of ethylbenzene in $CDCl_3$. The data were collected at a frequency of 300 MHz and a field strength of 7.1 T.

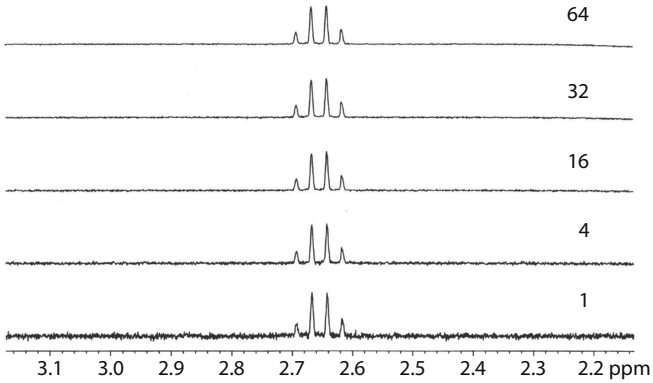

FIGURE 41.9 The effect of increasing the number of transients on the *S/N* ratio of the proton NMR spectrum of ethylbenzene.

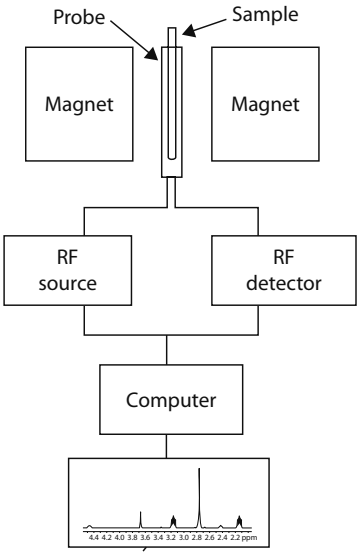

FIGURE 41.10 Schematic representation of an NMR spectrometer.

Instrument Design

Although NMR spectrometer design and construction vary from one manufacturer to the next, all spectrometers share the same basic components. Figure 41.10 shows a schematic representation of the basic features of a high-field NMR spectrometer. The heart of the spectrometer is the NMR probe that sits inside the superconducting magnet. The probe contains coils that transmit the RF pulses to the sample and receive the NMR signals that are emitted. Specialized probes for a variety of NMR experiments are available, and selection of the proper probe for the particular experiment is crucial to its success. They are exchangeable, and most NMR laboratories have more than one type. For in vivo spectroscopy, the probe may be surface coils placed in close proximity to the organ under study. The spectrometer contains an RF source for generating RF pulses and an RF detector for receiving

and amplifying the NMR signal. A computer that is also used for storing, processing, and displaying data controls the RF source and detector.

Experimental Design

The proper design of toxicological NMR studies depends on a number of factors. The first consideration when designing an NMR study is the nature of the sample. If the sample is a solution or cell suspension or can be dissolved in a suitable solvent, then a host of liquid-state NMR experiments is possible. If the sample is alive (perfused organs, laboratory animals, or humans), then in vivo spectroscopy using surface coils or imaging techniques is required. The choice of nuclei, type of NMR experiment, probe, solvent (if liquid-state analysis is required), and method of sample preparation will affect the outcome of the study. This section provides some guidance to the researcher in designing NMR studies. Particular emphasis is placed on liquid-state methods, as spectrometers for liquid-state analyses are more readily available than those for in vivo spectroscopy, and liquid-state methods are by far the most commonly used for toxicology research.

Choice of Nucleus

The most useful NMR-active nuclei for toxicology studies are ^{1}H, ^{13}C, ^{31}P, and ^{19}F. When the effects of heavy metal exposure are of interest, nuclei such as ^{199}Hg or ^{113}Cd can sometimes be used. Biologically derived samples are often complex mixtures, and NMR signals from the matrix can cause significant interference. This is particularly troublesome for ^{1}H and ^{13}C, as these nuclei are ubiquitous. With proper sample cleanup and utilization of appropriate NMR techniques, these matrix effects can be overcome. ^{1}H is the most sensitive NMR-active nucleus (in absolute terms) and is present in almost all organic compounds. If care is taken to minimize signal overlap from the matrix, it can provide qualitative and quantitative information on the metabolism and biochemical effects of xenobiotics. ^{1}H spectra can be obtained for compounds present in concentrations greater than about 50–100 μM in a reasonable amount of time. For these reasons, ^{1}H is the most commonly used NMR-active nucleus for studying mechanisms of toxicology. A number of studies of crude urine or urine extracts have been reported; however, biological fluids are usually concentrated and fractionated by HPLC prior to ^{1}H NMR analysis. In some cases, 2D NMR experiments such as correlation spectroscopy (COSY; discussed in the following text) can adequately resolve signals of interest with little or no sample cleanup.

^{13}C is of very low natural abundance (about 1%), and its magnetogyric ratio is four times lower than ^{1}H. As a result, its absolute sensitivity is over 5000 times less than ^{1}H. Despite this low sensitivity, ^{13}C NMR spectra can be obtained for compounds present at concentrations >10 mM in a reasonable amount of time. As in ^{1}H NMR studies, samples are most often concentrated and purified by HPLC prior to analysis. In some cases, isotopic enrichment with ^{13}C has been used to increase sensitivity. Enrichment to 90 atom-% ^{13}C results in a 90-fold increase in sensitivity, rendering direct analysis of crude urine for low-molecular-weight metabolites feasible. Combined with 2D NMR experiments such as INADEQUATE, isotopic enrichment with ^{13}C is a powerful tool for metabolite characterization. Noninvasive in vivo ^{13}C NMR spectroscopy has been used to study the pharmacokinetics of ^{13}C-enriched xenobiotics in the rat [134]. The high cost and limited availability of ^{13}C-enriched materials are the main disadvantages to isotopic enrichment.

^{31}P is a sensitive nucleus with a 100% natural abundance. As such, it is an attractive candidate for NMR studies. ^{31}P is one of the most commonly used nuclei for in vivo NMR spectroscopy and has been used extensively to determine the effects of xenobiotics on in vivo energy metabolism and intracellular pH [121]. It is much less useful for studies of xenobiotic metabolism, as very few xenobiotic metabolites contain phosphorous.

^{19}F is almost as sensitive as ^{1}H, and because it is not normally found in biological samples, ^{19}F NMR signals from the matrix are not a problem. If the xenobiotic of interest contains fluorine, useful information such as quantitative determination of fluorinated metabolites and excretion rates can be obtained from ^{19}F NMR. Most fluorinated compounds contain only a few fluorine atoms (one to three), so structural characterization of metabolites by ^{19}F NMR is rare. ^{19}F is a very good tracer for in vitro and in vivo spectral determination of organ perfusion and has been used to determine cerebral, hepatic, and muscular blood flows [135]. Using fluorophenols as model toxins, the microbial degradation of environmental pollutants has been examined in a detailed metabolomic study using ^{19}F NMR [19]. In practice, NMR studies often utilize more than one type of NMR-active nucleus. Characterization of metabolites is best accomplished with more than one type of NMR experiment. Typically, both 1D ^{1}H and ^{13}C spectra and a variety of 2D experiments are used.

Choice of NMR Experiments

Although a detailed discussion of modern NMR experiments is far beyond the scope of this chapter, several excellent books on the subject are available [45,47,127,166]. A brief description of those experiments that are likely to be most useful for studying mechanisms of toxicology is appropriate. NMR experiments can be divided into two main categories: 1D and multidimensional. One-dimensional NMR experiments are of the type discussed in the earlier section on basic theory. The output of a 1D experiment is a spectrum of intensity vs. frequency (or chemical shift in δ units). The ^{1}H spectrum of Figure 41.8 is a very good example. In principle, 1D spectra can be obtained for any spin-active nucleus. One-dimensional spectra are the type used most often for quantitative analysis.

For structural characterization of organic compounds, 1D proton and carbon spectra are almost always used. These spectra contain information about the chemical environments of the hydrogen and carbon atoms in the molecule. The proton spectrum, with its coupling information, also helps in assigning connectivities, as coupling constants and peak multiplicity can assist the spectroscopist in determining which hydrogens are coupled. Coupling constants also

help in assigning geometry in rigid systems (e.g., *cis* or *trans* isomers). Although a great deal of structural information can be obtained from these 1D spectra, it is often useful to obtain 2D spectra as well.

For 2D experiments, two or more RF pulses with variable delay times between them are used to excite the nuclei. The variable delay times introduce a second time domain, and after 2D Fourier transformation, a spectrum with two frequency domains is obtained. No attempt to explain the details of 2D NMR is made here; the interested reader should refer to other texts for such detail [127]. The 2D experiments, which are likely to be the most useful in toxicology research, are used to investigate spin–spin coupling by COSY. COSY spectra reveal ^{1}H–^{1}H spin–spin correlations and generate all connectivities between coupled protons. HETCOR (or HMQC and HMBC) spectra reveal proton–heteroatom correlations (usually ^{1}H–^{13}C correlations) and generate connectivities between protons and heteroatoms, such as carbon. INADEQUATE spectra (which are used much less frequently and most often for ^{13}C-enriched compounds) are used to generate connectivities between carbons. Two-dimensional experiments are very useful for resolving signals of interest from overlapping matrix signals in biological samples, and examples of their application in metabolism studies are presented in a later section.

Instrument Considerations

Ideally, the instrument with the highest available field strength (frequency) should be used to obtain maximum resolution and sensitivity. Commercial instruments with a field strength of 900 MHz are available. NMR but advances in magnet design should make even higher-field instruments possible. Price must be considered when purchasing an instrument. Fortunately, most academic NMR laboratories make instrument time available free or for a reasonable hourly rate.

In toxicology research, sample size is often limited. It may take many hours (or weeks) to isolate a few hundred micrograms of a xenobiotic metabolite, so sensitivity is a major concern. For a given field strength, the component that has the greatest influence on sensitivity is the probe. The most common probes are designed for 5 mm sample tubes and in many cases will give adequate sensitivity. Where sample size is limited, the smallest possible probe should be used. Microprobes designed for 3 mm sample tubes are available, and a 1.7 mm probe has been developed [126].

Probes are tuned to the resonance frequency of the nucleus under observation. Selectively tuned probes are designed to work over a very narrow frequency range. They are selected based on the frequency of the nucleus under observation. Selectively tuned probes for proton may be tuned slightly lower to observe fluorine but can never be tuned low enough to observe carbon. Broadband probes can be tuned over a very broad frequency range (a factor of 10 from lowest to highest), so one broadband probe can be used to observe most NMR-active nuclei. Although this sounds very attractive, there is a trade-off when using such a probe. Broadband probes are inherently less sensitive than selectively tuned probes. The S/N with a broadband probe will be at least a factor of two less than with a selectively tuned probe. For maximum sensitivity, it is best to use selectively tuned probes.

A powerful method for heteronuclear correlation experiments, called *indirect* or *reverse detection* [39,126], considerably increases the sensitivity of heteronuclear correlation experiments. It takes advantage of the sensitivity of proton NMR and the selectivity of heteronuclear NMR. It requires irradiation at the heteroatom frequency and observation at the proton frequency. Probes specifically designed for indirect detection are available in 5, 3, and 1.7 mm sizes. Using a 1.7 mm indirect detection probe reduces the sample size required for heteronuclear correlation experiments from ~50 μmol for a 5 mm probe to <0.05 μmol [126].

Sample Preparation

Interference from the sample matrix and the dilute nature of many biological samples often require a purification or preconcentration step prior to NMR analysis. HPLC, solid- or liquid-phase extraction, and TLC have all been used to purify xenobiotic metabolites. The method of choice will depend on the nature of the matrix, the chemical properties of the metabolites, and the sample size. If organic solvents are used for the purification, it is very important to remove as much of the solvent as possible by evaporation prior to NMR analysis. Residual solvents can add very large signals to proton and carbon spectra. These large signals often obscure significant portions of the spectra and, if large enough, can make detection of small signals difficult or impossible due to the limited dynamic range of most spectrometers.

Liquid-state NMR requires the sample to be dissolved in a suitable solvent. Solvents used for most NMR analyses are deuterated; that is, they contain deuterium (^{2}H) instead of protium (^{1}H). Deuterium is required for the spectrometer frequency lock, which corrects for field drift during spectral acquisition. For proton NMR, deuterated solvents are also desirable as they cut down on the intense solvent resonance that would appear in the presence of protiated solvents. Common NMR solvents, commercially available in >99 atom-% deuterium, are $CDCl_3$, CD_2Cl_2, d_4-methanol, d_6-DMSO, d_6-acetone, and D_2O. If samples in H_2O are to be analyzed, a small amount of D_2O (10%–20%) should be added for frequency locking, and a suitable solvent suppression method (discussed later) should be used.

When the sample has dissolved in a suitable solvent, it should be filtered to remove particulate matter. Even small particles can result in a loss of resolution, so filtration is particularly important when closely spaced signals or very small couplings are to be observed.

Solvent Suppression

Many biological samples are aqueous solutions. Acquisition of ^{1}H NMR spectra of such samples is complicated by the fact that the solvent water protons are present at a concentration of 110 M and the analytes of interest are often present in submillimolar concentrations. As a result, weak solute signals are often obscured or undetectable in the presence of

the very large solvent signal. It is essential to attenuate the water signal to observe weak signals in the ^{1}H NMR spectra of biological samples. A variety of so-called solvent suppression methods have been developed to reduce or eliminate the water peak in ^{1}H NMR spectra [80].

The simplest solvent suppression method is the removal of H_2O by lyophilization. The sample is lyophilized to dryness and reconstituted in D_2O. Typically, this is repeated several times to remove the last traces of H_2O prior to spectral acquisition. In practice, it is extremely difficult to eliminate the water peak completely because lyophilized biological samples such as urine are often quite hygroscopic and the sample invariably picks up moisture from the air. This method does, however, provide adequate reduction in the size of the water peak for most purposes. An additional advantage to this approach is that dilute samples can be concentrated to increase overall sensitivity.

Several instrumental methods are available for solvent suppression. Perhaps the simplest of these involves irradiation at the solvent resonance frequency to saturate the solvent signal. This irradiation may be continuous or it may be gated off during the pulse and acquisition. Suppression ratios of 1000 are possible for water. Unfortunately, peaks close to it are often attenuated or distorted. To suppress the water peak with less distortion of other resonances, selective relaxation methods based on T_1 relaxation differences between solvent and solute, such as the *water elimination Fourier transform* (WEFT) method, have been used. Although adequate water suppression is often achieved with WEFT, if signals from the molecule of interest have slow relaxation times, their intensities may be attenuated. If accurate integration is required for signals with a range of relaxation times, other solvent suppression methods should be used.

A powerful method for water suppression takes advantage of rapid proton exchange between water and an added chemical agent such as ammonium chloride. Rapid proton exchange greatly reduces T_2 for the water signal, which is suppressed using a special pulse sequence called *spin echo.* This pulse sequence allows the water signal to relax but still permits detection of the desired solute resonances that have longer T_2 relaxation times. This method, *water attenuation by T_2 relaxation* (WATR), can reduce the intensity of the water signal by factors of $>10^4$. WATR was employed in the determination of benzene and *N*-nitrosodimethylamine in aqueous solution by 500 MHz proton NMR with limits of detection of 35 and 510 ng/mL, respectively [57].

Recent advances in the theory and technology of NMR have led to the development of a host of pulse sequences designed specifically for solvent suppression. Fortunately, modern NMR computer control systems contain software for all common solvent suppression pulse sequences, and they are now routinely used with a minimum of operator involvement. A very powerful method, based on the 2D experiment NOESY, is called NOESYPRESAT. This method results in attenuation of the water signal by a factor of 10^5 or more. Using NOESYPRESAT (for 1D spectra), a series of 2D experiments, and a 750 MHz spectrometer, Nicholson et al. [141] were able to assign over 150 resonances in the ^{1}H spectrum of human blood plasma diluted with 10% D_2O.

Applications

Identification of Metabolites

NMR is often critical for elucidating the structure of unknown organic compounds. Both 1D and 2D NMR spectra were used to fully characterize *S*-(−)-cotinine-*N*-glucuronide, a previously unidentified metabolite of nicotine. The structure of this glucuronide was determined using 1D proton and carbon and 2D COSY and HETCOR spectra. The 1D spectra (Figure 41.11) were consistent with the proposed structure. The COSY spectrum shown in Figure 41.12A was used to establish connectivities of the coupled protons enabling the complete assignment of the proton resonances in the 1D spectrum. In COSY spectra, the contours that fall on the diagonal correspond to the 1D spectrum except they are seen as if the observer were looking down from above the spectrum. Contours that fall off the diagonal result from spin–spin coupling, so they reveal correlations between coupled protons. COSY spectra are usually presented with the 1D spectrum plotted on the top to facilitate interpretation. The HETCOR spectrum shown in Figure 41.12B was used to establish proton–carbon connectivities and enabled the complete assignment of the carbon resonances in the 1D carbon spectrum. HETCOR spectra may be thought of as a carbon spectrum plotted against a proton spectrum. Contours that appear in the HETCOR result from ^{1}H–^{13}C coupling and reveal proton–carbon connectivities—that is, which protons are bound to which carbons. This HETCOR spectrum is presented with the carbon spectrum (projection) plotted on the vertical axis and the proton spectrum plotted on the horizontal axis.

Perhaps the most common applications of NMR in toxicology research involve characterization of xenobiotic metabolites. Usually, NMR spectra of the purified metabolites are used in conjunction with mass spectral characterization and are included as final confirmation of the proposed structure. Such applications of NMR have contributed to the understanding of xenobiotic detoxification mechanisms. NMR analysis of crude or partially purified biological samples, although not frequently used, can be useful for metabolic studies. Typically, 1D proton NMR spectra of crude biological samples are very complex, with many overlapping signals. Nicholson and Wilson [139] have demonstrated the utility of 2D COSY NMR spectroscopy of crude urine for the simplification of such complex spectra. They collected urine from a human volunteer before and 3 h after ingestion of 1 g paracetamol. The urine was lyophilized, reconstituted in D_2O, and examined directly by 1D proton and COSY NMR. The 1D spectrum revealed considerable signal overlap in the aromatic region; however, in the COSY spectrum, well-resolved cross peaks for five paracetamol metabolites were immediately evident. They pointed out that 2D techniques are costly in terms of instrument time but can be used quite successfully when a

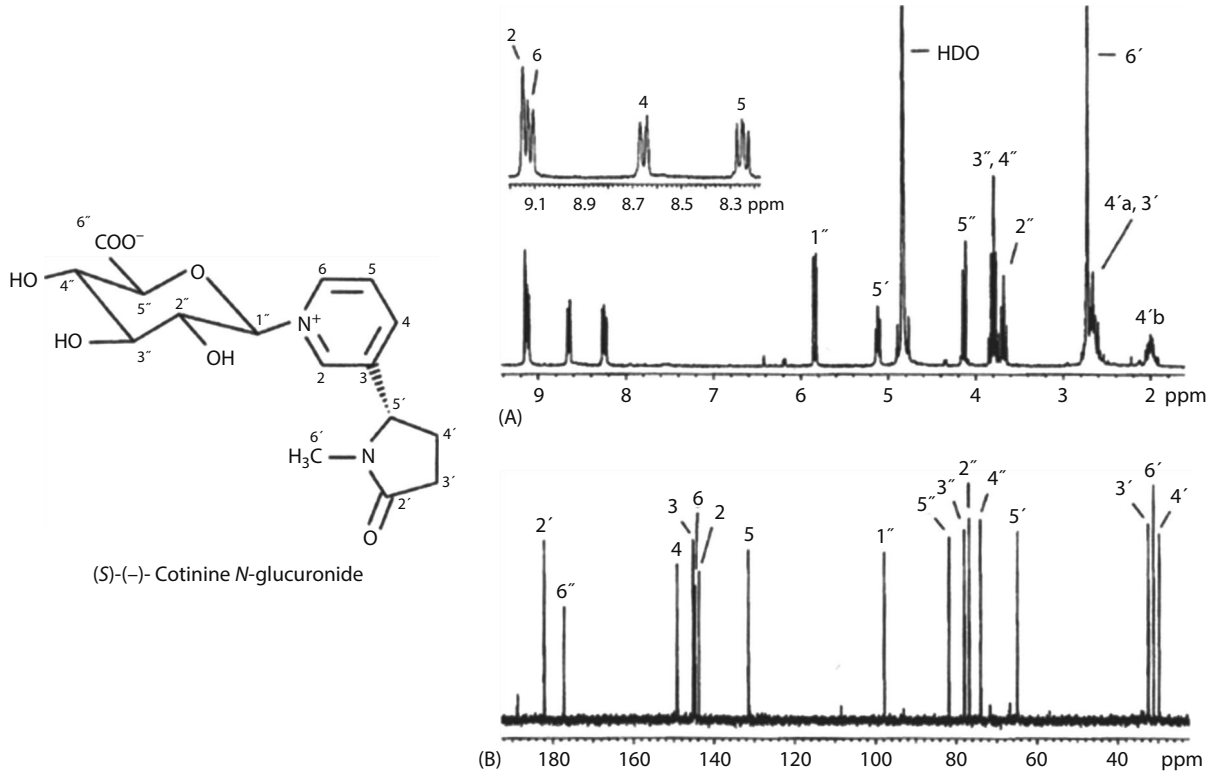

FIGURE 41.11 One-dimensional proton (A) and carbon (B) NMR spectra of (S)-(–)-cotinine-*N*-glucuronide.

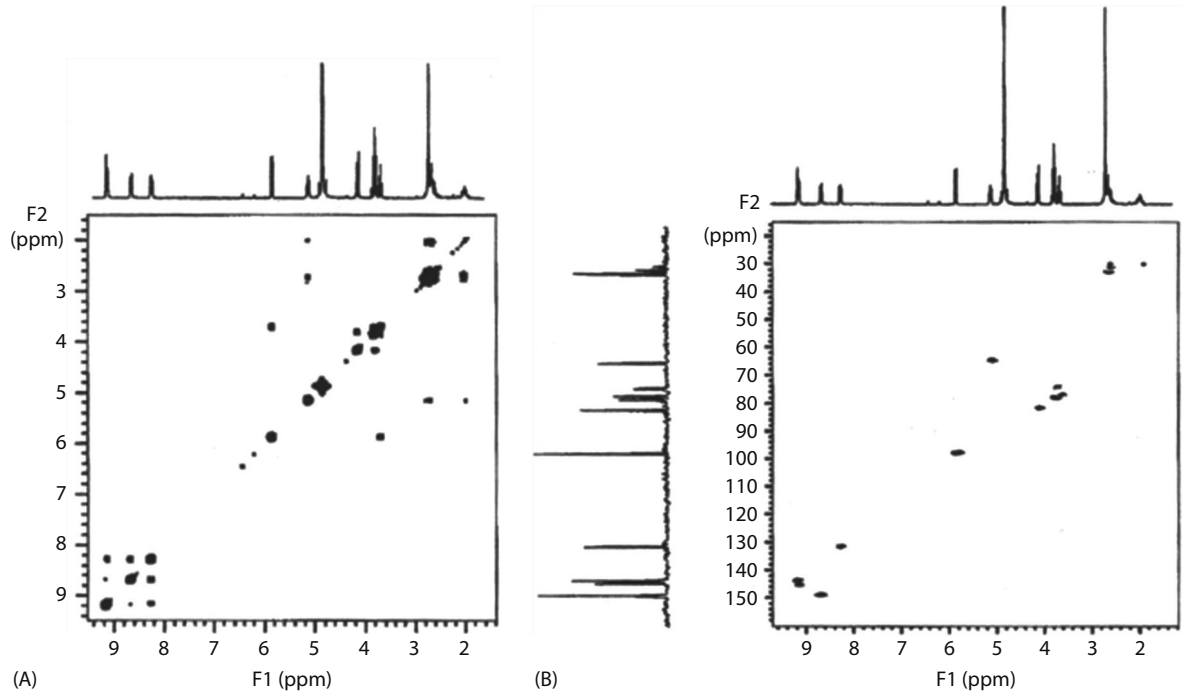

FIGURE 41.12 COSY spectrum (A) and HETCOR spectrum (B) of (S)-(–)-cotinine-*N*-glucuronide.

great deal of metabolic information is required from a few samples or spectral assignments are difficult from the 1D spectra alone.

When NMR spectroscopy is coupled with partial purification, structural characterization is often possible from 1D spectra alone. Solid-phase extraction (SPE) is a good method for the partial purification of biological samples on a moderately large scale and has been applied successfully to the characterization of ibuprofen metabolites by 1D proton and carbon NMR [139]. For this study, a normal, healthy male

volunteer was administered 400 mg ibuprofen and urine was collected predose, at 0–2 h, and at 2–4 h. The urine was lyophilized and reconstituted in D_2O, and 1D proton spectra were acquired. The samples were next applied to a C18 SPE column and eluted with a stepwise gradient of increasing methanol concentration. Fractions were collected, the solvent was evaporated, and the residue was reconstituted in D_2O. Spectra of each fraction were acquired to follow the progress of metabolite elution. Using this method, three ibuprofen metabolites, including the glucuronide, were recovered essentially free of impurities.

Mechanistic Studies of Xenobiotic Metabolism

To elucidate the mechanism of xenobiotic metabolism, detailed structural characterization of metabolites is required. Often, such detail is only available from NMR spectroscopic analysis. Because many metabolites are present in very small quantities, extremely sensitive NMR methods are required. A type of indirect detection called *proton-detected heteronuclear multiple quantum* coherence (HMQC) NMR provides detailed structural information (proton–carbon connectivities) with very good sensitivity. Using a novel 1D application of HMQC, Hackett et al. [66,67] have studied the microsomal hydroxylation of the herbicide triallate. They determined that cytochrome P450–catalyzed oxidation of triallate leads to the formation of an intermediate *allylic radical* (AR) that undergoes rearrangement leading to hydroxylation at two different positions (Figure 41.13). The structural characterization that led to such detailed mechanistic understanding was carried out on 20–45 μg of material isolated and purified by HPLC after microsomal incubation of ^{13}C-labeled triallate. Hackett et al. [66,67] used a 500 MHz NMR equipped with a 5 mm indirect detection probe. Even greater sensitivity could be realized with a higher-field NMR or a 1.7 mm probe. Their work has demonstrated the utility of 1D HMQC for mechanistic studies when sample size is quite limited and no other suitable analytical method is available.

Biochemical Changes Associated with Xenobiotic Toxicity

Exposure to toxins invariably leads to certain biochemical changes. These include changes in the urinary excretion of endogenous compounds such as carbohydrates, amino acids, and carboxylic acids. Mercury is known to accumulate in the kidneys of experimental animals after injection, causing damage to the proximal tubular epithelium and severe kidney failure. Mercury-induced nephrotoxicity is characterized by increased urinary excretion of amino acids, glucose, calcium, phosphate, bicarbonate, and low-molecular-weight proteins. Nicholson et al. [138] have studied changes in urinary and plasma levels of a large number of low-molecular-weight compounds in rats exposed to mercuric chloride by proton NMR and correlated these changes with histopathology and enzyme excretion. They quantified metabolites in untreated urine and plasma and observed dose-dependent decreases in urinary excretion of creatinine and citrate and increases in glucose, glycine, alanine, α-ketoglutarate, succinate, and acetate. They observed increases in plasma levels of lactate and creatinine. The observed changes were consistent with Hg^{2+} inhibition of certain citric acid cycle enzymes and intracellular, tubular acidosis. Their NMR data provided not only a sensitive measure of Hg^{2+}-induced nephrotoxicity but mechanistic insight as well. Similar quantitative NMR techniques may also prove useful for studying the mechanism of action of other toxins.

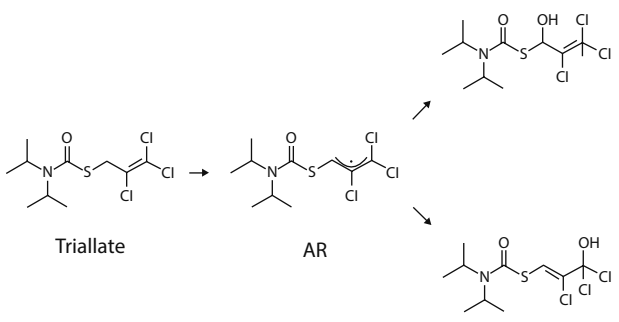

FIGURE 41.13 Cytochrome P450–catalyzed oxidation of triallate showing the AR intermediate.

Metabolomics

The systematic evaluation of biochemical responses to intoxication is the foundation of metabolomics. NMR spectroscopy of biological fluids and tissues, coupled with pattern recognition and chemometric analysis, is often essential to metabolomic studies [96]. Principal component NMR analysis of urine from rats treated with the phosphodiesterase inhibitor CI-1018 allowed researchers to differentiate between both vascular lesions and an independent inflammatory response secondary to CI-1018 treatment [181]. Indeed, for over a decade, NMR analysis of urine from humans and animals has been used to identify the metabolic perturbations associated with drug toxicity, such as the nephrotoxicity associated with ifosfamide treatment [54]. Metabolomic studies are certainly not limited to the study of toxic pharmaceuticals. The powerful combination of NMR and principal component analysis was employed in the development of a rapid throughput analysis of urine from rats treated with common hepato- and nephrotoxins [165]. Metabolomic analysis of urine components readily and reliably detected both the onset and reversal of toxicity associated with carbon tetrachloride, 4-aminophenol, and other compounds.

Hydrazine, a model hepatotoxin, is an excellent example of a compound that may be systematically studied using NMR-based metabolomics. The onset and recovery from hydrazine-induced liver toxicity in rats were easily monitored in a time- and dose-dependent manner using NMR analysis of urine, coupled with pattern recognition and pattern classification algorithms [187]. Similar analysis of urinary biomarkers, including lactate, acetate, taurine, and β-alanine, allowed researchers to distinguish between different rat strains and their biochemical response to treatment with hydrazine [77]. Predictive models for classifying the type of intoxication and the strain of intoxicated rat were highly accurate and reproducible. Interspecies variations in the biochemical responses to hydrazine intoxication are also readily discernible using

NMR-based metabolomics. Urinary metabolomic analysis of hydrazine metabolites and biochemical markers, including citrulline and trimethylamine-*N*-oxide, was very effective for detecting and differentiating between hydrazine toxicity in rats and mice [20].

Lipids are a particularly useful group of metabolomic markers for toxicological studies. Often, changes in lipid profiles in biological matrices are both pronounced and readily detectable by NMR after intoxication. Metabolomic studies of urinary dicarboxylic aciduria, which combined NMR with multivariate statistical analysis, were very useful for detecting and characterizing the mechanism of liver toxicity attributed to MrkA, an experimental therapeutic [132]. The effect of rosiglitazone, an antidiabetic compound, on several metabolic pathways was characterized by NMR metabolomics. Changes in liver–plasma lipid exchange, de novo fatty acid synthesis, lipid levels in the peroxisome, cardiac lipid metabolism, and lipid metabolism in adipose tissue were all detected using this method, which served as a phenotypic model for assessing clinical response to the drug [209]. Metabolomic analysis of urine from rats treated with chloroquine, amiodarone, or DMP-777, an elastase inhibitor, showed distinct differences in phospholipidosis and phenylacetylglycine levels, depending on which drug was administered [50]. This allowed the authors to correctly determine drug exposure, as well as differentiate between tissue-specific toxicity, based on the unique metabolomic profile of each compound.

Metabolomic studies employing NMR may also be very useful for the environmental toxicologist. Both acute and chronic environmental stresses, including toxin exposure, may be detected by metabolomic analysis of field samples. Principal component analysis of extracts from abalone foot muscle, digestive gland, and hemolymph allowed toxicologists to distinguish between the biochemical profiles of healthy, stunted, and diseased abalone, an important commercial shellfish [203]. In addition to providing a means of assessing the presence of toxins and other environmental stressors, this metabolomic approach also allowed the researchers to diagnose withering syndrome, a common disease in abalone. High-resolution NMR was employed in a metabolomic study of earthworms treated with 3-fluoro-4-nitrophenol, a model toxin. Changes in the relative concentrations of organic acids (in particular, acetate and malonate) served as a reliable marker of toxin exposure [23].

As discussed, the intensity and resolution of NMR spectra may be compromised by a number of factors, including pH, solvent effects, and overlapping peaks. These may limit the reliability and robustness of NMR-based metabolomics. Accordingly, several specialized systems and mathematical approaches have been developed to overcome these limitations. Variations in peak position might be overcome by employing orthogonal projection, back-scale plots, and weighted variable peak position data in NMR plot analysis [113]. Two-dimensional J-resolved spectra, combined with precision spectral preprocessing and logarithmic transformation, afford proton-decoupled projected 1D spectra with reduced peak congestion and more accurate integration of metabolomic peaks [202]. Magic-angle spinning NMR is a more sophisticated technique that has been successfully employed for identifying and correlating metabolomic analytes with histopathological changes in thioacetamide toxicity [208]. Advances in NMR technology for metabolomic applications have been reviewed by Griffin and Bollard [63]. Advances in the mathematical approaches used to interpret metabolomic NMR spectra include advanced pattern recognition algorithms, hierarchical cluster analysis, and nearest neighbor classification systems [11,62].

In Vivo Spectroscopy and Imaging

Advances in NMR theory and hardware have enabled the study of morphological and metabolic changes in isolated organs and whole animals and humans based on NMR principles. Magnetic resonance imaging (MRI), widely used in clinical medicine, relies mainly on the detection of hydrogen nuclei in water and fat to construct high-resolution images. The contrast in these images results from different T_1 and T_2 relaxation times for hydrogen nuclei in different tissue environments. In vivo magnetic resonance spectroscopy (MRS) is used to obtain spectral information (such as chemical shift and intensity) on chemical compounds within living tissues and can be used to monitor metabolic changes resulting from disease or xenobiotic exposure. MRI and MRS are based on the same principles as liquid-state NMR—that is, the behavior of nuclei in a magnetic field under the influence of RF pulses—but the hardware, pulse sequences, and data processing are somewhat different. Several good reviews describe these differences in detail [9,131,193].

In vivo ^{31}P MRS has been used to monitor energy metabolism in tumors in laboratory animals during growth [190] and following hyperthermia [201], treatment with interleukin-1α [35], and endocrine therapy [189]. In many cases, the changes seen in phosphorous metabolites (ATP, ADP, PCr, P_i, β-nucleoside triphosphate, phospholipids) correlate well with tumor growth. These results demonstrate the potential for noninvasive monitoring of tumor development by in vivo NMR spectroscopy.

Both MRI and MRS have been used to study liver damage induced by hepatotoxic halocarbons. Locke and Brauer [116] monitored the response of rat liver in situ to bromobenzene by proton MRI and ^{31}P MRS. They found that a sublethal dose of bromobenzene-induced acute hepatic edema and decreased energy metabolism. Both effects had an onset of 15–20 h and were maximal at 25–60 h. These effects were blocked by Trolox C, a potent inhibitor of lipid peroxidation.

In vivo NMR spectroscopy has tremendous potential as a tool for studying mechanisms of toxicology. MRI and MRS techniques provide detailed information on the response of specific organs to toxicants and can also be used to monitor xenobiotic metabolism in vivo. In addition, they could greatly reduce the number of animals required for toxicology studies, as a single animal could be followed over an extended period of time to monitor internal changes. As spectrometers for in vivo NMR become more readily available to the practicing

toxicologist, in vivo NMR spectroscopy will almost certainly become the method of choice for many toxicology studies.

LC-NMR

Hyphenated techniques coupling MS and various separation methods such as GC, LC, and CE are standard analytical tools widely used in toxicology research. Advances in the design of NMR probes and in methods for adequate solvent suppression together with the availability of high-field NMR spectrometers have enabled the coupling of NMR with LC. This hyphenated method takes advantage of HPLC separations and the wealth of structural information provided by 1D and 2D NMR spectra. This section provides a brief overview of LC-NMR instrumentation, methods, and applications in toxicology research. For more detailed information, the interested reader is referred to several very good reviews of the topic [113–115,137].

The complex nature of most biological samples makes direct analysis by NMR spectroscopy difficult. Spectra of crude biological samples typically contain many overlapping resonances, which greatly complicate their interpretation. One approach to the simplification of such samples is the removal of endogenous components and the separation of the compounds of interest. The use of SPE followed by 1D NMR analysis (SPE-NMR) for the isolation and characterization of ibuprofen metabolites from human urine has already been mentioned [139]. Although SPE-NMR can be quite effective, it suffers from several limitations. SPE is a relatively low-resolution separation method. It is inadequate for very complex mixtures or mixtures of very similar compounds. SPE-NMR is a tedious and time-consuming technique involving the collection of samples, solvent removal, and reconstitution of samples in an appropriate NMR solvent. It also requires a relatively large amount of sample. Although this is usually not a problem for the analysis of human urine, it can be limiting in some circumstances. These limitations are largely removed by hyphenation of NMR with HPLC.

Although reports of successful LC-NMR experiments date back to the late 1970s, the limited sensitivity of NMR and the technical hurdles associated with suppression of signals from the protonated solvents commonly used for LC greatly limited the utility of LC-NMR. With the advent of micro NMR probes, the greater availability of high-field NMR spectrometers (>300 MHz) with increased dynamic range, and the development of truly effective solvent suppression methods, LC-NMR has come into its own as a widely used analytical technique. Hardware and software making LC-NMR a relatively routine method are now commercially available from major vendors.

Although the configurations of LC-NMR systems vary from vendor to vendor and laboratory to laboratory, all systems share the same basic components. The design of a typical LC-NMR system is shown schematically in Figure 41.14. The LC pumps, injector, column, and detector are all standard equipment. From the detector, the flow enters the LC-NMR interface, which contains components for flow control and peak sampling. The LC-NMR interface can send the flow directly to the NMR probe, divert the flow to waste, or store peaks detected by the in-line detector for subsequent NMR analysis. The NMR probe contains a flow cell that replaces the standard glass NMR tube and typically has a volume between about 50 and 250 μL. The probe can be of the selectively tuned type for direct detection of ^{1}H, ^{19}F, ^{31}P, etc., or it can be of the indirect detection type. Broadband probes are inherently less sensitive than selectively tuned or indirect probes and therefore are rarely, if ever, used for LC-NMR. The most commonly used LC-NMR probe is the dual $^{1}H/^{13}C$ indirect probe. Instruments configured as shown in Figure 41.14 can be used in the continuous-flow mode if peaks eluted from the LC column pass through the LC-NMR interface to the flow cell for detection in real time. This so-called online LC-NMR works well for fairly concentrated samples (mass detection limits >10 μg in the flow cell), but because the residence time in the flow cell is limited, it is difficult to obtain

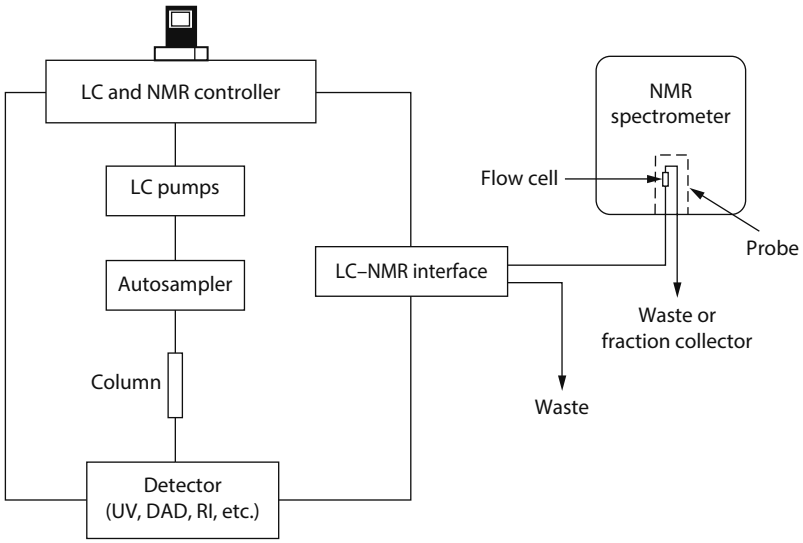

FIGURE 41.14 Schematic representation of an LC-NMR system.

more than 32–64 transients, and 2D experiments are not possible. When higher resolution or sensitivity is required, the LC-NMR interface can be programmed to stop the flow once a peak of interest enters the probe. Such stop-flow or static LC-NMR offers the opportunity to acquire as many transients as necessary for adequate S/N in 1D experiments and is compatible with 2D experiments as well. Using stop flow, samples of ~1 μg or less can be analyzed. A variation of the stop-flow technique involves programming the LC-NMR interface to store peaks of interest in sample loops and then pass them one at a time to the probe. Once a peak enters the probe, the flow is stopped and the peak is scanned.

A major development in the evolution of routine LC-NMR was in the area of solvent suppression. Because most LC separations of biological samples depend on reversed-phase columns, they utilize solvent mixtures containing water and protonated organic solvents such as methanol or acetonitrile. The most common LC-NMR solvent mixtures are D_2O/acetonitrile or D_2O/methanol. The use of D_2O reduces the effective concentration of solvent protons and provides a source of deuterium for the instrument lock. Because D_2O is relatively inexpensive, it is a very good alternative to H_2O. As discussed previously, the use of protonated solvents (such as acetonitrile) requires attenuation of the solvent signals to observe the weak signals from solute molecules. Fortunately, LC-NMR control systems contain computer software for solvent suppression pulse sequences such as NOESYPRESAT. The suppression of signals from mixed solvents is now a routine operation requiring minimal operator involvement.

Major NMR vendors offer systems for fully automated LC-NMR. Naturally, such systems are capable of automated online LC-NMR, but they are also capable of automated stop-flow LC-NMR. A typical system can

- Autodetect an LC peak
- Transfer the peak to the flow cell
- Stop the flow
- Shim the NMR magnet
- Optimize solvent suppression
- Acquire the spectrum
- Restart the LC pump
- Send the peak to waste or a fraction collector
- Repeat the procedure for any desired peaks in the chromatogram

Such a system can also automatically store peaks of interest in the LC-NMR interface for subsequent analysis.

The most common applications of LC-NMR involve the characterization of xenobiotic metabolites. An early example, reported by Spraul et al. [186], was the characterization of ibuprofen metabolites in human urine. For this study, a normal, healthy male volunteer was administered 400 mg ibuprofen and urine was collected for the period from 0 to 4 h after dosing. The urine was lyophilized and reconstituted in a D_2O–d_3-acetonitrile mixture prior to LC-NMR analysis. Gradient-elution LC was accomplished with (1) potassium dihydrogen phosphate in D_2O and (2) acetonitrile. A linear gradient from 2% to 45% acetonitrile at a flow rate of 1 mL/min was used for stop-flow analysis. For online analysis, a flow rate of 0.5 mL/min was used. The 500 MHz NMR was equipped with a commercial selectively tuned ^1H flow probe with a 60 μL flow cell, and solvent suppression was accomplished using the NOESYPRESAT pulse sequence. For online analysis, 16 transients were collected for each peak, giving a time resolution of 12 s. For stop-flow analysis, both 1D (256 or 512 transients) and 2D TOCSY experiments were performed. Using online analysis, Spraul et al. [186] were able to identify five metabolites, including three glucuronide conjugates. The stop-flow analyses provided high-resolution 1D ^1H spectra and 2D TOCSY spectra that permitted unambiguous characterization of the five metabolites. On-column detection limits were 10 μg for the online analyses and 1 μg for the stop flow. Using LC-NMR, the authors were able to identify one metabolite, the dicarboxylic acid metabolite of ibuprofen, which was not observed using SPE-NMR.

Glucuronic acid conjugates of carboxylic acids can undergo an isomerization reaction known as *acyl migration*, where the carboxylic acid moiety migrates from the 1 position of the glucuronic acid to the 2, 3, or 4 position. Such isomerizations can occur in vivo and in vitro at physiological pH and under mildly alkaline conditions. The resulting acyl-migrated positional isomers can exist as either β- or α-anomers. Lenz et al. [111] used LC-NMR to characterize the glucuronic acid conjugates of the nonsteroidal anti-inflammatory drug 6,11-dihydro-11-oxodibenz(*b*,*e*)oxepin-2-acetic acid in human urine and study the pH dependence of the acyl migration reaction in that matrix. They used a 400 MHz NMR with a 120 μL flow cell in the stop-flow mode to obtain ^1H spectra of the α- and β-anomers of all possible acyl migration products. For one metabolite, they used a 600 MHz NMR to obtain higher-resolution spectra that aided in full characterization. This work is noteworthy in that the α- and β-anomers were not separated by the LC and yet using NMR as a detector allowed the unambiguous characterization of the metabolite anomers. The high-resolution and rich information content of NMR spectra can often be used to characterize closely related metabolites that coelute in the liquid chromatogram.

The use of very high-field NMR spectrometers (800 MHz) greatly increases spectral resolution and can aid tremendously in metabolite characterization by LC-NMR. Sidelmann et al. [179] used SPE-NMR at 400 MHz and stop-flow LC-NMR at 800 MHz to identify the major phase II metabolites of tolfenamic acid in human urine. They identified glucuronide conjugates of the parent compound and five metabolites, the first report of the direct identification of these phase II metabolites in biofluids. The 800 MHz NMR was particularly useful in determining the exact position of hydroxylation of the aromatic ring of tolfenamic acid. The increase in spectral dispersion obtained at ultrahigh field provided very high-resolution 1D ^1H spectra, thus permitting unambiguous assignments.

LC-NMR has also been applied to studies of in vitro microsomal metabolism. Corcoran et al. [38] identified the metabolites of two phenoxypyridines obtained from incubation with

rat microsomes using stop-flow LC-NMR at 750 MHz. They were able to characterize one metabolite that was 6% of the total and another that was only 0.6%. The unequivocal identification of these metabolites without the use of radiolabeled substrates or synthetic metabolite standards demonstrated that LC-NMR can be used for metabolite characterization in in vitro systems and could be used in a high-throughput mode for lead optimization in drug discovery.

In an effort to further reduce the sample size required for hyphenated separation NMR techniques, some laboratories have explored microbore or capillary LC as well as other separation methods such as CE and capillary electrochromatography (CEC) coupled to NMR. Wu et al. [222] developed a theoretical model for predicting the S/N ratio as the NMR flow-cell volume is scaled down. Their model predicted only a twofold reduction in S/N for a 400-fold reduction in flow-cell volume. They constructed a 50 nL flow cell by wrapping narrow-gauge copper wire around a fused silica capillary column. Using this microflow cell and a 300 MHz spectrometer, they realized a mass limit of detection of ~1 μg on-column in an online microbore LC-NMR analysis. The most serious limitation to their approach was the relatively broad line widths in the online NMR spectra. When the analysis was performed in the stop-flow mode, they were able to optimize the NMR shims and other instrument parameters and reduce the line widths to <1 Hz. As a result, 2D experiments such as COSY and NOESY were possible.

Pusecker et al. [158] constructed a 240 nL capillary flow cell coupled to a packed fused silica microbore column. This column could be used for CE, CEC, or capillary HPLC separations. Using a 600 MHz NMR, they realized a mass limit of detection of ~300 ng in the stop-flow mode. They also reported that in the online mode, the limit of detection was adequate for all three microseparation techniques. A major advantage to this system is the ease of changing from one separation method to another. When this microflow cell was used in an online CE analysis of paracetamol metabolites in an extract of human urine (at 600 MHz), the mass limit of detection was ~10 ng [171]. Online CEC analysis afforded a similar mass limit of detection. Identification of metabolites by NMR could be accomplished with nanoliter sample volumes. Improvements in flow cell and probe design are likely to reduce limits of detection even further, and microseparation methods coupled to online NMR detection will almost certainly become routine tools for metabolite identification. Metabolomic studies are routinely performed using NMR-LC systems; for example, a high-throughput metabolomic profiling system that used a short monolithic column, a rapid gradient, and a high flow rate to analyze large numbers of urine samples was recently reported [148].

LC-NMR-MS

The hyphenated techniques of LC-MS and LC-NMR are both very powerful tools for characterization of xenobiotic metabolites, but they both suffer from unavoidable limitations. Neither technique can, by itself, provide the unequivocal assignment of chemical structure in all cases. MS often cannot distinguish between positional isomers, but NMR is very good for this application. Certain functional groups do not contain NMR-active nuclei and are invisible in NMR spectra. An example of an NMR-invisible functional group is the sulfate group of sulfate conjugates. In such cases, MS can be used to characterize the sample. Because MS and NMR are complementary techniques and both have been successfully hyphenated with LC, it was inevitable that these techniques would be merged to form LC-NMR-MS. The first report of successful LC-NMR-MS appeared in 1995 [156] and was soon followed by reports of its application for characterization and quantification of xenobiotic metabolites [24,42,79,167–169,178,215]. The advantages of LC-NMR-MS are obvious in these reports. By capitalizing on the strengths of NMR and MS in one method, it affords rapid identification of unknown compounds in complex mixtures such as urine in a single chromatographic run. With the increasing availability of LC-NMR instruments that can be coupled to mass spectrometers, more reports of LC-NMR-MS in toxicology research are sure to appear.

Limitations

Although NMR spectroscopy is a very powerful tool for studying mechanisms of toxicology, it is subject to a number of significant limitations. Perhaps the greatest limitation of NMR is its sensitivity. As discussed earlier, even the most sensitive spectrometers require nanograms of sample for NMR analysis. Advances in magnet, flow cell, and probe design have greatly reduced the amount of sample required for NMR, but it would be unrealistic to assume that NMR will be as sensitive an analytical technique as MS in the foreseeable future. Another limitation of NMR is cost and, hence, availability. NMR spectrometers are quite expensive, and their price goes up dramatically with field strength. High-field spectrometers are not common laboratory instruments, so instrument time must be purchased from a local (or regional) NMR laboratory or acquired through collaboration. NMR spectroscopy is a specialized discipline requiring a significant amount of expertise. Operation of NMR spectrometers and interpretation of spectra are not straightforward and require a great deal of training and experience. A toxicologist lacking this training and experience would do well to establish an active collaboration with an NMR spectroscopist to carry out sophisticated NMR studies.

EPR Spectroscopy

A free radical is a chemical species that contains an unpaired electron. Extremely reactive, free radicals play a role in the mechanisms of tissue injury and toxicity of many chemicals [7,68,133]. Free radicals are often formed during xenobiotic metabolism by enzymes such as the cytochromes P450 and peroxidases. In the Haber–Weiss reaction, superoxide forms the extremely reactive hydroxide radical (HO·) in the presence of ferrous ion. The toxic effects of iron overload have been attributed to the hydroxyl radical. Free radicals are also involved in lipid peroxidation, leading to LDL oxidation and

atherosclerosis. Because free radicals are so reactive, they have short lifetimes and are present at very low concentrations in biological systems; consequently, they are usually difficult to detect. Although indirect methods such as product analysis, inhibition by antioxidants, and other radical scavengers and photolysis have been used to detect free radicals in biological systems, these methods provide little information on the nature of the radical. *Electron paramagnetic resonance* (EPR) spectroscopy, also known as *electron spin resonance* (ESR) spectroscopy, is the most versatile and information-rich method for free-radical analysis. The following sections provide a concise discussion of the theory of EPR spectroscopy, as well as several examples of its application in toxicology research. The basic phenomenon of magnetic resonance is common to both EPR and NMR; thus, the reader should be familiar with the preceding theoretical discussion of NMR spectroscopy. More detailed descriptions of the theory and instrumentation of EPR are available [3,6,153].

Basic Theory of EPR Spectroscopy

Like a proton, an electron rotates about its axis and has a magnetic moment (μ). In an applied magnetic field, B_o, this magnetic moment will precess around the axis of B_o with a characteristic precessional frequency (υ). Because the spin quantum number (*S*) of an electron is 1/2, it exists in two energy states in a magnetic field. The energy difference (*E*) between these two spin states is given by

$$E = h\nu = gB_o \frac{eh}{4\pi m_e c} \tag{41.5}$$

where

m_e is the electron mass
e is the electronic charge
c is the speed of light
h is Planck's constant

The proportionality constant (*g*), the spectroscopic splitting factor, is the ratio of the magnetic moment to the angular momentum and has a value of 2.0022319 for an unbound electron.

The exact precessional frequency of an electron in a radical and the position of its resonance signal depend on its chemical environment. In NMR spectroscopy, the position of the resonance signal is denoted by the chemical shift (δ). In EPR, resonance positions are expressed as *g* values. Tables of the *g* values of common radicals are available [59]. Because the magnetic moment of an electron is approximately 700 times greater than that of a proton, the energy difference between spin states is correspondingly higher in EPR than in NMR. Accordingly, EPR is more sensitive than NMR, and EPR spectra can be obtained from radicals present in low micromolar concentrations. The larger energy difference between radical spin states also means that EPR requires more energy than NMR spectroscopy. The frequencies used for EPR are in the microwave region of the electromagnetic spectrum. In a typical EPR spectrometer operating at a field strength of 0.34 T, the precessional frequency of an electron is approximately 9.5 GHz.

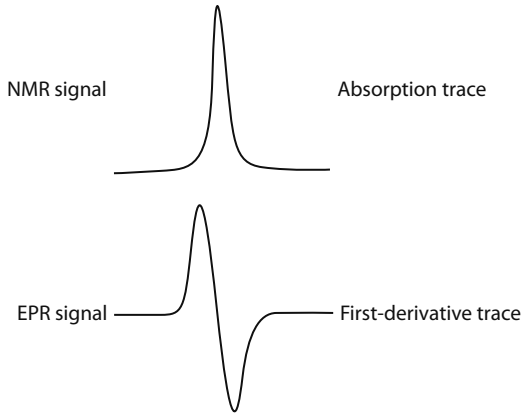

FIGURE 41.15 NMR spectrum plotted as an absorption trace and an EPR spectrum plotted as a first-derivative trace.

EPR signals are typically much broader than NMR signals. Assignment of EPR resonance positions (*g* values) is facilitated by plotting first-derivative traces. Although NMR spectra are plotted as absorption vs. frequency, EPR spectra are plotted as the rate of change of absorption vs. frequency. These two types of spectral traces are shown in Figure 41.15. Because radicals have only one unpaired electron, the EPR spectrum of a single radical species will generate only one signal. If a sample contains more than one radical species, multiple signals will appear. The total area under a peak in an EPR spectrum is proportional to the number of unpaired electrons in a sample associated with that peak. For quantification of unknown radical concentrations in a sample, direct comparison to a radical standard of known concentration is made. Common radical standards include diphenylpicrylhydrazyl (DPPH), which contains 1.53×10^{21} unpaired electrons/g.

In a radical, the unpaired electron is not associated with only one atom. The electron spin is distributed over several atoms in the radical and may interact with any spin-active nuclei with which it is associated. The interaction of electron and nuclear spins leads to spin–spin coupling called *hyperfine splitting*, similar to the coupling seen in NMR. An EPR signal will be split into $2nI + 1$ peaks, where *n* is the number of equivalent nuclei of spin *I*. The hyperfine splitting constant is denoted a_i, where *i* is the atomic symbol of the nucleus to which the electron is coupled. Hyperfine EPR splitting constants are typically measured in gauss (G) or in hertz (Hz). Figure 41.16 shows a computer-simulated spectrum of the methyl radical. The unpaired electron is coupled to three equivalent protons, so the signal appears as a quartet. The magnitude of the hyperfine splitting constant is directly proportional to the electron spin density at the coupled nucleus. It is thus related to the probability of finding the unpaired electron associated with the coupled nucleus. Hyperfine splitting constants are often applied in the interpretation EPR spectra. Tables of a_i values are available for reference [59].

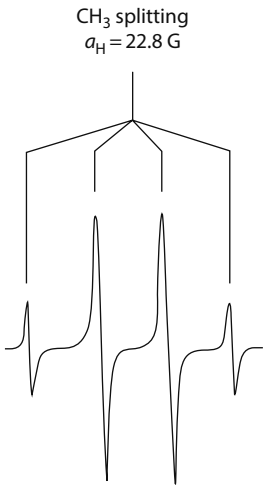

FIGURE 41.16 Computer-simulated EPR spectrum of the methyl radical showing the 22.8 G hyperfine splitting caused by coupling of the unpaired electron to the three spin 1/2 protons.

Instrument Design

EPR spectrometers contain the same basic components as NMR spectrometers: a probe, a magnet, a source of electromagnetic microwave radiation, a transmitter and receiver with amplifiers, and a data collection system. In conventional EPR, the microwave frequency is typically held constant, and the magnetic field is varied during spectral acquisition. Most conventional EPR spectrometers operate with a field strength of 0.34 T with field swept over a several hundred G range. The microwave frequency for a field strength of 0.34 T is about 9.5 GHz. FT methods and frequency pulse techniques are also employed in EPR. Multipulse 1D experiments and sophisticated 2D experiments may be performed.

Spin Trapping

Some free radicals are stable enough in solution to be directly detected by EPR; however, most free radicals of interest to the toxicologist are quite unstable, so an indirect detection method is often required. Spin trapping is the most commonly used method for indirect free-radical detection. Spin trapping entails adding a spin trap (typically a nitrone or nitroso compound) to the sample prior to radical generation. When the radical is generated, it rapidly reacts with the spin trap to produce a secondary radical or spin adduct, more stable than the parent free radical and detectable by EPR. Although the detectable species is not the parent radical, the nature of the parent radical may often be determined by comparison with model reactions between known radicals and the spin trap. Detailed descriptions of in vitro and in vivo EPR experiments are available [25,26].

Applications

Model Studies

The EPR study of appropriate model systems in aqueous solutions may yield important insights into related in vivo radical reactions and pathways. Such model systems are often used to study free-radical processes thought to play a role in radical toxicity. Compounds containing cobalt or other transition metals have been linked to free-radical generation by reaction of the metal ions with lipid hydroperoxides. An examination of the in vitro reaction of Co(II) with H_2O_2 and lipid hydroperoxides in the presence of various biological ligands and the spin trap DMPO revealed that, in the presence of sulfhydryl-containing molecules, Co(II) can react with H_2O_2 and lipid hydroperoxides to generate free radicals [176]. This observation may be important for understanding Co(II)-mediated toxicity and carcinogenicity. High-field EPR and site-directed spin labels have been used to study the structure and conformational dynamics of toxic proteins, thereby providing detailed information on transient intermediates and their relationship to biological action [130]. D-Mannitol has neuroprotectant properties and reduces sensory neurological disturbances associated with ciguatera intoxication. In vitro EPR analysis of rat neurons exposed to the toxin and several potential neuroprotectants and antioxidants revealed that toxin-induced changes in neuronal excitability could not be attributed to toxin-mediated hydroxyl radical generation alone. The authors suggest that the neuroprotectant effects of D-mannitol are more complex than simple osmotic reduction in neuronal swelling and may be due to reduced toxin association with ion channels [16].

Spin Trapping

Direct evidence for in vivo free-radical generation in response to xenobiotic exposure may be obtained with EPR spin-trapping techniques. Ozone exposure is believed to cause pulmonary damage as a result of lipid peroxidation in lung tissue. Kennedy et al. [95] employed a spin trap with EPR in ozone-exposed rats and detected a spin adduct in lipid extracts of lung tissue obtained from exposed animals. The concentration of the radical spin adduct in lung extracts correlated well with ozone exposure, suggesting both free-radical production and toxicity in ozone-exposed lungs.

Nitric oxide is an important endogenous metabolite, vasoactive substance, and neurotransmitter in many organisms. At higher concentrations, nitric oxide may have toxic effects, and its production may be induced by exposure to xenobiotics. Nitric oxide contains an unpaired electron and is a relatively stable free radical. Nitric oxide binds to Fe(II)-containing molecules such as hemoglobin and endogenous iron–sulfur proteins, thereby forming stable complexes readily detectable by EPR. EPR is useful for studying nitric oxide–Fe(II) complexes in iron-containing biomolecules generated in response to environmental toxins and disease. The administration of an endogenous spin trap has also been extensively employed for EPR studies. Kubrina et al. [98] used an Fe(II)–diethyldithiocarbamate (Fe-DETC) complex, which forms a characteristic radical spectrum with nitric oxide, to clearly demonstrate that nitric oxide originates from the guanidine nitrogens of L-arginine in vivo. ^{15}N-labeled L-arginine (L-guanidineimino-^{15}N-arginine) was administered to rats along with the Fe-DETC complex and lipopolysaccharide.

EPR analysis of the livers from sacrificed animals revealed a very characteristic doublet hyperfine splitting associated with ^{15}NO complexed with the iron. This study clearly demonstrated the application of both spin trapping and stable isotope labeling in EPR while providing the first direct evidence that L-arginine is the ultimate precursor of endogenous nitric oxide.

EPR Imaging

Sensitive and specific, EPR is readily adaptable to in vitro and in vivo imaging of free radicals in biological samples [185]. Measurements of in vivo blood oxygenation in rats [101] and in vivo imaging of kidneys [100] and tumor heterogeneity and oxygenation in mice [102] are some examples. A murine model of septic shock has been developed using EPR for in vivo monitoring of real-time nitric oxide generation secondary to intoxication with bacterial lipopolysaccharide [84]. The authors employed both surgically implanted in situ radical probes and a soluble systemic probe with EPR to monitor and localize both oxygen and nitric oxide. In vivo EPR techniques are also extensively employed in the pharmaceutical industry. Pharmacokinetic and pharmacodynamic interactions, as well as the metabolism of nitroxides and metals, have been measured noninvasively in vivo with EPR [120]. An in vivo evaluation of adriamycin nephropathy in rats [145] employed EPR with a nitroxide radical to monitor the reducing ability of renal tissues in adriamycin-exposed rats. The decay rate of the EPR signal intensity correlated with renal reducing activity and the extent of drug-induced nephropathy.

Limitations

EPR analysis in toxicology is limited by the very nature of the species under study, the free radical. Unstable free radicals are generally detected indirectly by spin-trapping techniques. The spin trap itself may have toxic effects independent of the toxic mechanisms under study. Because spin adducts are secondary radicals, it is not always possible to determine the structure of the parent radical by EPR analysis of the spin adduct. EPR is nonetheless a very important tool for studying free-radical mechanisms of toxicity and may be combined with other analytical techniques, such as stable isotope labeling. EPR remains the method of choice for detecting and monitoring free radicals both in vitro and in vivo.

UV-VIS Spectrophotometry

Principles

UV and visible (VIS) absorption spectrophotometry has been the principal methods of chemical analysis for many years. It involves the measurement of light absorption by substances in the wavelength region from 190 to 380 nm for UV absorption and from 380 to 900 nm for VIS light absorption [41,48,173,184]. Light absorption in these regions arises from electronic transitions within the molecule. The frequency of absorption depends on the energy difference between the normal or ground state of an electron and that of the excited

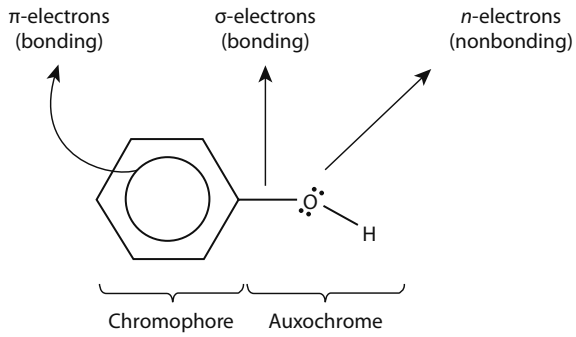

FIGURE 41.17 Some basic terminologies in UV spectrophotometry.

state (higher energy level). Absorption of UV or VIS light is also accompanied by vibrational and rotational transitions that result in relatively broad band characteristic of UV-VIS spectra.

A molecule containing electrons in σ, π, and *n* orbitals (see Figure 41.17) may absorb light energy and be promoted from the ground state to higher energy states. Antibonding orbitals (σ* and π*) exist in the excited state for the bonding electrons, and *n* electrons may be associated in the ground state with heteroatoms that do not participate in bonding yet can absorb energy and be promoted to either σ* or π* orbitals. From a practical consideration, the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions are of most utility because these transitions occur in the useful range (200–750 nm) of the UV-VIS spectrum; $\sigma \rightarrow \sigma^*$ transitions require more energy and usually occur at wavelengths of less than 200 nm. Compounds that contain nonbonding electrons on oxygen, nitrogen, sulfur, or halogen atoms can undergo $n \rightarrow \sigma^*$ transitions; however, these are of lower energy than $\sigma \rightarrow \sigma^*$ transitions. The absorption due to $n \rightarrow \sigma^*$ transitions is of limited utility because it is very weak and in most cases occurs at wavelengths too short to be easily measured on conventional instruments—for example, trimethylamine (λ, 277 nm; ε, 227) and methanol (λ, 183 nm; ε, 150). Molecules that contain oxygen, nitrogen, sulfur, or halogen atoms usually show an intense absorption, known as *end absorption*, around 200 nm due to $n \rightarrow \sigma^*$ transitions.

The energy required for the $\sigma \rightarrow \sigma^*$ transition is very high; consequently, compounds in which all valence shell electrons are involved in single-bond formation, such as saturated hydrocarbons, do not show absorption in the ordinary UV region. An exception is cyclopropane, which shows a wavelength of maximum absorption (λ_{max}) of about 190 nm (propane shows λ_{max} about 135 nm). Transitions to antibonding π* orbitals are associated with unsaturated centers in the molecule such as alkenyl, carbonyl, imino, and azo groups. These transitions are of relatively lower energy requirements and occur in the useful part of the UV spectrum—for example, C=O: ~285 nm (low intensity, $n \rightarrow \pi^*$) and 185 nm (high intensity, $\pi \rightarrow \pi^*$). The $\pi \rightarrow \pi^*$ transitions lie between $n \rightarrow \sigma^*$ and $n \rightarrow \pi^*$ transitions in terms of energy content. Figure 41.18 illustrates in a nonempirical manner the relative electronic excitation energies for the previously mentioned electronic transitions.

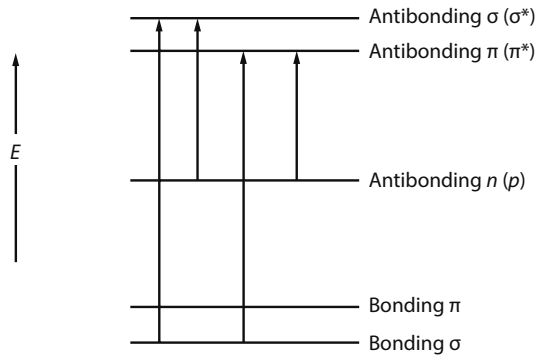

FIGURE 41.18 Summary of electronic energy transitions.

Quantitative Aspects of UV-VIS Spectrophotometry and the Beer–Lambert Law

When UV light traverses a cell containing an absorbing solute dissolved in a suitable solvent, the light intensity is diminished by reflection at the inner and outer surfaces of the cell, by light scattering by any particles in the solution, or by absorption of light by the molecules of the solute. The intensity of the light absorbed can be expressed as

$$I = I_{\text{absorbed}} - I_0 - I_T \tag{41.6}$$

where

I_0 is the original intensity incident on the cell
I_T is the reduced intensity transmitted from the cell

The transmittance (T) is the ratio I_T/I_0 and the percent transmittance ($\%T$) is given by

$$\%T = \frac{100 I_T}{I_0} \tag{41.7}$$

The *absorbance* (A) is the common logarithm of the reciprocal of T:

$$A = \log \frac{I_0}{I_T} \tag{41.8}$$

It can be shown that the intensity of a beam of parallel monochromatic radiation decreases exponentially as it passes through a medium of homogeneous thickness. Or, alternatively, the absorbance is proportional to the path length (b) of the solution. This is the basis of Lambert's law.

Beer's law states that the intensity of a beam of parallel monochromatic radiation decreases exponentially with the number of absorbing molecules, or more simply the absorbance is proportional to the concentration (c). A combination of the two laws yields the Beer–Lambert law:

$$A = \log \frac{I_0}{I_T} = abc \tag{41.9}$$

The proportionality constant (a) is the absorptivity. The name and value of a depend on the units of concentration. When c is in mol/L, the constant is called *molar absorptivity* or the *molar extinction coefficient* (ε). Thus,

$$A = \varepsilon bc \tag{41.10}$$

The molar absorptivity at a specified wavelength of a compound in solution is the absorbance at that wavelength of a 1 mol/L solution in a 1 cm cell. The units of ε are therefore L/mol/cm. Expressing the absorptivity in terms of a 1 mol/L solution facilitates the comparison of the light-absorbing abilities of compounds with widely differing molecular weights. Substances that have ε values less than 100 are weakly absorbing; those with ε values above 10,000 are intensely absorbing. Many absorbing xenobiotics and drugs have ε values at their wavelengths of maximum absorption of $10^{3.5}$–$10^{4.5}$.

Another form of the Beer–Lambert proportionality constant is the *specific absorbance*, which is the absorbance of a specified concentration in a cell of specified path length. The most common form is the A (1%, 1 cm) value, which is the absorbance of a 1 g/100 mL (1% w/v) solution in a 1 cm cell. The Beer–Lambert equation therefore takes the following form:

$$A = A_{1\text{ cm}}^{1\%} bc \tag{41.11}$$

where

c is in g/100 mL
b is in cm

Whenever an analyte is involved in an equilibrium, such as protonation or deprotonation, tautomerism, dimerization, or complex formation, the material added to the solution will be distributed among several forms, and the apparent concentration (amount of material dissolved/volume) will not be proportional to the actual concentration of the parent substance. A deviation for Beer's law will be observed under these circumstances unless the absorptivity is identical for all the species present or the equilibrium is controlled in some manner. If only two species are present and their spectra are not too different, useful measurements following Beer's law can be made by measuring at the isosbestic point rather than at λ_{max}. The isosbestic point is the wavelength at which the UV spectra of the two species cross when measured at equal molarities or, equivalently, the wavelength at which their molar absorptivities are equal. Deviations arising from acid–base equilibria can be avoided by carefully buffering the solutions because the ratio of protonated to deprotonated analyte will be constant at constant pH. A variety of other equilibria can be controlled in a similar manner.

Absorption spectra of compounds with conjugated chromophores or aromatic moieties in their structure show maxima shifts to longer wavelengths (bathochromic shifts) when compared to the wavelength of individual chromophores. This is due to increased stability of the π-electron system, which requires less energy for the $\pi \rightarrow \pi^*$ transition. This bathochromic shift is usually accompanied by an increase in intensity of the absorption (a hyperchromic shift).

Instrument Design

Single-Beam Spectrophotometers

The arrangement of the components in a commercially available, single-beam, UV-VIS spectrophotometer is shown in Figure 41.19. The essential characteristic is that the light travels in a single continuous optical path between the light source and the detector. Single-beam instruments are relatively inexpensive and are satisfactory when many samples are being assayed by a simple measurement of absorbance at the same wavelength. A major disadvantage is the need to reset the 100% transmission value at each wavelength to compensate for the large variation of intensity of light from the lamp at each wavelength.

Double-Beam Spectrophotometers

In this type of instrument (Figure 41.20), the monochromatic light is split by a rapidly rotating beam chopper into two beams that are alternately directed in rapid succession through a cell containing the sample and one containing only solvent. If there is greater absorption of light in the sample cell than in the reference cell, the recombination of the beams at the detector produces a pulsating current that is converted

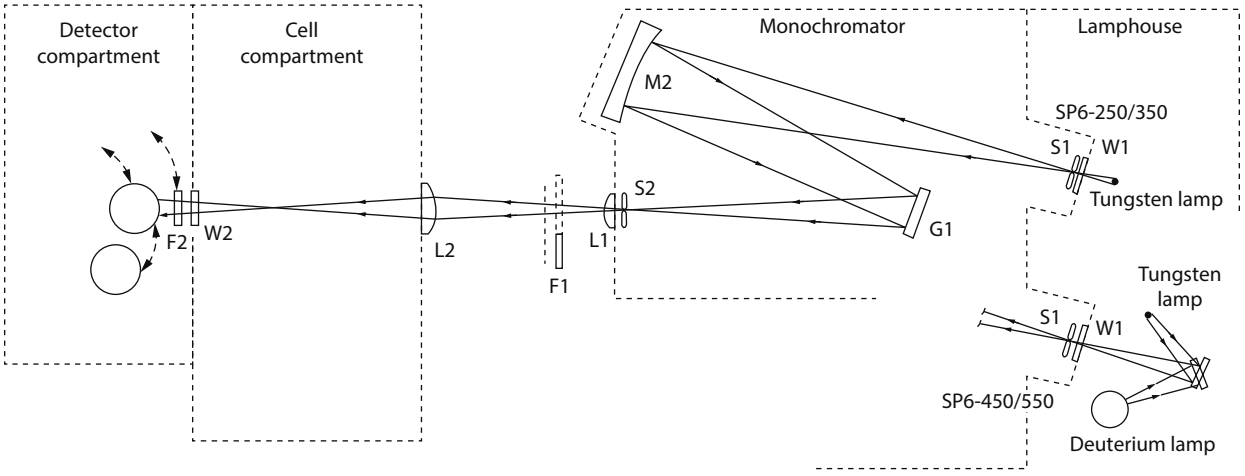

FIGURE 41.19 Optical diagram of a single-beam UV spectrophotometer; abbreviations: F = filter, G = grating, L = lens, M = mirror, S = slit, and W = window. (Courtesy of Pye Unicam, Ltd.)

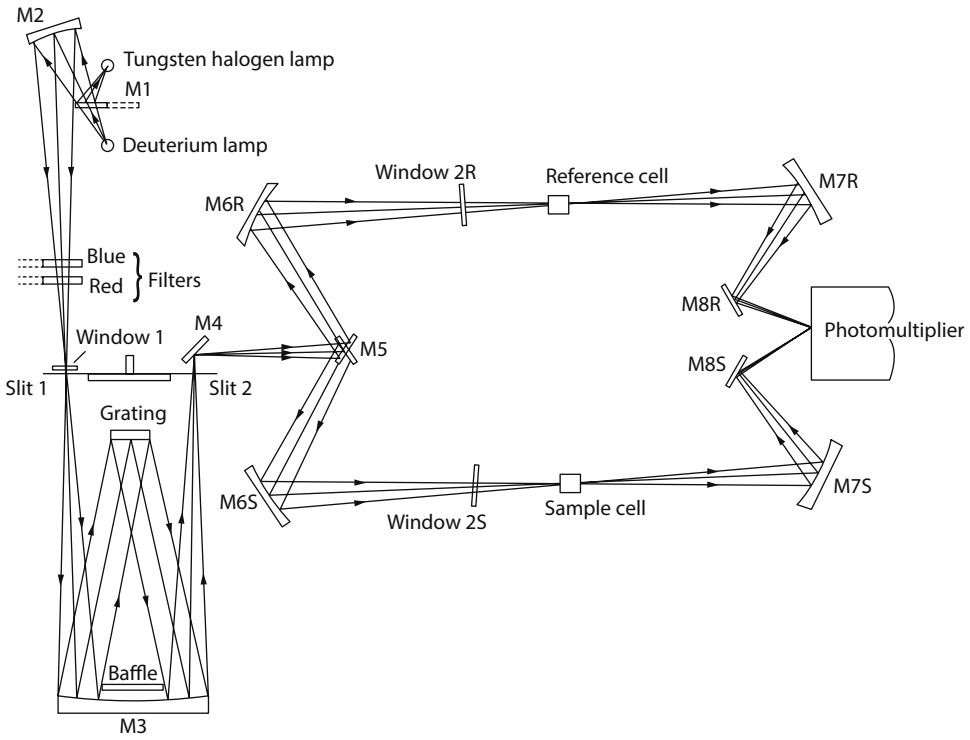

FIGURE 41.20 Optical diagram of a double-beam UV-VIS spectrophotometer. (Courtesy of Pye Unicam, Ltd.)

into two dc voltages proportional to the light intensities I_0 and I_T transmitted by the reference solution and the sample solution, respectively. The ratio of voltages is recorded as a transmission. Double-beam optics, therefore, automatically compensate for variation of I_0 with wavelength. Recording spectrophotometers are double-beam instruments equipped with a wavelength scanning device that enables rapid automatic scanning of spectra.

Solvents and Sample Conditions

Solvents

The solvent of choice in UV-VIS spectrophotometry is determined by several factors, including the solubility of the analyte and the absorption of the solvent at the wavelength employed for analysis. The solvent should be available in a purity grade suitable for carrying out spectrophotometric work, devoid of contaminants that are either fluorescent or absorb at the analytical wavelength. Moisture-sensitive compounds require nonhygroscopic solvents that are easily dried. Recovery of the analyte after analysis requires the use of more volatile solvents.

It is important to note that the analyte may be sensitive to pH changes; for example, acid–base equilibria, tautomerism, complex formation, and other equilibria are often pH dependent. In such systems, then, a strongly acidic or basic solvent may be indicated to ensure that the analyte is present in solution as a single species. Nonabsorbing buffers may also be used in UV analysis for this purpose.

The exact wavelength of a particular electronic transition depends not only on the chromophore but also on the solvent, on substituents present on the chromophore, and on chromophore geometry. The solvent effect arises because solvation alters the electronic energy levels of a chromophore, and the degree of solvation is frequently different for the ground and excited states. If the ground state is solvated more strongly than the excited state, the energy difference between the levels is increased. The increase in energy difference is reflected in a shift of the absorbance to shorter wavelengths (hypsochromic or blue shift) than those observed in the gas phase where there is no solvation. If the excited state is solvated more strongly, the energy difference decreases and absorbance is shifted to a longer wavelength (bathochromic or red shift). Absorption due to $n \rightarrow \sigma^*$ and $n \rightarrow \pi^*$ transitions is usually shifted to shorter wavelengths in more polar solvents.

If a group is more polar in the ground state than in the excited state, the nonbonding electrons in the ground state are stabilized (relative to the excited state) by hydrogen bonding and other electrostatic interactions with a polar solvent. The absorption is shifted to shorter wavelengths (higher energy) with increasing solvent polarity. Conversely, if the group is more polar in the excited state, the nonbonding electrons of the excited state are stabilized (relative to the ground state) by interaction with a polar solvent, and the absorption is shifted to longer wavelengths (lower energy) with increasing solvent polarity. Thus, polar solvents generally shift the $n \rightarrow \pi^*$ and $n \rightarrow \sigma^*$ bands to shorter wavelength and the $\pi \rightarrow \pi^*$ band to longer wavelength.

Cells

UV-VIS cells (also called *cuvettes*) may be made of glass (for use down to about 360 nm) or silica. Disposable plastic cells are also available. They usually have a transmission cutoff at about 320 nm and may not be suitable for high-precision work. Modern cells are fused and may be square, rectangular, or (rarely) cylindrical in section. Silica is substantially transparent between about 190 and 1000 nm, and special grades extend this range downward to below 180 nm and upward to about 2000 nm. For precision work and operation near the wavelength limits, the use of these purer silicas is recommended.

Path Length and Concentration

Optimum accuracy and precision in UV spectrophotometric analyses are obtained when the absorbance is about 0.9; however, in practice, absorbencies in the range of 0.3–1.5 are sufficiently reliable, and the combination of cell path length and concentration of analyte should be adjusted to give an absorbance within this range.

Correlation of Molecular Structure to UV-VIS Absorption

An isolated functional group not in conjugation with any other group is said to be a *chromophore* if it exhibits absorption of a characteristic nature in the UV or VIS region. If a series of compounds has the same functional group and no complicating factors are present, all of the compounds will generally absorb at nearly the same wavelength and will have nearly the same molar extinction coefficient. Thus, the spectrum of a compound, when compared to published spectra for known compounds, can be a valuable aid in determining the functional groups present in the molecule.

Auxochromes are groups that do not in themselves show selective absorption above 200 nm but that, when attached to a given chromophoric system, usually cause a shift in the absorption to longer wavelength and an increased intensity of the absorption peak. Common auxochromic groups are hydroxyl, amino, sulfhydryl (and their derivatives), and some of the halogens. These groups all contain nonbonding electrons; transitions involving these *n* electrons are responsible for these effects; for example, the absorption band at the longest wavelength of *trans*-pethoxyazobenzene is shifted 65 nm to longer wavelengths and is about twice as intense as that of the corresponding band of *trans*-azobenzene. Benzene shows λ_{max} 255 nm and ε 230, and aniline shows λ_{max} 280 nm and ε 1430. (Interestingly, the anilinium ion, which has no nonbonding electrons, shows λ_{max} 254 nm and ε 160.) Some functional groups that do not contain nonbonding electrons, such as alkyl groups, can also be considered as auxochromes due to weak inductive or hyperconjugative effects.

If two or more chromophoric groups are present in a molecule and they are separated by two or more single bonds, the effect on the spectrum is usually additive, as there is little electronic interaction between isolated chromophoric groups. However, if two chromophoric groups are separated by only one single bond (a conjugated system), a large effect on the

spectrum results because the π-electron system is spread over at least four atomic centers. When two chromophoric groups are conjugated, the high-intensity ($\pi \rightarrow \pi^*$ transitions) absorption band is generally shifted 15–45 nm to a longer wavelength as compared to the unconjugated chromophore.

Many colorimetric analyses developed for UV-absorbing drugs, xenobiotics, and metabolites have been based on the formation of an analyte-specific, multiple conjugated chromophoric system that readily absorbs in the visible range of the spectrum. This helps to avoid interfering UV absorption from impurities in the sample observed when the underivatized analyte is analyzed.

Compounds containing an extensively conjugated chromophore will appear colored to the eye if they absorb above 400 nm. As UV absorption peaks are frequently broad, the absorption of a peak with a λ_{max} of approximately 350 nm will generally extend into the VIS region. Usually, if a compound appears to be colored, it will contain not less than four and usually five or more conjugated chromophoric and auxochromic groups.

Substitution of aromatic chromophores with auxochromic groups is worthy of mention. When benzene is substituted by halogen or alkyl groups, only a slight shift with a small increase in extinction coefficient is seen; however, substitution by groups carrying nonbonding electrons or π-electrons (e.g., –OH, $–NH_2$, –CHO) causes a pronounced wavelength shift and a greatly intensified absorption relative to benzene.

In aromatic and conjugated structures where an auxochromic group may function as an acid or base, the effect of pH on the absorption spectrum of the conjugated system can be qualitatively useful; for example, the conversion of phenol (PhOH) to the phenolate ion (PhO^-) by addition of base results in an additional electron pair and a formal negative charge being located on the auxochromic group. The interaction of these electrons with the conjugated system results in a bathochromic–hyperchromic shift when compared to the neutral phenol spectrum. Adjusting the pH of the solution reverses this process, such that the phenol is regenerated. The conversion of aniline ($PhNH_2$) to the anilinium ion ($PhNH_3^+$) by lowering the pH of the medium results in a hypsochromic–hypochromic shift that is reversible by increasing the pH of the medium.

In polyaromatic compounds, as the number of fused rings increases, the absorption band is shifted to longer wavelengths. Extensively conjugated polyaromatics, such as naphthacene (λ_{max}, 480 nm; ε, 11,000—yellow) and pentacene (λ_{max}, 580 nm; ε, 12,000—blue), absorb in the VIS region of the spectrum. The spectra of simple heterocyclic aromatic compounds such as pyridine, pyrrole, indole, furan, thiophene, and their derivatives generally resemble the spectra of analogous benzenoid or naphthalenoid structures.

UV Spectrophotometry: Direct and Indirect Methods

When carrying out a quantitative spectrophotometric assay, the analyte is dissolved in a solvent that is transparent in the wavelength region to be examined. The wavelength normally selected is that at which the analyte exhibits maximum absorption (λ_{max}). The usual procedure is to obtain the absorbance value of the solution under nonscanning conditions (i.e., with the monochromator set at the analytical wavelength). Alternatively, if a recording double-beam spectrophotometer is used, the absorbance may be read from a recording of the spectrum. This latter procedure is generally utilized for qualitative purposes and in assays in which absorbances at more than one wavelength are required.

The measurement of absorbance is generally carried out using one of three methods:

1. Comparison with a standard absorptivity value
2. Use of a calibration curve
3. Single- or double-point standardization

Use of a standard absorptivity value is generally restricted to compounds that exhibit broad absorption bands and are not significantly affected by variation in instrumental parameters. An available value obviates the need to prepare a standard solution for absorptivity determination if the reference analyte is difficult to obtain.

The use of a calibration curve is a common procedure for carrying out quantitative spectrophotometric assays. The absorption of four or more standard solutions of the reference compound at concentrations above and below the expected concentration of the analyte is determined. A concentration vs. absorbance graph is then constructed. The concentration of the analyte in the sample solution is then read from the graph as the concentration corresponding to the absorbance of the solution. Calibration data are essential if the absorbance has a nonlinear relationship with concentration, if it is necessary to confirm the proportionality of absorbance as a function of concentration, or if the absorbance or linearity is dependent on the assay conditions. In certain VIS spectrophotometric assays of colorless substances, performed by converting the analyte to a colored derivative by heating it with one or more reagents, slight variation of assay conditions, such as pH, temperature, and time of heating, may cause a significant variation in absorbance. Here, experimentally derived calibration data are required for each set of samples.

Single- or double-point standardization is often used in place of a calibration curve. In the single-point procedure, the absorbance of a solution of the sample and that of a standard solution of the reference substance (the concentration should be close to that of the sample solution) are determined. The concentration of the test compound is calculated as follows:

$$C_{test} = \frac{A_{test} \times C_{std}}{A_{std}} \tag{41.12}$$

where

C_{test} and C_{std} are concentrations in the sample and standard solutions, respectively

A_{test} and A_{std} are the absorbances of the sample and standard solutions, respectively

This method is best suited for those compounds that obey Beer's law and for which a reference standard of acceptable purity is readily available.

Occasionally, a linear but nonproportional relationship between concentration and absorbance occurs, which is indicated by a significant positive or negative intercept in a Beer's law plot. A two-point bracketing standardization is therefore required to determine the concentration of the sample solutions. The concentration of one of the standard solutions is greater than that of the sample, while the other standard solution has a lower concentration than the sample. The concentration of the substance in the sample solution is given by the following equation:

$$C_{\text{test}} = \frac{(A_{\text{test}} \quad A_{\text{std}_1})(C_{\text{std}_1} \quad C_{\text{std}_2}) + C_{\text{std}_1}(A_{\text{std}_1} \quad A_{\text{std}_2})}{A_{\text{std}_1} \quad A_{\text{std}_2}} \qquad (41.13)$$

where std_1 and std_2 refer to the more concentrated standard and the less concentrated standard, respectively.

Direct spectrophotometric analysis of a xenobiotic or metabolite may not be possible for several reasons. The natural absorption of the analyte may occur at too low a wavelength to be useful, the molar absorptivity may be too small to give the required sensitivity, or other materials contaminating the analyte may absorb at the same wavelength. These problems can be overcome in many cases by chemical modification of the analyte to change its absorption characteristics. Some examples of useful derivatization procedures for spectrophotometric utility include (1) diazotization and coupling of primary aromatic amines, (2) condensation reactions (e.g., between amines or hydrazines and carbonyl compounds), (3) reduction of tetrazolium salts in the presence of an α-ketol group (–CHOH–C=O), (4) ion pairing of amines with ionized acidic dyes, (5) oxidation of the side chains of weakly absorbing compounds containing an aromatic ring, and (6) metal–ligand complexation. It is also possible to measure a substance by the change in absorbance when a chromophore is destroyed.

Difference Spectrophotometry

This method of spectrophotometric analysis is useful for obtaining selective and accurate analytical data on solutions of analytes containing absorbing interferants. Basically, the technique measures a difference absorbance (ΔA) between two equimolar solutions of the analyte in different chemical forms that exhibit different spectral characteristics. The method is valid provided that *reproducible* changes are induced in the spectrum of the analyte by the addition of one or more reagents and that the absorbance of the interfering substance is not altered by the reagents.

The simplest and most commonly employed technique for altering the spectral properties of the analyte is the adjustment of the pH by means of aqueous solutions of acid, alkali, or buffers. The UV-VIS absorption spectra of many substances containing ionizable functional groups, including phenols, aromatic carboxylic acids, and amines, are dependent on the state of ionization of the functional groups and consequently on the pH of the solution.

The pHs chosen must quantitatively form single species with at least 99% spectral purity. This can be achieved with monofunctional analytes (e.g., aromatic amines or aromatic carboxylic acids and phenols) by simply working at a pH at least 2 pK_a units above the pK_a of the analyte. The difference spectrum is obtained by placing one form of the analyte in the sample cell of the spectrophotometer and the other form in the reference cell and plotting the observed absorbance against wavelength. The value of difference spectrophotometry is that it provides a reference solution that contains both analyte and interfering substances in the same concentrations but at a pH different from that of the analyte solution in the sample cell. Interferants present in the sample should not be affected by the pH changes, and their contribution to the total absorbance is therefore cancelled.

The absorption spectra of the drug benzthiazide are shown in Figure 41.21 in both acidic and basic media [54]. The difference absorption spectrum is plotted as the difference in absorbance between the basic solution and the acidic solution against pH. The spectrum may be generated automatically using a double-beam recording spectrophotometer with the basic solution in the sample cell and the acidic solution in the reference cell. At 255 and 287 nm, both solutions have identical absorbance and consequently exhibit zero difference absorbance. Such wavelengths of equal absorptivity are the isosbestic or isoabsorptive points. Above 287 nm, the basic solution absorbs more intensely than the acidic solution and the ΔA is positive. Between 255 and 287 nm, ΔA has a negative value. A maximum in the difference spectrum occurs at

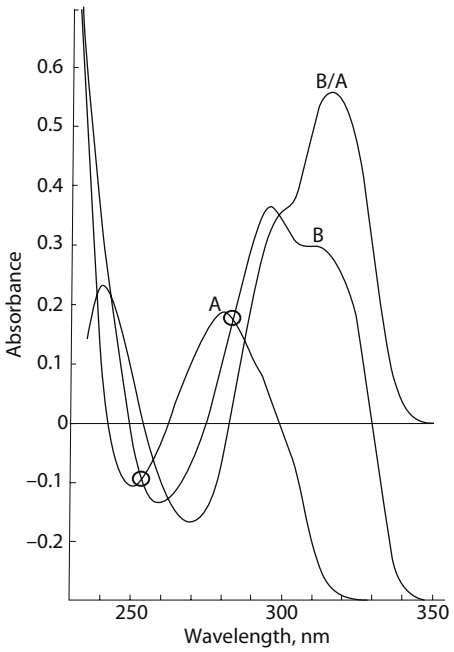

FIGURE 41.21 Conventional and difference spectra of benzthiazide (15 g/mL) in acidic solution (A) and basic solution (B) and the difference spectrum of basic vs. acidic solutions (B/A). (Courtesy of American Pharmaceutical Association.)

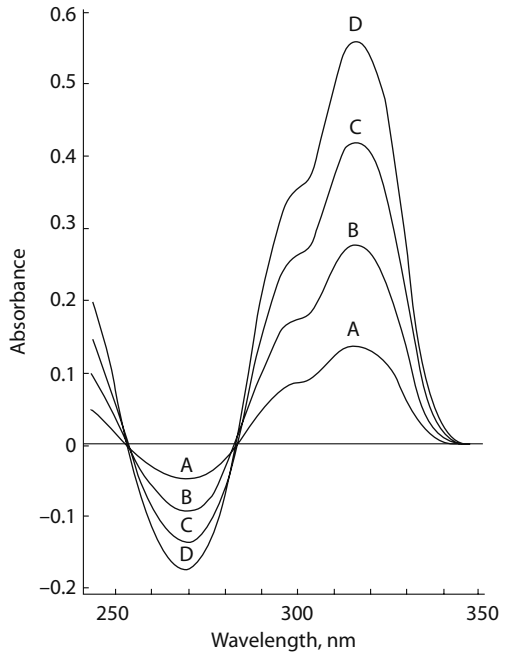

FIGURE 41.22 Difference spectra used to verify Beer's law for benzthiazide. Plots are basic vs. acid difference spectra for concentrations of 5 (A), 10 (B), 15 (C), and 20 g/mL (D). (Courtesy of American Pharmaceutical Association.)

313 nm and a minimum at 271 nm. The absorbance difference at these two wavelengths is termed the *amplitude*. A plot of absorbance differences vs. drug concentration will obey Beer's law and can be used for quantitative determinations. The isosbestic points are useful indicators of whether the absorbance of interferants in the sample is affecting the measurement of the absorbance of the drug. Figure 41.22 shows the isosbestic points for benzthiazide generated from several concentrations of the analyte. Any sample containing the drug or any standard curve concentration should show zero absorbance at the isosbestic wavelengths unless an interfering compound is present. If this is not observed, an alternative assay procedure or removal of the interferant is indicated.

Difference spectrophotometry is useful in the analysis of macromolecules such as proteins, peptides, and nucleic acids, as conformational effects are likely to be accompanied by changes in the environment of aromatic residues, which will in turn lead to small wavelength shifts as well as other perturbations. A difference spectrum in a protein may be generated by unfolding or by proteolytic degradation, as well as by more localized changes engendered by conformational adjustments or subunit association or dissociation, or, in favorable cases, the binding of a ligand, as well as a change in bulk solvent. The latter effect can be achieved simply by adding a benign perturbant, such as glycerol, sucrose, or D_2O, or by a change of temperature below the region of denaturation. Such a change in solvent character will in general affect only those aromatic residues in contact with solvent and therefore provides a means of determining, at least in a semiquantitative way, what proportion of aromatic residues are so exposed and following any change in this degree of exposure.

Special Considerations in the Spectrophotometric Determination of Xenobiotics and their Metabolites in Biological Matrices

The determination of xenobiotics and their metabolites in biological matrices, such as blood, tissues, or urine, is a more challenging procedure than the simple quantitation of aqueous solutions of analytes. Often, only a very small quantity of the xenobiotic or metabolite is present in a large volume of blood, urine, or tissue. Accordingly, solvent extraction of the analyte may be required. This nonspecific extraction may produce an extract that contains, in addition to the xenobiotic, endogenous compounds such as pigments and proteins that may render optical methods of analysis subject to additional error. Care should be taken in developing the analytical methodology, including choice of solvent, the pH of extraction, and the purification procedure. Solvent extractions for the quantitative recovery of the analyte may be further complicated by emulsification induced by surface-active components, as well as managing large volumes of solvent for evaporation. These problems may be ameliorated by utilizing a large solvent-to-sample ratio and by carrying out control analyses with biological matrices spiked with the analyte. Extraction procedures must account for xenobiotics that may be present both free and as polar, water-soluble conjugates such as glucuronide or sulfate. Another complicating factor for many drug molecules is protein binding. This can lead to poor recoveries of the drug, thereby necessitating the inclusion of a protein denaturation step in the overall extraction procedure.

Direct spectrophotometric procedures, even after purification of the sample by solvent extraction or chromatographic cleanup, often lack the sensitivity and selectivity required for the assay of low concentrations of drugs that are found in body fluids after the administration of therapeutic doses. Modified spectrophotometric techniques, however, such as those involving chemical derivatization or difference spectrophotometry, are sufficiently discriminating and sensitive for the assay of many drugs and other xenobiotics.

Flow-Through UV Detection

Many of these difficulties associated with the spectrophotometric determination of xenobiotics and metabolites in biological matrices can be overcome by using a tandem separation method with flow-through UV detection. CE, affinity columns, size-exclusion chromatography, and HPLC are routinely employed in flow-through UV detection systems. Indeed, because many xenobiotics exhibit characteristic UV spectra, the most common HPLC detector for toxicological applications is a UV spectrophotometer. UV detectors may be fixed-wavelength spectrophotometers operating at a single predetermined wavelength, variable-wavelength spectrophotometers that can be tuned to the λ_{max} of the analyte of interest, or scanning spectrophotometers that can collect spectra over the entire UV-VIS range. In all cases, the eluent from an HPLC column passes through a small flow cell placed between the light source and the photodetector. Flow-cell volume is typically on the order of 1–10 μL, so chromatographic resolution is not lost due to a large dead space. The

output of a UV detector is a chromatogram that plots change in absorbance vs. time. Perhaps the most significant development in the design of flow-through UV detectors is the rapid scanning multiwavelength photodiode array detector. This detector permits collection of spectra over the entire UV-VIS range during a chromatographic run. The data may be plotted as typical chromatograms at a wavelength of choice; absorption spectra of eluted peaks; or as 3D projects of absorbance, wavelength, and time. The use of HPLC with photodiode array detection has greatly facilitated the identification of xenobiotics and metabolites in biological samples.

Applications

Changes in the production of glycolytic metabolites may be useful biomarkers for monitoring pathophysiology, toxic injury, and clinical therapeutics. A metabolomic analysis of glycolysis cycle metabolites in human erythrocytes was developed using a tandem CE-UV analytical method [123]. The nine components of the glycolytic metabolome of 22 volunteers were readily and reproducibly assayed in micromolar quantities with minimal error. Temporal changes in the microbial metabolome may also be monitored using tandem UV methods. Over 400 fungal metabolites, including important mycotoxins, have been analyzed and identified in culture using chromatographic UV-MS systems [140].

Martin et al. [125] developed an in vivo VIS spectrophotometric assay to simultaneously monitor the nitric oxide, hemoglobin, and deoxyhemoglobin contents in discrete areas of the rat brain. The overlapping absorption of spectra of these oxidized hemoglobin chromophores has complicated their analytical resolution in biological matrices. Here, the combination of VIS spectroscopy with least-squares mathematical analysis allowed the researchers to study the interaction of the important biomolecule and potential toxin, nitric oxide, with hemoglobin status in brain tissue.

Tandem HPLC-phased diode array analysis has simplified the rapid detection and identification of drugs, toxins, and their metabolites in a variety of systems. This may be particularly useful for the clinical toxicologist [155]. When integrated with a database of known retention parameters and UV spectra, tandem HPLC-phased diode array analysis may be one of the most effective techniques for the rapid, systematic toxicological analysis of biological samples.

UV spectroscopy is often combined with other detection methods for specialized toxicological analyses. Domoic acid is a potent neurotoxin that causes amnesic shellfish poisoning in humans who have ingested contaminated shellfish. The presence of domoic acid in bivalve mollusks was determined for the first time in commercial shellfish harvested in Ireland using an LC-photodiode array UV-MS system [87]. Another application of tandem UV analysis in the area of food toxicology was conducted using size-exclusion chromatography with UV-MS detection for the determination of toxic metals in edible mushrooms [223]. The association of silver, arsenic, cadmium, mercury, lead, and tin with the high- and low-molecular-weight fractions of edible mushroom components was strongly influenced by mushroom species and growth medium composition.

IR Spectroscopy

Basic Technique

IR spectroscopy [146] deals with the absorption of electromagnetic radiation in the wavelength range from 0.8 μm (800 nm) to 1000 μm (1 mm). This range can be subdivided into the near-IR region (0.8–2 μm), the middle or fundamental IR region (2–15 μm), and the far-IR region (15–1000 μm). The fundamental IR region is the one that provides the greatest amount of information for the elucidation of molecular structure. Most IR spectrophotometers are designed to carry out measurements in this wavelength range.

When a molecule absorbs IR radiation, changes in the vibrational energy in the ground state of the molecule occur. All but the simplest of molecules have a large number of accessible vibrational and rotational energy levels with a correspondingly large number of allowed transitions. The energies of these transitions are strongly influenced by molecular structure; thus, IR spectra are highly structured, with unique features that are ideally suited for the identification of xenobiotics, drugs, and other organic substances. A typical IR spectrum of the antibacterial drug sulfacetamide is illustrated in Figure 41.23.

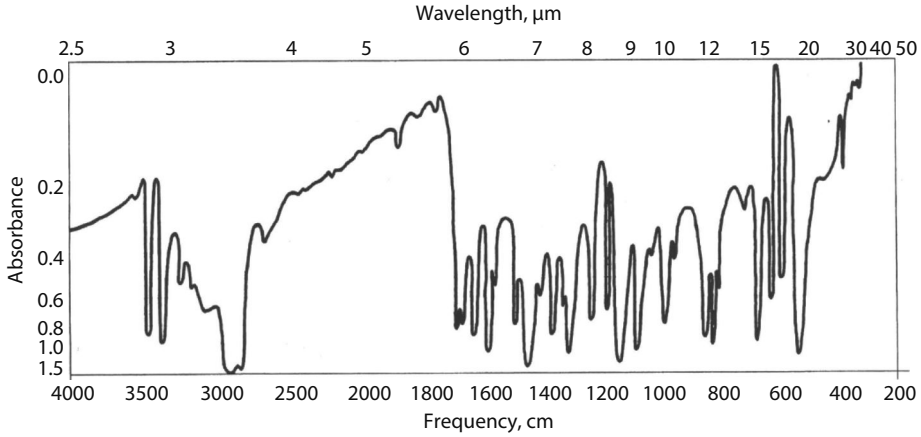

FIGURE 41.23 IR spectrum of sulfacetamide.

For energy to be transferred from the IR source to the molecule, the frequency of vibration of both must coincide, and energy transfer must be accompanied by a change in the dipole moment of the molecule. Molecules that contain certain symmetry elements will display more simplified spectra; for example, the C=C stretching vibration of ethylene and the symmetrical C–H stretching of the four C–H bonds of methane do not produce an absorption band in the IR region. For nonlinear polyatomic molecules, the number of fundamental modes of vibration is $3n - 6$ ($3n - 5$ for linear molecules), where n is the number of atoms. Certain groups within a molecule, such as –OH, –C=O, $–NH_2$, –CN, and –CC–, have characteristic absorption frequencies known as *group frequencies*. These frequencies are generally independent of the structure of the rest of the molecule and can therefore be used diagnostically to confirm the presence of the functionality in a molecule of unknown structure.

In practice, it is usually not possible to observe the calculated number of peaks in the spectrum of a known compound. This may be due to the superimposition or coalescing of absorptions that are too close to be resolved. A fundamental band may be too weak to be observed. Alternatively, additional (nonfundamental) bands may be observed that are either overtones and harmonics that occur with greatly reduced intensity or combination and difference bands.

Molecules have two types of fundamental vibrations. Stretching occurs when the distance between two atoms increases or decreases but the atoms remain in the same bond axis. Polyatomic molecules may produce in-phase (symmetric) or out-of-phase (asymmetric) stretching vibrations (see Figure 41.24). Bending (or deformation) occurs when the position of the atom changes relative to the original bond axis.

The stretching frequency (ν) of a bond is related to the masses of the two atoms involved (M_a and M_b, in g), the velocity of light (c), and the force constant of the bond (k, in dynes/cm). An approximate value for the stretching frequency can be calculated from the following equation:

$$\nu\,(\text{in cm}^{-1}) = \frac{1}{2\pi c}\sqrt{\frac{k}{M_a M_b/(M_a + M_b)}} \qquad (41.14)$$

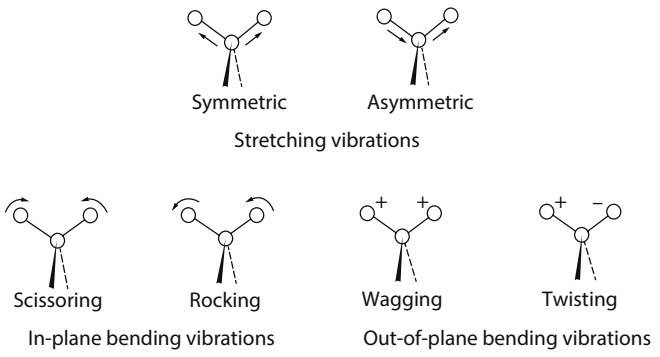

FIGURE 41.24 IR vibration modes of groups of atoms (+ and – refer to vibrations above and below the plane of the paper, respectively).

Note that the force constants for sp^3, sp^2, and sp bonds have values of 5, 10, and 15 $\times 10^5$ dynes/cm, respectively.

IR spectra are usually recorded as plots of sample absorbance (or percent transmittance) vs. wavelength or frequency in reciprocal cm (wave numbers). The relationship between wavelength and wave number is

$$\text{Wavenumber}\,(\text{cm}^{-1}) = \frac{1\times 10^4}{\lambda\,(\mu\text{m})} \qquad (41.15)$$

Unlike UV-VIS spectra, IR spectra are by convention plotted with zero absorbance (or 100% transmittance) at the top of the spectrum—that is, in an inverted mode relative to UV-VIS spectra.

Qualitative Uses and Interpretation of Spectra

The complexity in the IR spectra of organic molecules is a valuable tool that can be used for the unambiguous identification of unknown compounds if an authentic standard is available. If all the bands in the IR spectrum of the unknown structure are identical in all respects (i.e., in their wave number value and their relative intensity) when compared with an IR spectrum of an authentic standard, then the two compounds are identical. The region 1430 to 910 cm^{-1} contains many absorptions caused by bending vibrations as well as absorptions caused by C–C, C–O, and C–N stretching vibrations. As a molecule has many more bending vibrations than stretching vibrations, this region of the IR spectrum is particularly rich in absorption bands and shoulders and has been termed the *fingerprint region*. Although similar molecules may show very similar spectra in the region 4000 to 1430 cm^{-1}, there will nearly always be discernible differences in the fingerprint region. Spectral comparisons are best carried out in the solution state. Compounds can often be prepared in different polymorphic forms, both crystalline and amorphous, depending on the conditions of crystallization. Polymorphic forms of the same compound may show significant differences in the fingerprint region of the spectrum; therefore, if spectral comparisons are to be made in the solid state, then both the unknown and an authentic standard should be recrystallized from a specific solvent in the same manner.

A second important use of qualitative IR spectroscopy is that it gives structural information about an unknown molecule. The previously mentioned group frequencies, together with frequencies of other characteristic bands, can be utilized in the form of comprehensive frequency correlation charts that have been compiled for easy reference [59]. This type of compilation is invaluable as a means of confirming the presence or absence of a particular functionality in an unknown structure. In this respect, the region between 4000 and 1500 cm^{-1} is probably easier to interpret than that between 1500 and 650 cm^{-1}, as the latter includes many skeletal vibrations, typical of molecules as a whole, which are not diagnostic.

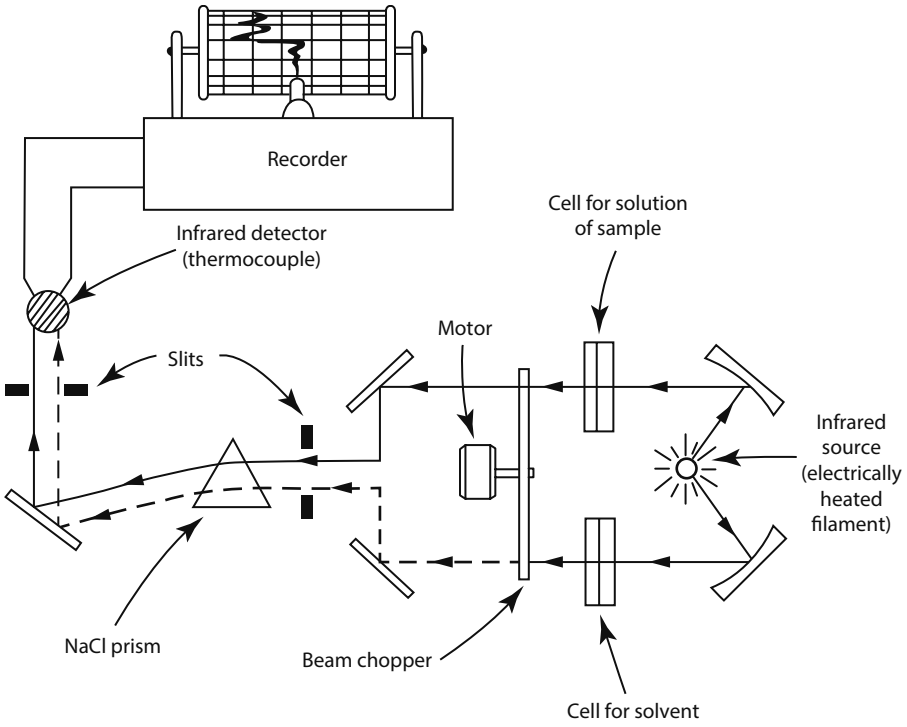

FIGURE 41.25 Schematic representation of a double-beam recording IR spectrophotometer.

Instrument Considerations

Dispersive IR Spectrophotometers

The arrangement of a typical double-beam recording IR spectrophotometer is shown in Figure 41.25. The beam chopper, which is a rotating mirror, permits radiation passing alternately through sample cell and solvent cell to reach the IR detector. The difference in absorption by solute and solvent is measured as an alternating electric current from the thermocouple. The system operates on the optical null principle with the recorder pen linked mechanically to a "comb" (not shown), which is placed across the solvent cell beam and moved by a servomechanism to reduce or increase the solvent cell-beam intensity. The servomechanism is actuated by the amplified thermocouple output to make the solvent beam intensity equal to the solution beam intensity, which reduces the detector output to zero or the null point. The spectrum can be scanned through the various wavelengths by rotation of the prism in synchronization with the motion of the recorder drum or chart. Most modern instruments now utilize a diffraction grating in place of the prism. Sodium chloride prisms can be used for the entire region from 4000 to 650 cm^{-1} but suffer from the disadvantage of low resolution at 4000 to 2500 cm^{-1}. The use of a grating monochromator provides a better overall resolution throughout the range 4000 to 625 cm^{-1}.

Fourier Transform IR Spectrometers

FTIR spectrometers are widely used in chemical analysis [2,64]. The FTIR spectrometer is built around an interferometer rather than a monochromator (see Figure 41.26). The beam from the light source passes through the chopper and is collimated and directed to the beam splitter via mirror C. The beam splitter is a half-silvered mirror that reflects 50% of the incident light onto movable mirror F and allows 50% to pass through fixed mirror E. The beams reflected from

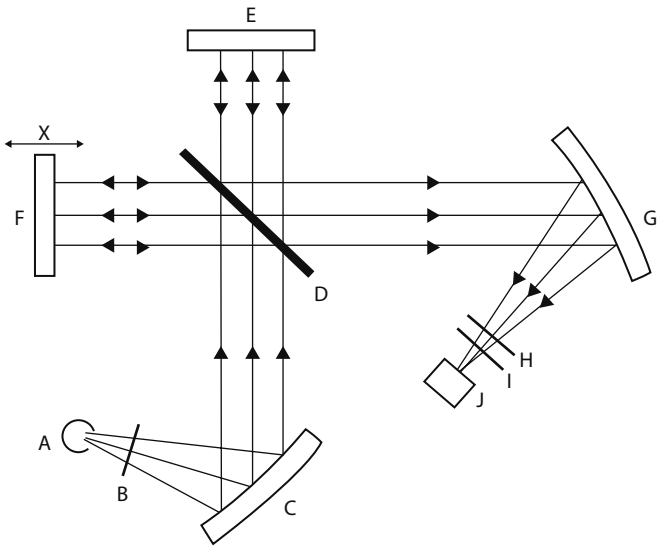

FIGURE 41.26 Layout of the optics of an FTIR spectrometer.

mirrors F and E are then combined at the beam splitter, so an interference pattern results, and this is focused by mirror G onto detector J. The sample to be analyzed is located in the combined beams between spectral filter H and the detector.

The interferogram of the source is obtained by driving the moveable mirror over a fixed distance and determining the interference pattern as a function of path length difference traveled by the light beams in the two arms of the interferometer. Each frequency of light that passes through the interferometer produces its own interference pattern. Because all of the frequencies generated are observed all at once by the detector, the spectrum is said to be multiplexed, and no grating is required to disperse the radiation. The increased energy reaching the detector allows the relatively rapid accumulation of data, so repetitive scans can be carried out.

Extensive data analysis must be undertaken to extract the frequency information from the interferogram. The principle computation required is Fourier transformation of the interferogram. Because data that have been transformed to the frequency domain are available in digital form, manipulations such as substitution of solvent background and smoothing are simplified.

FTIR analysis offers considerable advantages over conventional prism or grating instruments. The S/N under the conditions of the FT experiment is increased from the conventional apparatus by $N^{1/2}$, where N is the number of resolution elements in the spectrum; thus, for a 1000 cm^{-1} scan with 2 cm^{-1} resolution, the S/N advantage is $(1000/2)^{1/2}$ or about 22. These high S/N values permit the accurate subtraction of backgrounds due to liquid H_2O. Accordingly, one principle application of FTIR spectrometry is in the acquisition of protein spectra in aqueous media. In terms of sample preparation and analysis time, FTIR is considered to be a truly high-throughput analytical technique.

Quantification of IR Bands

Quantitative analysis of compounds by IR spectrophotometry utilizes the same basic principles involved in UV-VIS spectrophotometry; however, the complexity of the spectra obtained allows the selection of several bands for quantitative work. Generally, the selection of a fairly strong band for each component in a mixture is made, so no interference occurs between components. The areas of absorption bands are typically integrated; however, with sharp IR bands, peak heights may also be used for quantitative calculations. A calibration curve of absorbance vs. concentration can then be constructed. If Beer's law is obeyed, a direct comparison of the sample absorbance with that of a standard can be made.

In IR studies, very short path lengths (0.025–0.1 mm) are used, and solution concentrations of analytes are often in the 10% region. All solvents absorb in some part of the IR spectrum. Given this high concentration of solute, the accurate cancellation of solvent absorption is difficult to achieve. For this reason, the baseline technique is usually applied (see Figure 41.27). This technique assumes that absorption due to solvent (or a second component) is constant or varies linearly with wavelength over the region of the absorption band. The determination of small amounts of impurities or low amounts of solutes in a preparation can also be improved by introducing the major component, or the solvent, into the reference beam of the spectrophotometer, thereby compensating for the absorption of this component.

Due to the higher concentrations of samples required for IR analysis, deviations from Beer's law are encountered much more frequently in IR spectrophotometry than in UV-VIS spectrophotometry; thus, significant intermolecular hydrogen-bonding effects may be observed that increase as the concentration of the solute increases. Notably, the absorbance at the λ_{max} of a free OH group actually decreases with increasing concentration of the analyte.

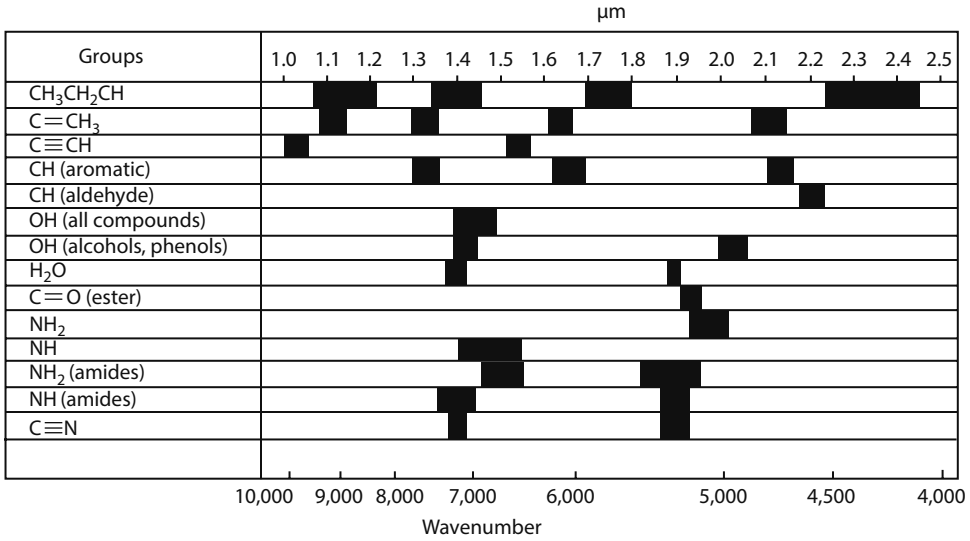

FIGURE 41.27 Characteristic group frequencies in the near-IR spectrum.

Sample Preparation and Sample Cells

Although IR spectra have been obtained on materials in every physical state from solids to gases, most analyses are carried out on the neat liquid analyte, solutions of the analyte in organic solvents, suspensions, Nujol suspensions, and KBr disks. Solution spectra are preferred for quantitative analysis, as errors arising from nonhomogeneous samples and path lengths are minimized. KBr disks are often used for qualitative analysis because subtle structural differences such as polymorphism can often be observed in this medium.

Cells for quantitative IR analysis of solutions consist of a pair of sodium chloride plates with a thin metal spacer between them. This unit is housed in a metal frame along with protective washers. The plate spacing is ~10^{-2} cm. Considerable care in handling and storing the IR cells should be exercised, because the windows are easily fogged by traces of moisture and are easily scratched. When not in use, cells should be stored in a desiccator.

Neat liquids and Nujol suspensions or mulls for qualitative analysis are usually placed as a drop on an unmounted circular sodium chloride plate, and a second plate is pressed on top until the liquid is spread into a thin film with a thickness on the order of 1×10^{-4} to 50×10^{-4} cm. The plates are held in a frame, while the spectrum is scanned. Nujol mulls are prepared by grinding the solid analyte in a small mortar with mineral oil until a milky emulsion is obtained. Other suspending agents such a perfluorokerosene, hexachlorobutadiene, or other heavy liquids can also be used. The spectrum obtained with mulls will consist of bands from the analyte superimposed upon bands from the mulling agent. The C–H regions of the spectrum will be obscured when mineral oil mulls are used, and the C–F or C–Cl regions will be obscured when the halogenated mulls are used.

Qualitative analysis of solid analytes is typically performed using KBr disks. Here, the analyte (0.3–9 mg) is ground with 300 mg of spectral-quality KBr (~400 mesh). Between 100 and 300 mg of the grind is then pressed into pellets or disks at between 20,000 and 100,000 psig using a stainless steel die and a vacuum pump. The finished pellet is transparent and produces excellent spectra. Unlike mulling agents, KBr does not contribute extraneous bands in the IR spectrum; however, KBr is slightly hygroscopic and may pick up moisture during the disk preparation. This will lead to characteristic water bands at 3300 and 1640 cm^{-1}.

Applications

High S/N spectra and sophisticated data-reduction techniques have made FTIR a versatile, high-throughput analytical technique. Advances in the acquisition of FTIR spectra from preparations such as monolayers, tissues, and samples in situ include fiber-optic waveguides for beam handling and remote field sensing. Metabolic fingerprinting, a process where crude metabolite mixtures are rapidly screened and analyzed by multivariate mathematical models, is central to metabolomic studies. FTIR is critical to these studies, wherein mixed sample profiles are correlated to biological origin, metabolic status, and exposure to chemical or environmental stimuli.

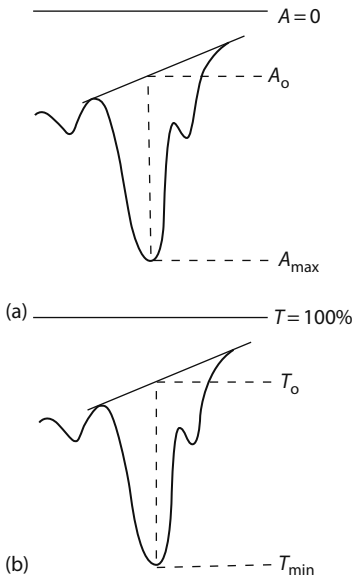

FIGURE 41.28 Measurement of IR band intensities using the baseline technique. (a) When the scale is linear in absorbance, peak absorbance = $A_{max} - A_o$. (b) When the scale is linear in transmittance, peak absorbance = $-\log (T_{min}/T_o)$.

Near-IR spectroscopy is particularly useful as a noninvasive in vivo analytical tool. Virtually every organic compound has a near-IR spectrum that can be measured. Near-IR spectra consist of overtones and combinations of fundamental mid-IR bands. These IR spectra are characterized by good penetration of light into tissues and a high capacity to identify organic compounds. Figure 41.28 illustrates some of the structural moieties that can be determined by near-IR and their absorption characteristics.

The application of in vivo FTIR microspectroscopy in the diagnosis of disease has been reviewed [91,108]. FTIR spectroscopy is used for studying human arteries, cancers and tumors, and brain tissues from stroke and Alzheimer's patients [109,110,122]. Wetzel et al. [211] utilized FTIR microspectroscopy for deuteration studies investigating the metabolic activity in various layers of the cerebellum. Some specific examples of the use of FTIR spectroscopy in disease diagnosis and toxicological research follow.

Analysis of Solid Human Tumor Cells by FTIR Microspectroscopy

Early detection of human tumors often allows for a higher survival rate. Cells from normal and neoplastic human lung tissues have been analyzed by means of FTIR microspectroscopy [14]. Reliable spectra that can differentiate between normal and neoplastic cells may be obtained. Neoplastic cells show an increase in the intensity of the bands corresponding mainly to the PO_2 symmetrical and asymmetrical vibrations (1080–1540 cm^{-1}) of DNA compared to normal cells. This analytical method may be useful for recognizing early neoplastic transformation that is usually not possible with traditional procedures. Similar results have been observed in the FTIR spectra of sections of normal and malignant human colon tissues and in other malignant tissues such as stomach,

esophagus, skin, liver, cervix, and vagina [219]. The results suggest that, in cancerous tissues, most PO_2 groups become hydrogen bonded and the intermolecular packing among PO_2 groups becomes closer. Nucleic acids may be the molecules primarily responsible for the observed changes in the $\nu_s(PO_2^-)$ and $\nu_{as}(PO_2^-)$ bands.

Analysis of Stroke-Induced Changes by Near-IR Spectroscopy

Historically, near-IR spectroscopy has been used to monitor fat and protein in agricultural products. Near-IR spectroscopy is also well suited for animal work because tissue penetration by near-IR light is good, excellent S/N values can be obtained in near-IR measurements, and discrimination between various types of tissue constituents is possible because the near-IR signals arise from combinations and overtones of the fundamental IR bands of these constituents. The gerbil brain is an established animal model of stroke. The gerbil skull is relatively thin, making near-IR spectroscopy of the brain readily achievable in vivo with common spectrometers of moderate light intensity. In addition, the gerbil brain is enriched in polyunsaturated fatty acids. Dramatic changes occur in fatty acid metabolism during the ischemia and reperfusion stages. Near-IR techniques have been used to examine stroke-induced changes in the lipids and proteins of whole gerbil brains [28]. The changes parallel the hypothesized series of free-radical and altered enzymatic events that occur during transient ischemia and reperfusion in the human brain.

To understand the early changes in lipid and protein metabolism following stroke and trauma, animal models have been developed to recreate these changes. The examination of whole brains has been made possible by a combination of hardware modifications and mathematical techniques designed to make the sample presentation to the spectrometer quite reproducible. A refrigerated sample compartment with dry nitrogen purge can be constructed for analysis of whole frozen brains. The cooled compartment enables repeated scans of frozen brains to be collected over time without thawing. The spectrophotometer itself can be purged with dry nitrogen gas, eliminating spectral artifacts associated with lipid/protein oxidation and atmospheric water vapor or other gases. A geometric noise filter removes spectral variations arising from positional variation of the brain. The BEST and extended BEST algorithms, which scale spectral vectors in multidimensional hyperspace with a directional probability, can be used with a supercomputer to analyze the spectra collected.

In addition to changes observed in stroke, age-related changes occur in the polyunsaturated fatty acid pool and in the state of protein oxidation within the central nervous system. Near-IR spectral analysis has many applications to aging and stroke research, including

- Determination of age from brain spectra
- Prediction of short-term memory deficit from the spectra of injured brains
- Simultaneous multicomponent analysis of lipids and proteins
- Quantification of edema
- Transcranial scanning of the brain in vivo

Near-IR scanning of brains in vivo simplifies the testing of antiepileptic drug candidates by reducing the number of subjects required, by allowing each subject to be used as its own control, and by eliminating variance due to outlier subjects, such as those that have had a stroke before the experiment.

Near-IR Fiber Optics as Arterial Probes for Studying Cardiovascular Disease

Another tool for studying mechanisms of toxicology couples near-IR with fiber-optic arterial probes. Fiber-optic catheters have been used to locate atherosclerotic lesions, but the techniques merely distinguish lesions from healthy arterial tissue. Near-IR fiber optics may be used to spatially map lesions and their chemical constituents [30]. Chemical analysis of lesions in vivo permits the kinetic study of atherogenesis and contributes to the understanding of lesion formation and growth. The chemical imaging power of this technique permits the testing of hypothetical mechanisms of lesion formation, growth, and regression, including those involving toxic injury and antioxidant protection.

Toxicological Studies

IR spectroscopy is extensively employed in toxicological research. The toxic effects of selenium exposure on the external cell membranes of living bacteria may be efficiently analyzed by FTIR directly in the biomass [53]. FTIR spectroscopy was employed to assess the hepatotoxic effects of a single dose of carbon tetrachloride in rat liver [40]. Dynamic chemical changes in liver samples were monitored over time by analyzing the IR spectra in the lipid (1800 to 1000 cm^{-1}) and C–H stretching (3000 to 2400 cm^{-1}) regions and comparing them to untreated controls. Changes in band shape and intensity were correlated with toxicity. To develop a high-throughput metabolomic toxicology assay, researchers hypothesized that FTIR could be used to detect differences between urine collected from rats treated with lipopolysaccharide, a potent inflammatory agent, and urine obtained from untreated controls [69]. In addition, cotreatment with ranitidine, a drug often associated with idiosyncratic hepatotoxicity, was performed to determine whether or not the FTIR method would be useful for predicting this idiosyncratic toxicity. The results of this pilot study suggest that similar methods might be applied for the rapid metabolomic screening of drug toxicity. In the occupational and environmental toxicology settings, FTIR analysis may be useful for the continuous monitoring of the presence of airborne toxins. Over a 2-year period, 39 selected air toxins were measured at 11 different petrochemical sites using open-path FTIR [31]. The data enabled the researchers to calculate the hazard indices for both acute and chronic health effects attributable to these toxins over time and by location, thereby estimating potential health risks to workers.

Specialized Instrumentation

Several modified approaches for performing IR spectroscopic imaging microscopy have been developed. One instrument integrates an acousto-optic tunable filter (AOTF) and charge-coupled device (CCD) detector with an infinity-corrected microscope for operation in the near-IR spectral regions [198]. Images at moderate spectral resolution (2 nm) and high spatial resolution (1 μm) can be rapidly collected. Data can be presented with 128 × 128 pixels. The CDD is a true imaging detector, with wavelength selection provided by the AOTF and quartz tungsten halogen lamp to create a tunable source. The instrument can be utilized for both absorption and reflectance spectroscopies.

Synchrotron FTIR (SFTIR) microscopy, which does not require sample mounting or specialized preparation, has been used to probe the molecular responses to toxic injuries in intact, living cells [76]. SFTIR allows the observation of several types of molecular responses and lesion development induced by stressors such as radiation and toxic compounds in the same cell over time. Real-time changes in nucleic acids and proteins in live human cells treated with dioxin were studied using this technique.

Portable FTIR units are available and broadly deployed for field use. Potential applications include the rapid detection of toxins and suspected bioterror agents at remote locations. To evaluate the utility of these portable systems for the field-based characterization of biological threats, a series of peptide and nonpeptide toxins were analyzed and compared to libraries of known spectra [172]. In the case of pure compounds, this spectral searching technique allowed the researchers to discriminate between aflatoxin, mycotoxin, and strychnine at a 99% confidence level. Nonpeptide toxins were readily identified in chemical mixtures, and peptides such as ricin were correctly identified in mixtures at a 95% confidence level. FTIR analysis is being rapidly developed as a versatile tool for on-site identification of chemicals, toxins, and biological threats.

Raman Spectroscopy

Raman spectroscopy is closely related to IR spectroscopy, in that the information about molecular vibrational frequencies provided by the latter technique is of the same kind as that provided by the Raman vibrational spectrum [27,146]. In molecules with a center of symmetry, however, vibrational transitions that are allowed in the IR spectrum are forbidden in the Raman effect, and vice versa, providing useful information about molecular symmetry. Structurally symmetrical diatomic molecules such as H_2 and O_2 are also electrically symmetrical and do not give IR absorption spectra. These molecules do afford Raman spectra due to excitation of symmetrical vibrations. In a molecule such as tetrachloroethylene (CCl_2=CCl_2), the double-bond stretching frequency is symmetrical and the molecule does not show a double-bond stretching frequency in the IR spectrum; however, this vibration appears strongly in the Raman spectrum of tetrachloroethylene and provides evidence of a symmetrical structure (see Figure 41.29). Thus, the two techniques are complementary to each other.

The Raman effect is a scattering process in which the interaction between photon and the molecule occurs in a very short period of time, and the Raman peaks obtained correspond to photons that have bounced inelastically off the molecule. The Raman spectrum arises as a result of the light photons being captured momentarily by molecules in the sample and giving up (or gaining) small increments of energy through changes in the molecular vibrational and rotational energies before being emitted as scattered light. These changes in vibrational and rotational energies result in changes in wavelength of the incident light. The convention in Raman spectra is to quote the positions of vibrational peaks as the difference between the absolute wave numbers of the exciting line and the absolute wave numbers of the resulting scattered photons. The Raman effect is extremely weak, and only a minute portion of the incident photons are useful emergent photons; thus, relatively high-power lasers must be used to create a high photon flux. Sophisticated optical and electronic equipment is also required to detect the scattered photons.

One of the major advantages of Raman spectroscopy is that spectra may be obtained for molecules in aqueous solutions, as water has a weak Raman spectrum that interferes only minimally with the spectrum of the solute. Analyte concentrations in the range of 0.1 to 0.01 M in water are normally used. In resonance Raman spectroscopy, concentrations of chromophoric molecules in the range 10^{-4} to 10^{-6} M can be used, making this technique particularly useful for biochemical studies.

Basic Optics of the Raman Experiment

In a standard Raman experiment, intense monochromatic radiation provided by a CW laser is focused onto or into the sample. Some of the resultant scattered light is collected by optics and directed to a dispersing system that is usually a monochromator. The monochromator separates the scattered light on the basis of frequency, and these frequencies are then detected and recorded either by single-channel (scanning) or multichannel detection. Figure 41.30 illustrates the optics involved in a conventional Raman experiment.

Sampling Techniques and Problems

Samples for Raman spectroscopy may be examined in any physical state. Liquid samples are usually measured in a quartz (1 cm) cuvette similar to the type used in fluorescence spectroscopy; however, because the incident laser beam travels longitudinally down the length of the liquid column, the cell bottom must be transparent. Capillary cells are often used for biological samples, especially when material is limited in availability. Single crystals and fibers can be analyzed by mounting on a goniometer head. Solid crystalline or polycrystalline materials can be pressed into pellets, prepared as KBr disks, or packed into capillary microprobes. Samples in the form of thin films can also be examined. Potential problems include the breakdown of photolabile analytes during laser irradiation. This can be reduced or eliminated for liquid samples by utilizing a spinning cell, a cell in which the liquid sample is continually moved through the laser beam, or a

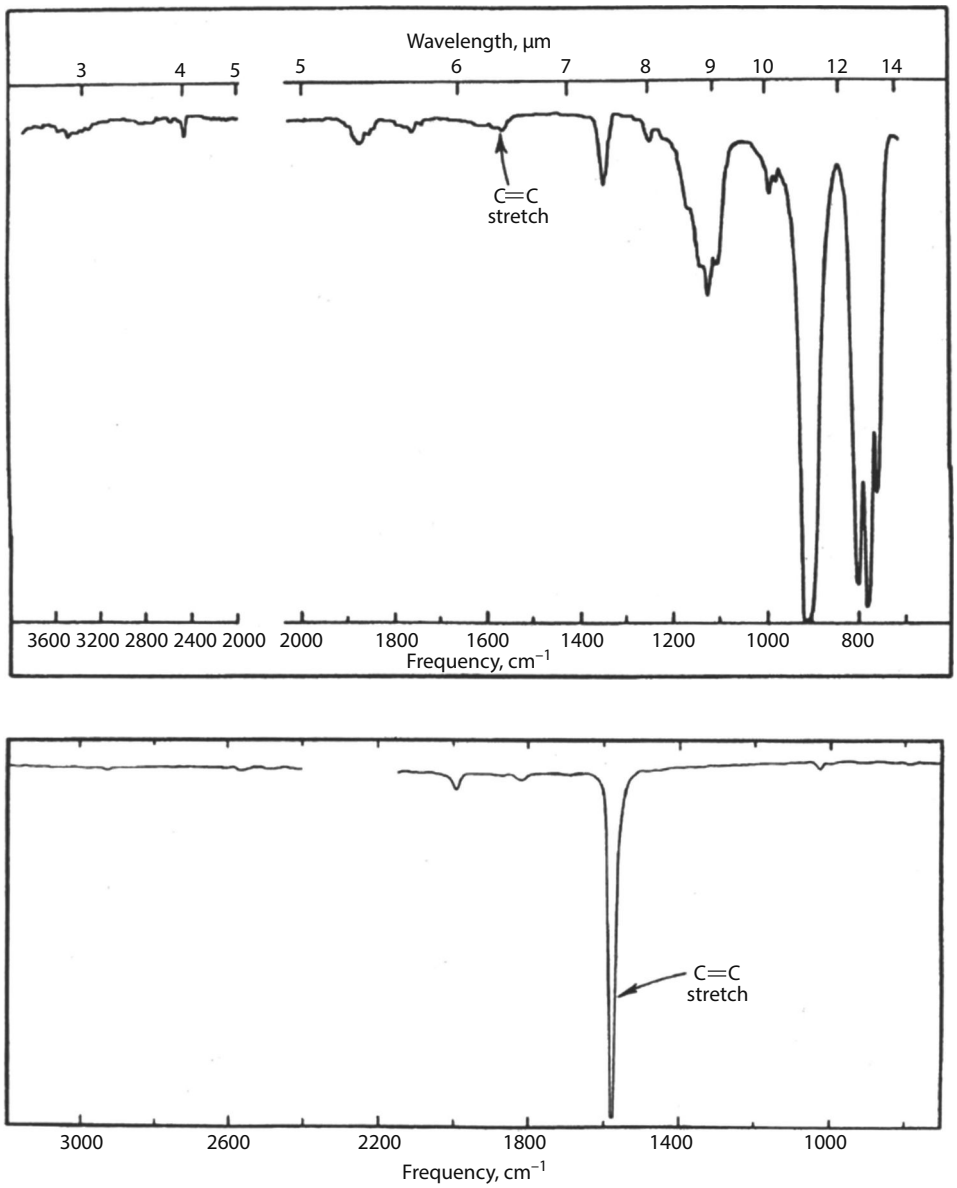

FIGURE 41.29 IR (top) and Raman (bottom) spectra of tetrachloroethylene.

cell in which the liquid is continually stirred with a magnetic stirrer. These procedures reduce the buildup of degradation products usually observed with static sample analysis.

In Raman spectroscopy, it is important for the analytes to be optically homogeneous, especially in the case of biological samples. Particulate matter in solutions should be removed either by centrifugation or filtration; otherwise, hot spots may occur in the sample on irradiation, leading to possible degradation. Luminescence, which may often obliterate the Raman effect, may occur if the sample or an impurity in the sample has a chromophore. Luminescence can often be reduced or eliminated by changing the wavelength of excitation or adding a quenching agent such as KI.

Protein Conformation Determination

Protein molecules are classical examples of the application of Raman spectroscopy to biomolecules. This technique can probe structural details such as average peptide backbone conformation, as well as the side chains of some amino acids, such as tyrosine and tryptophan. Protein spectra are usually obtained in the 450–650 nm region. UV-excited resonance Raman spectra of proteins containing aromatic amino acids of interest are often conducted with excitation below 300 nm. The normal Raman spectrum of proteins contains diagnostic amide I (C=O stretch) and amide III (N–H in-plane bending) bands that can be utilized to characterize the secondary structure of the protein or peptide backbone. Table 41.3 gives approximate positions for the amide I and amide III bands in both the IR and Raman spectra of various polypeptide conformations. Characterization of the secondary structure of a protein depends on the determination of characteristic amide I and III frequencies in the Raman spectrum for α-helical, β-sheet, and random protein conformations. This is often achieved by using polypeptide models and proteins

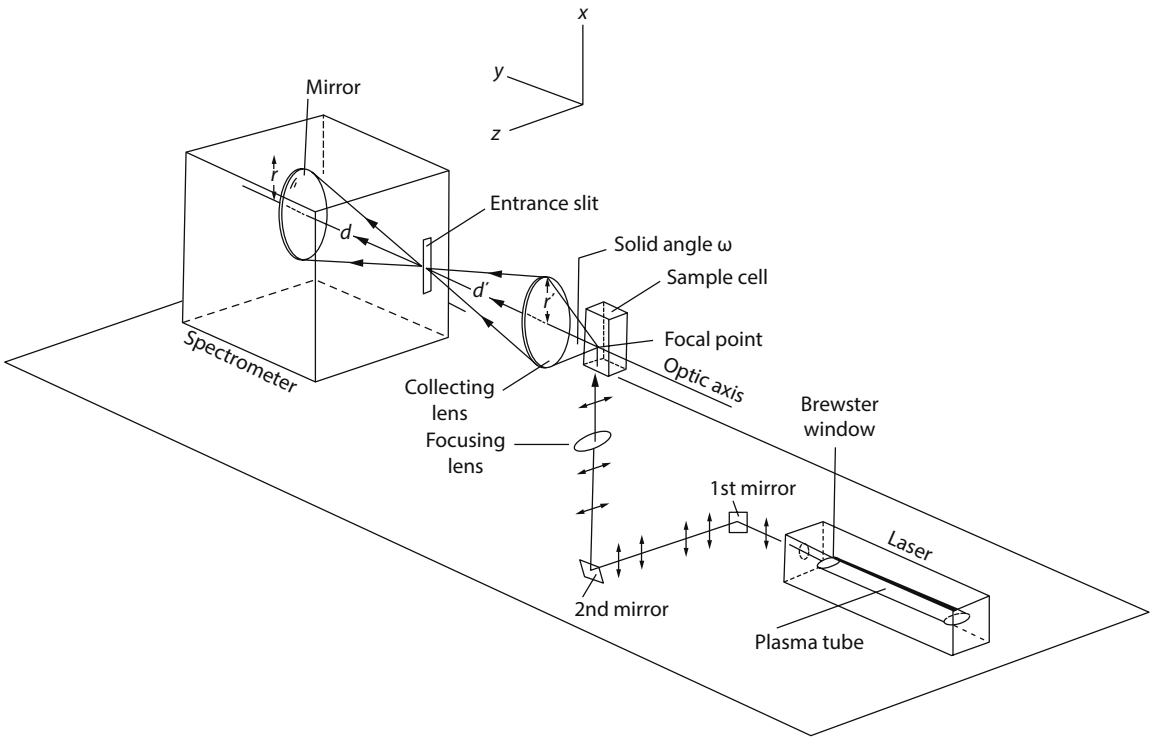

FIGURE 41.30 The optics of a conventional Raman spectrometer.

TABLE 41.3
Approximate Positions (cm^{-1}) of the Most Intense Amide I and Amide III Bands in Raman Spectra and the Amide I Band in IR Spectra for Various Polypeptide Conformations

	Amide I		Amide III
Conformation	**Raman**	**IR**	**Raman**
α-Helix	1645–1660	1650.00	1265–1300
β-Sheet	1665–1680	1632.00	1230–1240
Unordered	1660–1670	1658.00	1240–1260

of known conformation. Figure 41.31 illustrates the Raman spectra, run in water, of native and denatured ribonuclease A and shows the change in the amide III band in the disordered protein [33].

Resonance Raman Labels

Resonance Raman labels are chromophores that have been carefully designed to mimic natural biochemical components and that are themselves biologically active molecules. They provide detailed vibrational and electronic spectral data when in the vicinity of a biologically important site. Extrinsic protein-bound chromophores, such as methyl orange bound to bovine serum albumin, have been studied in detail. Other systems that have been studied include protein–ligand interactions, such as drug–enzyme and hapten–antibody complexes where the drug or hapten is the chromophoric resonance Raman label. This method is useful for studying enzyme–substrate complexes and can provide vibrational spectra of the substrate during enzyme catalysis.

RNA and DNA Structural Analysis

Analysis of polynucleotides by Raman spectroscopy affords bands (~30) primarily attributable to purine or pyrimidine ring modes. In addition, the phosphate group shows interesting features in the spectrum. The sugar moieties in DNA and RNA molecules and the related polynucleotides are poor Raman scatterers. From the Raman spectrum, it is possible to obtain a semiquantitative estimate of the relative population of bases in the polynucleotide molecule. Base protonation in DNA has been studied using Raman spectroscopy. Metal–nucleotide binding has also been investigated. The mode of binding of ions such as Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, and Mg^{2+} to adenosine triphosphate over a wide pH range has also been studied. Raman spectroscopy can detect the disruption of base-pairing and base-stacking interactions [105]. In Figure 41.32, raising the temperature of poly(rA) · poly(rU) from 32°C to 82°C results in thermal disruption of the helix. Important features in the difference spectra are the radical changes in the carbonyl region (1650–1700 cm^{-1}) due to the disruption of H bonding in the Watson–Crick model. Phosphate backbone conformation in nucleic acids has also been studied by monitoring the –O–P–O– symmetrical stretching vibration (800 cm^{-1}) and the $–PO^{2–}$ symmetrical stretch motion (1100 cm^{-1}). The interaction of nucleic acids with proteins is an area of active study; for example, the stabilizing effect of the viral capsids on the secondary structure of the viral RNA has been investigated. Also, Raman data on DNA–histone interactions have indicated that the

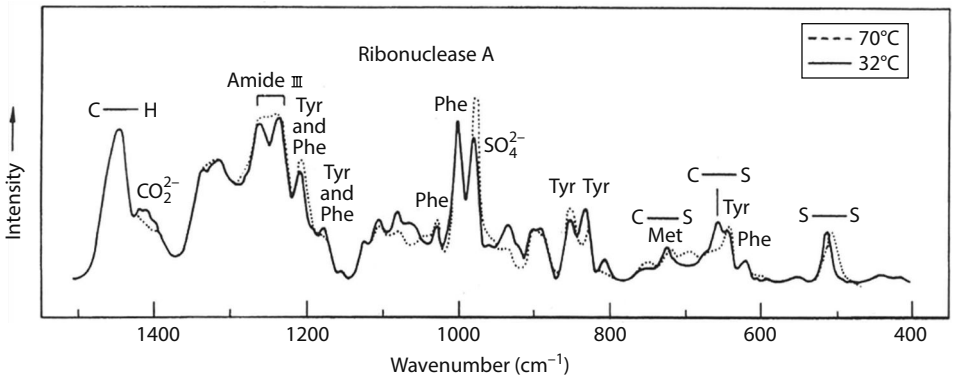

FIGURE 41.31 Raman spectra of native and denatured ribonuclease A, at 32° and 70°, respectively, after correction for the water background and being normalized to the intensity of the methylene deformation mode at 1447 cm^{-1}. Protein concentrations of about 10% were used with typical spectral conditions of 488 nm excitation, 200 mW power, and 7 cm^{-1} spectral slit. (From American Chemical Society. With permission.)

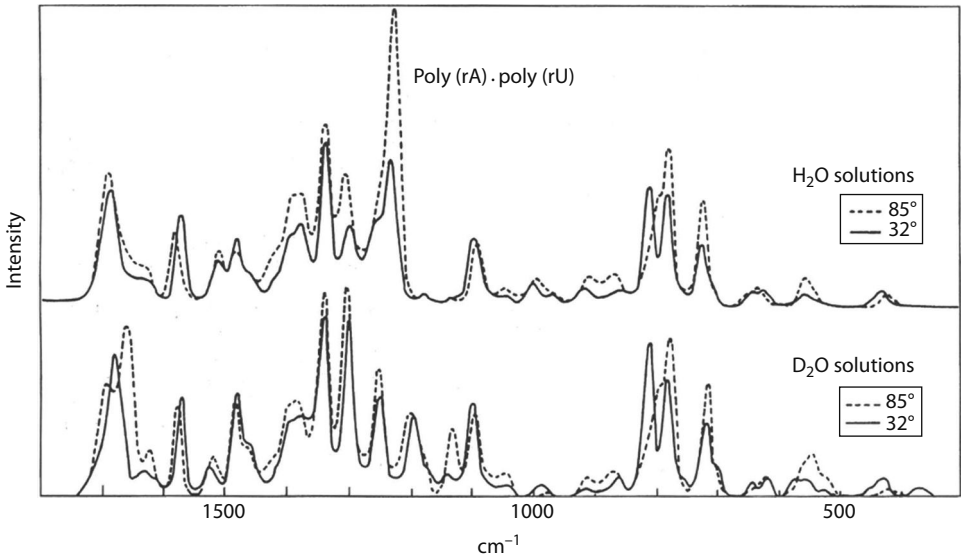

FIGURE 41.32 Raman spectra of H_2O and D_2O solutions of poly(rA)poly(rU) at 32°C and 85°C, respectively. The background of Raman scattering by the solvent has been subtracted from each spectrum. (From Lafleur, L. et al., *Biopolymers*, 11, 2423, 1972 [77]. With permission.)

sites of DNA–protein interactions are probably located in the grooves running along DNA.

Lipids and Membranes

Raman spectroscopy is ideally suited for the study of lipids and membranes and has some advantages over other techniques. The analysis time frame of fractions of a picosecond provides instant snapshots, thereby eliminating the line broadening commonly seen in magnetic resonance spectra. No probe molecule is required, and both gel and liquid-crystal hydrocarbon regions can be monitored. The most useful regions are 1000–1150 cm^{-1} (C–C accordion stretch) and the 2800–3000 cm^{-1} (C–H stretching mode). Transitions from gel to liquid crystal in membranes are indicated by marked changes in the bands assigned to the C–C stretch mode, and the C–H stretch region is extremely sensitive to conformational change within the individual fatty acid chains.

Applications

Raman spectroscopy has become the tool of choice for many chemical and biological applications [6]. Extremely sensitive to minute structural changes, Raman techniques require minimal sample preparation and are extensively employed in noninvasive studies. Raman microspectroscopy also offers high spatial resolution for analyzing discrete regions of biological matrices. These characteristics make Raman spectroscopy particularly useful for exploring biological processes in living tissues and in individual living cells and organelles [157]. Continuous Raman spectroscopy monitoring of living A549 human lung cells was employed to evaluate the effects of nonionic surfactant exposure on biochemical processes and the sequence of events in cell death. The molecular mechanisms of cell death were strongly associated with specific decreases in Raman peaks associated with nucleic acids and proteins [143]. Western blotting analysis supported

the conclusions derived from the Raman spectroscopy data. Raman spectroscopy analysis of specific, time-dependent biochemical changes might be applied to tissue engineering and high-throughput toxicity screening.

Evans et al. [51] combined coherent anti-Stokes Raman scattering (CARS) with video-rate microscopy for real-time monitoring of dynamic processes in lipid-rich tissues of living mice. CARS imaging of the methylene stretching vibrational band afforded exceptional subcellular resolution in tissues such as adipocytes, corneocytes, and the sebaceous glands. Important processes such as in vivo cellular transport, xenobiotic diffusion, and transcorneal absorption might be monitored using the CARS method.

Several reports on the biomedical applications of in vivo Raman spectroscopy (IV-RS) have been published [91,107,177]. These include the in vivo diagnosis of certain diseases in their very early stages [177]. In vivo Raman scattering has been explored as a means of measuring levels of the antioxidant molecules lutein and zeaxanthin in the retinas of young and older adults [58]. The results indicated that the concentration of these protective pigments decreased with increasing age, even in normal eyes. The authors suggest a role for Raman screening of retinal carotenoid levels in populations at risk for macular degeneration, a leading cause of blindness. The potential use of IV-RS for diagnosing arterial disease and cancer in gynecological tissues, soft tissues, breast, colon, bladder, and brain has been discussed by Manoharan et al. [122]. Lawson et al. [107] reviewed the application of Raman spectroscopy to the study of human arteries, tumors, gallstones, hair, and nails. Brain tissues have also been investigated using FT-Raman spectroscopy, and spectra from the cerebral cortex, cerebral white matter, caudate–putamen, thalamus, synaptosomal fraction, and myelin fractions have been recorded [128,129]. Brain tumors have also been studied using FT-Raman spectroscopy [128].

Ong et al. [144] studied the substantia nigra of the rostral midbrain in monkeys using Raman microspectroscopy to determine differences between white and gray matter. As compared to the laser spot size of 20 μm in IR microspectroscopy, the 1 μm laser spot size in Raman microspectroscopy afforded much greater spatial resolution in sample analysis. The white and gray matter could be clearly distinguished and their relative proportions evaluated from Raman frequencies in the 3000 cm^{-1} region.

Singer et al. [180] evaluated Raman confocal microscopy as a means of assessing environmental pollutant bioavailability and toxicity in a cell culture system. Phenanthrene, dodecane, 3-chlorobiphenyl, and pentachlorophenol (four common environmental pollutants) were detected and quantified using this potential pollution bioassay.

Along with IR spectrometers, portable Raman spectrometers have also been developed for remote field use. Raman analysis with portable instrumentation was used in a blind field test to analyze a matrix of 58 unknowns for comparison with a standard hazardous materials response library of known spectra [70]. Despite the inclusion of multiple solvents and compounds with uncataloged spectra, over 97% of the samples were correctly identified with no false positives. Freebase crack cocaine and its hydrochloride salt were readily identified and distinguished from common street adulterants using fiber-optic Raman spectroscopy [29].

Raman spectroscopy systems have been developed for the detection of toxins associated with bioterror, including chemical and microbial weapons. The capacity of a human lung cell–based Raman spectroscopy system to detect and differentiate between sulfur mustard and ricin exposure was assessed noninvasively over time [142]. Differences in cellular Raman spectra correlated with characteristic changes in cellular biochemistry and structure. Damaged cells were detected with high sensitivity and specificity, and toxic agent identification was reasonably accurate for ricin (71.4%) and sulfur mustard (88.6%).

Isotopic Labeling

The use of isotopes to study the fate of xenobiotics in vitro and in vivo developed initially with the use of radioisotopes. The development of methods for detecting and quantifying energy emitted from radioisotopes led to synthetic xenobiotics containing radioactive isotopes that could be tracked or traced in organisms. The development of sophisticated NMR and mass spectral methods has made the use of stable isotopes for studying the fate of xenobiotics fairly common. Stable isotopes are popular because they often yield a great deal of structural information about xenobiotic metabolites without the hazards to the organism (often humans) inherent in radioisotopes. The following sections provide an introduction and overview of isotope methods. More comprehensive information is available [89,99,212,218].

Radioisotopes

Most elements exist as several isotopes that vary in atomic weight. Some of these isotopes are radioactive, spontaneously decaying to form an atom of another element. This decay is accompanied by the emission of radiation. The radiation emitted is of three distinct types: alpha (α), beta (β), and gamma (γ). Alpha particles are actually helium nuclei (^{4}He), β-particles are electrons, and γ-rays are high-energy electromagnetic radiation. Isotopes that emit β-particles, so-called β-emitters, are less hazardous to laboratory workers than γ-emitters because β-particles do not possess sufficient energy to penetrate the skin. Beta emitters are only hazardous if ingested or where they otherwise come in contact with cells. In contrast, γ-emitters are more hazardous to laboratory workers because γ-rays are highly energetic and can easily penetrate the skin. Special precautions such as lead shielding are often required when working with γ-emitters.

Radionuclides are quantified in terms of their *specific activity*, which is the radioactivity per unit mass of material. Specific activity can be given in curies per mole (Ci/mol) or becquerels per mole (Bq/mol). A becquerel is defined as 1 disintegration per second (dps) and 1 curie = 3.7×10^{10} dps; thus, 1 Ci = 3.7×10^{10} Bq. The most commonly used radionuclides are ^{3}H and ^{14}C. Both of these isotopes are weak β-emitters that emit fast-moving electrons that can penetrate up to 50 cm in air and up to 0.5 cm in aluminum. Both ^{3}H- and ^{14}C-labeled

compounds can be handled easily and safely with the use of glass containers, protective gloves, and safety glasses. ^{14}C β-particles carry significantly more energy (β^- = 155 keV) than ^{3}H β-particles (β^- = 18.6 keV). This difference enables mixtures of radionuclides (e.g., double-labeled $^{3}H/^{14}C$-containing compounds) to be measured simultaneously (see later discussion). Although ^{14}C is a more energetic nuclide and therefore a more easily detected tracer atom than ^{3}H, its maximum specific activity when incorporated into a drug molecule is only 62.4 mCi/milliatom of carbon, compared to a maximum specific activity for ^{3}H of 29.1 Ci/milliatom of hydrogen.

The energy emitted when radioactive isotopes decay is easily traced, as the radionuclide itself is not metabolically altered by the biological system under study. The radioisotopes most commonly used in biological systems are shown in Table 41.4.

Xenobiotic Disposition Studies Using Radiolabeled Tracers

The most common use of radiolabeled xenobiotics in toxicology is the study of the fate of a chemical in animal models. These studies are often referred to as absorption, distribution, metabolism, and excretion (ADME). The successful ADME study requires

- A radiolabeled xenobiotic with the most appropriate isotope and position of the label
- An analytical method for separating the parent compound and its hypothesized metabolites
- Methods for quantifying the amount of radioactivity

Choice and Location of Label

The radioisotope of choice for xenobiotic ADME studies is carbon-14 (^{14}C) because it is a radioactive form of the element that forms the backbone of most xenobiotics. In addition, ^{14}C has a very long half-life (over 5000 years), and as a weak β-emitter, it poses fewer health risks to laboratory workers. The position of the label must be carefully selected to ensure that the label is not lost upon metabolism. Researchers using ^{14}C will often uniformly label one of the aromatic rings of a xenobiotic if it is thought not to undergo ring opening during metabolism. These positions are preferable to ^{14}C labeling in a methyl group attached to an oxygen or a nitrogen, because both of these carbons will undergo demethylation reactions catalyzed by either cytochrome P450 or flavin-containing monooxygenase. Sometimes there are limitations for placing the label, based on synthetic concerns. Every effort should be made to locate the label in a chemically and metabolically stable position.

Tritium (^{3}H), the radioactive form of hydrogen, is often used as a tracer because of the high specific activity that can be obtained with this isotope. High specific activity increases the researcher's ability to detect smaller amounts of the xenobiotic. Receptor binding assays typically use tritiated ligands for this reason; however, the use of tritium has some shortcomings, including a relatively short half-life (12.33 years) that requires adjustment of the specific activity over time. In addition, because tritium is an isotope of hydrogen, it undergoes exchange with nonradioactive protium (^{1}H) in solvents. This so-called *solvent exchange* must be taken into account when positioning a label on the xenobiotic. Positions that readily undergo solvent exchange are not good candidates for labeling. These positions are referred to as *labile positions* (e.g., protons attached to oxygen, nitrogen, or sulfur atoms are labile). Tritium may also be lost due to metabolism of the xenobiotic, and an experienced investigator will usually avoid inserting labels at positions in the molecule where they may be lost during biotransformation; for example, ^{3}H labeling at a carbon adjacent to a heteroatom or at a hydroxylation site on a phenyl ring or a ^{3}H-labeled *N*-methyl group, which may often be lost by oxidation, should generally be avoided.

Radiochemical Purity

The purity of labeled compounds is usually critical to the success of an experiment. When following (tracing) a radiolabeled compound, there is no specificity to the radioactivity emitted by the radioactive isotope. Chemical impurities containing the radioisotope will be indistinguishable from the xenobiotic of

TABLE 41.4
Radioactive Isotopes Commonly Used to Study the Fate of Xenobiotics

Atomic No.	Element	Atomic Weight	Half-Life	Radiation (MeV)
1	Hydrogen	3	12.33 years	β^- (0.019)
6	Carbon	11	20.4 min	β^+ (0.96)
		14	5730 years	β^- (0.156)
15	Phosphorus	32	14.28 days	β^- (1.71)
		33	25.3 days	β^- (0.25)
16	Sulfur	35	87.5 days	β^- (0.167)
17	Chlorine	36	3×10^5 years	β^- (0.71)
37	Rubidium	87	4.8×10^{10} years	β^- (0.272)
53	Iodine	125	60.14 days	γ (0.035)
		131	8.040 days	β^- (0.607, 0.336) γ (0.080, 0.284, 0.364, 0.637, 0.723)

interest; therefore, the original material administered to the organism should be of the highest purity possible to ensure that the radioactivity detected is derived from the parent compound. Radiochemical purity should be confirmed by a suitable method, such as radiochromatography (discussed later), prior to using a radiolabeled compound for an ADME study.

In addition to radiochemical purity, enantiomeric purity may also be important. Many biological processes are stereoselective, with either the S or R enantiomer of a biologically active compound having significant activity. That is, only one of the stereoisomers of a compound is active in the system under study; for example, the (S)-(–) enantiomer of nicotine is responsible for most of its pharmacological activity. The affinity of the (R)-(+) enantiomer for high-affinity nicotinic acetylcholine receptors is 60-fold less than the (S)-(–) enantiomer. With the recognition that many toxic responses are receptor mediated, the enantiomeric purity of radiochemicals has become more important.

The solvent selected for storage of a radiolabeled compound is an important consideration; for example, radiolabeled peptides and proteins that have been stored in water may undergo extensive degradation. Solvent exchange can also be a problem for tritiated compounds, as described earlier. The selection of a solvent is usually a compromise between adequate solubility and a minimum of inherent reactivity with the radiolabeled compound.

Route of Administration

A primary consideration when conducting an ADME study with radiolabeled xenobiotics is the route of administration. The xenobiotic is usually administered to the animal model by a route appropriate to either the anticipated major route of exposure to the species of interest (usually humans) or by a route of administration that is consistent with the goals of the research; for example, if the xenobiotic of interest is a component of a consumer product applied to the skin, then the appropriate route of delivery may be dermal administration. If, however, the objective of the ADME experiment is to determine the pharmacokinetics of the xenobiotic in blood and tissues, then the intravenous route of administration would be chosen over the dermal route. The solubility of the compound in an appropriate delivery vehicle must also be considered when deciding on a route because many vehicles are not compatible with intravenous administration.

Liquid Scintillation Spectrometry

Total xenobiotic-derived radioactivity can be determined in all excreta including urine, feces, and expired air to quantify the routes of excretion for a xenobiotic and its metabolites. Total radioactivity in aqueous samples is relatively easy to quantify using liquid scintillation spectrometry. This method for quantifying radioactivity involves mixing an aliquot of the sample with a *liquid scintillation mixture* (LSM) that uses an organic compound or mixture of compounds that are scintillators, compounds that give off light when they absorb radioactive energy. Traditionally, these scintillators were dissolved in toluene-based mixtures that enabled counting both organic and aqueous samples. For aqueous samples, a typical mixture of two-part sample to eight-part LSM formed a gel that was counted. These LSMs could disperse no more than about 20% water. Newer LSMs that are less hazardous, can disperse more water, and can be discarded down the sanitary sewer have replaced toluene-based LSMs for most applications. The mixture of the radioactive sample and the liquid scintillation mixture is placed in special vials that are highly efficient at passing light without absorbing radioactive energy. The vials are counted by placing them in a liquid scintillation counter uses two opposed photomultiplier tubes to detect light-emitting events triggered by the radioactive decay of the isotope. Coincidence circuitry is used to separate random events from radioactive isotope–driven events. The counting region is lined with lead to shield the vial from extraneous environmental radiation. The sample is compared to a blank or background vial that contains everything but the radioisotope-containing sample. The radioactive counts from the background vial must be subtracted from the sample to obtain net radioactivity.

The liquid scintillation counter expresses data in counts per minute (CPMs) that must be corrected to disintegrations per minute (DPMs) to account for inefficiencies in capturing all of the radioactive energy emitted. The method employed in most liquid scintillation counters today involves the use of external standard calibration. Here, a radioactive source housed in the instrument is placed automatically near the vial containing the sample, and the photomultiplier tubes detect the resulting light emitted from the scintillator within the sample vial. The CPMs detected by the instrument are then automatically compared to the known DPMs of the external standard source, and a counting efficiency is determined. The counting efficiencies for ^{14}C are usually much higher than for the less energetic β-emitter tritium.

In addition to the inefficiencies in transferring the energy of radioactive decay into light energy, there is also *quenching* of the light emitted from the scintillator. Many solvents and biological molecules can quench the light emitted by the scintillator, so the researcher must correct for the amount of quenching within the sample by comparing the expected DPMs to a quench curve and determining the actual DPMs present in the quenched sample. This is usually accomplished automatically by the liquid scintillation counter by first running a set of vials containing a known amount of radioactivity with increasing amounts of a quenching agent added to each vial. The resulting quench curve is stored in the counter memory and used to determine the DPMs of the sample.

Radioactive samples containing scintillant are often sensitive to external light, especially sunlight, which results in excitation and abnormally high CPMs on analysis. This phenomenon, called *chemiluminescence*, is usually more of a problem with ^{3}H-containing rather than ^{14}C-containing radiolabeled samples and can usually be minimized or eliminated by storing scintillation fluids and samples containing scintillant in the dark for 30–60 min before analysis.

Combustion Techniques

Many samples are not amenable to liquid scintillation counting because they are either solids or otherwise incompatible

with LSMs. Total radioactivity in tissue samples and fecal samples can be determined by combustion of the sample to carbon dioxide and water. ^{14}C in the original sample is converted to $^{14}CO_2$ and tritium is converted to tritiated water. Combustion instruments automatically combust samples on a platinum electrode covered with a glass chimney. The products of combustion are swept away by an airstream and trapped in appropriate solvents. Liquid scintillation mixture is added and sample radioactivity is measured in a liquid scintillation counter.

Autoradiography

Autoradiography is the production of an image by the emission of radioactive decay energy from a radionuclide. It provides a qualitative visual image of the tissue distribution of a xenobiotic and has been used to provide quantitative distribution data, as well. ^{14}C is probably the most often used radionuclide for autoradiography because of its long half-life and relatively high energy. Tritium is used when greater resolution is required because it is a weaker emitter. Radioisotopes of nitrogen and oxygen do not have sufficiently long half-lives to permit development of an image. Iodine, sulfur, chlorine, and phosphorous are also used for autoradiography.

Whole-body autoradiography is an excellent technique for visualizing the distribution of xenobiotic-derived radioactivity in an animal model. This technique involves administration of the xenobiotic to the animal; following anesthesia, the animal is quick frozen by immersion in hexane or acetone with dry ice. The time interval between administration and freezing must be selected based on some knowledge of the rate of elimination of the compound. After complete freezing, the animal is placed in a block of carboxymethylcellulose ice on the stage of a microtome, an instrument that uses a sharp blade to shave thin sections off one side of the block. Sections varying in thickness from 5 to approximately 80 μm are captured from the microtome blade using an adhesive matrix. The section is then placed on x-ray film and stored in a freezer, while the radiation emitted by the isotope exposes the film. The resulting pictures (autoradiograms) illustrate where in the body the xenobiotic-derived radioactivity has distributed. Highly perfused organs such as the kidney, liver, and heart are the first to light up with the compound [206]. Over time, it becomes obvious which tissues sequester the compound.

Digital imaging techniques have enabled quantitative analysis of whole-body autoradiograms providing absolute concentrations of xenobiotic-derived radioactivity in tissues. Zane et al. [225] have shown that autoradiography with quantitative digital image analysis compared extremely well with the more traditional combustion method. For this comparison, they treated rats with ^{14}C-labeled CGS 18102A and sectioned the animals as described earlier. They also took 16 tissue samples from each animal for combustion analysis. The sections were placed on x-ray film along with a series of calibration standards of known radioactivity. After 3 weeks of film exposure, the autoradiograms were analyzed with a digital imaging system. The concentrations obtained from digital analysis of the autoradiograms compared very well to those obtained by combustion analysis. Jacob et al. [85] used quantitative whole-body autoradiography (QWBA) with digital image analysis to study the transplacental uptake and covalent binding of [^{14}C]-chloroacetonitrile (CAN) to various tissues in normal and glutathione-depleted pregnant mice. Their finding that covalent binding of CAN to maternal and fetal tissue was elevated in glutathione-depleted mice suggested a modulatory role of glutathione in CAN distribution and transplacental toxicity and demonstrated the utility of QWBA in mechanistic studies.

Although x-ray film provides excellent resolution and has been used successfully for QWBA, it is not necessarily the medium of choice. Exposure times for x-ray film are typically weeks to months. Also, x-ray film often does not provide a linear calibration over the wide range of radioactivity (optical densities) common in QWBA. To avoid these limitations, storage phosphor screens are commonly employed for QWBA instead of x-ray film. Storage phosphor screens trap the energy released by radioactive samples. The energy is stored by the phosphor screen and is only released when the screen is scanned with a laser beam. The released energy appears in the form of blue light, which is detected by a photomultiplier tube and converted to a digital signal that is stored in a computer file. To quantify the levels of radioactivity in the whole-body sections, a series of standards are analyzed and the response of each is determined. A regression equation, derived for the standard responses, is used to determine the absolute concentration of radioactivity in the whole-body sections.

The autoradiograms can also be visualized by converting the digital data to a graphics file and printing the results. Commercially available hardware and software are available for QWBA using storage phosphor screens. Storage phosphor screens overcome the major limitations of x-ray film. They have a linear response over a 5 log-unit range and can be developed in hours to days. It has been estimated [214] that a 2-week exposure to x-ray film is equivalent to a 24 h exposure to a storage phosphor screen. For a more comprehensive introduction to storage phosphors for QWBA, the reader is referred to the work of Wilson and Kraus [214] and Herman and Chay [71].

An interesting application of storage phosphor QWBA is illustrated in Figure 41.33. For this study, Sprague Dawley albino rats received [^{14}C]-RJR-2403 at a dose of 15 mg/kg by gastric gavage. Animals were euthanized at specific time points and sectioned (30 μm) as described earlier. Sections were exposed to storage phosphor screens for 7 days, and data were collected using a PhosporImager SF (Molecular Dynamics). Figure 41.33A shows an autoradiogram obtained at 30 min, and Figure 41.33B shows an autoradiogram obtained at 4 h after dosing. Table 41.5 lists the tissue concentrations by tissue for each time point. Note that radioactivity was extensively absorbed and distributed throughout the tissues of the rat at 30 min after the dose. Tissue concentrations were highest in the kidney and liver. High levels were also detected in the stomach, heart, lung, and spleen. By 4 h postdose, tissue concentrations decreased considerably, with

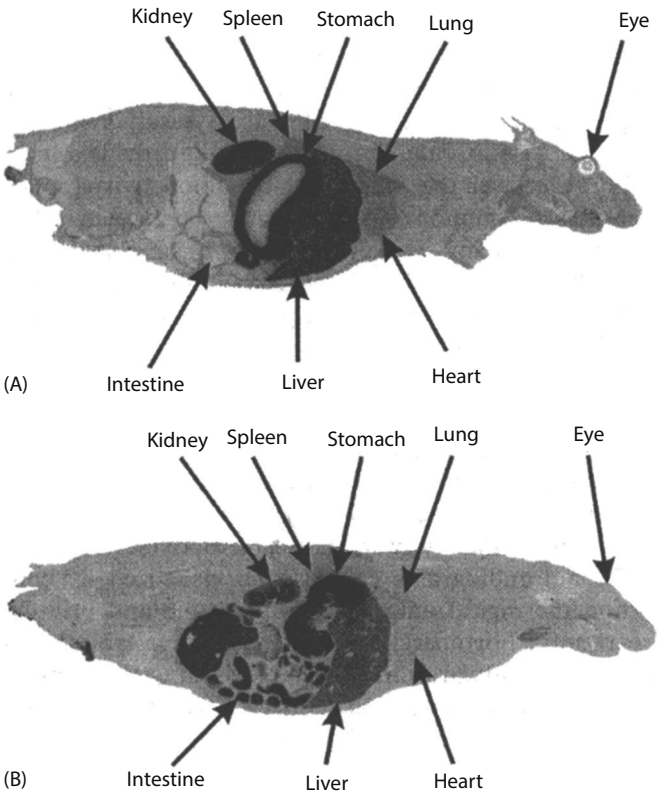

FIGURE 41.33 Quantitative whole-body autoradiograms of male albino rats after administration of [^{14}C]-RJR-2403 at a dose of 15 mg/kg by gastric gavage. (A) 30 min after dosing and (B) 4 h after dosing.

TABLE 41.5
Tissue Concentrations of Total Radioactivity after Single Oral Administrations of [^{14}C]-RJR-2403 to Male Albino Rats

Tissue	30 min	4 h
Eye	1.01	0.67
Heart	5.39	2.46
Kidney	39.17	39.15
Large intestine wall	3.28	Not measured
Liver	26.12	17.53
Lung	6.49	3.52
Spleen	5.18	4.96
Stomach wall	15.38	7.53

Note: Target dose is 15 mg/kg; results are expressed as μg equivalents/g.

high levels seen in tissues associated with metabolism and excretion such as the liver and kidney.

Analytical Methods for Determining Chemical Form

Total radioactivity data are of limited value because of their lack of specificity. When major routes of excretion have been determined, it is important to characterize the chemical form of the excreted radioactivity. As an example, expired CO_2 can be selectively trapped in base such as sodium hydroxide or ethanolamine, thereby providing insight into how extensively a xenobiotic is metabolized. Acrylic acid and ethyl acrylate, two monomers used extensively in the plastics industry, are rapidly metabolized, and 70% of the compounds are eliminated from the body as CO_2 via normal catabolic biochemical pathways that degrade the carbon skeleton of the molecule [43]. On the other hand, many xenobiotics are metabolized to polar compounds by selective metabolism of functional groups on the molecule without catabolic degradation of the carbon skeleton. HPLC is useful for quantifying metabolites in biological matrices because metabolites that are relatively hydrophilic and nonvolatile can be chromatographed easily. Other methods for separating metabolites from the parent compound include LC-MS, LC-NMR, TLC, capillary zone electrophoresis, chemical reaction interface MS, and GC-LC.

The simplest approach to characterizing the radioactivity in excreta is to inject urine directly onto an HPLC. Usually the urine is filtered first by passing it through a centrifugal ultrafilter with a small pore size (0.2–0.45 μm). HPLC guard columns are used to protect the analytical column from the high concentration of other organics such as creatinine and salts that are found in urine. The metabolites of the parent compound are detected after adequate separation of the radioactivity in excreta on the HPLC column. Radioactive peaks eluting from the HPLC column can be collected in scintillation vials as a series of fractions and the radioactivity counted in a liquid scintillation counter. A histogram of radioactivity in the fractions should reveal distinct peaks of radioactivity. The radioactive peaks can be coeluted with synthetic standards of putative metabolites by injecting the standards along with the sample onto the HPLC column. Radioactive effluent from the HPLC column can also be detected using commercially available flow-through radioactivity detectors [147], which have replaced the more labor-intensive fraction collection method in many laboratories. Radioactive flow detectors use two types of flow cells. Solid scintillator cells use glass beads, calcium fluoride, or yttrium silicate and permit the recovery of unadulterated effluent. Using this type of detector, peaks containing radioactivity can be collected and analyzed by some other method such as MS to determine the identity of the radiolabeled metabolite. Liquid scintillator cells mix liquid scintillation mixture with the column effluent prior to passing in front of the photomultiplier tubes where the radioactivity is detected. The mixture of effluent and LSM must be discarded as radioactive waste. The choice of the type and volume of the flow cell depends on the isotope and specific activity of the label. Optimization of these parameters permits rapid and accurate determinations.

Double-Label Techniques

The use of doubly labeled compounds in metabolic experiments is often necessary to obtain in-depth information on metabolic pathways and biotransformation mechanisms; thus, one part of a xenobiotic molecule may be labeled with ^{14}C, and another group in the molecule may contain a ^{3}H label. Because ^{3}H and ^{14}C have different maximal β-energies

(see earlier discussion), this allows the quantitative measurement of both ^{3}H and ^{14}C within the same sample. Similarly, other β-emitting nuclides, such as ^{35}S, can also be measured in the presence of ^{3}H or ^{14}C. The determination depends on the fact that there will be a region in the energy spectrum of the mixture where the β-particles from only one of the nuclides will be present. By measuring this region and comparing it with a nuclide standard, the appropriate isotope content in the mixture can be obtained. Some very elegant experiments have been conducted using doubly labeled xenobiotics. Pool and Crooks [152] used ^{3}H and ^{14}C to determine the in vivo stability of (R)-(+)-[^{3}H–N′–CH_3; ^{14}C–N–CH_3]-*N*-methylnicotinium ion, a primary nicotine metabolite in the guinea pig.

Positron Emission Tomography

Positron emission tomography (PET) was introduced in the early 1970s as a noninvasive diagnostic technique to study in vivo physiological processes in both animals and humans [161,194]. The process is an imaging technique that provides quantitative, regional measurements and kinetics of specific biochemical and physiological processes in living animals or human subjects. PET is similar to x-ray computerized axial tomography and MRI in that images of cross-sectional slices of the body are produced. The technique involves the use of a substance with the desired biological activity containing a positron-emitting radioactive isotope. Positron-emitting nuclides are neutron deficient compared to their stable isotopes and decay by spontaneous conversion of a proton to a neutron. This conversion is accompanied by release of a positron, which travels a small distance before encountering an electron, resulting in antimatter–matter annihilation. This annihilation event releases energy in the form of two 511 keV tissue-penetrating γ-ray photons radiating at approximately 180° from one another. A circular array of scanners consisting of scintillation crystals arranged so that opposing crystals are grouped in coincidence circuits are placed around the subject to detect the paired γ-rays as they simultaneously arrive on opposite sides (see Figure 41.34) [60]. The PET scanner's coincidence circuits enable the localization of the source of each annihilation. A computer then uses this information to reconstruct an image of radionuclide distribution within the body. Several million coincidences may be assimilated during a 1–15 min scan interval. In this way, a tomographic image can be obtained, illustrating the spatial distribution of the radionuclide. When images are recorded at appropriate intervals after the administration of the radionuclide, quantitative measurements reflecting the dynamic process under study can be obtained. Images can also be color coded to show differences in the levels of activity from one time point to the next.

Positron-Emitting Isotopes

The radionuclides most commonly used in PET are carbon-11, nitrogen-13, oxygen-15, and fluorine-18. All of these isotopes decay exclusively by positron emission, producing readily detectable tissue-penetrating γ-ray photons, but have relatively short half-lives (see Table 41.6). Their brief existence means that they must be manufactured close to the detection site, and more importantly, fast chemical reaction techniques and isolation procedures must be developed to ensure the production of a useful radionuclide. In addition, the use of such labeled compounds is limited to biochemical processes with rapid rates of turnover.

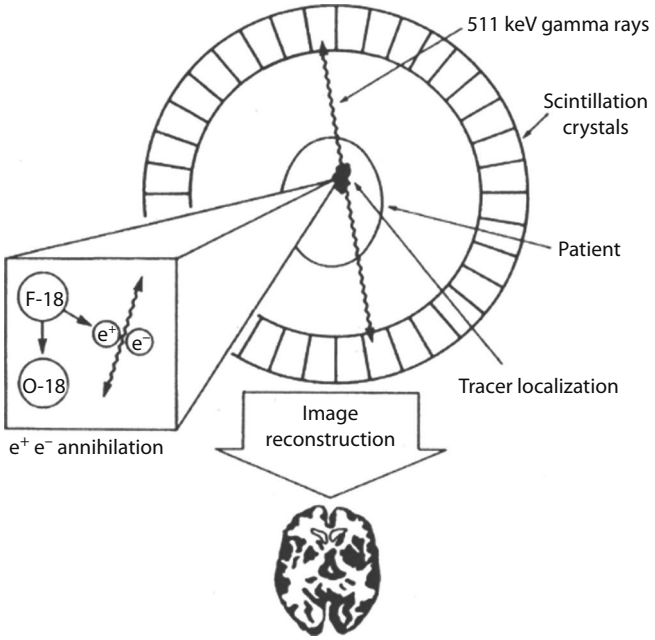

FIGURE 41.34 Key features of a PET scan: radiopharmaceutical localization, radioisotope decay by positron emission and immediate positron–electron annihilation; detection of body-penetrating 511 keV annihilation radiation by an external circular assay of scintillation crystals; and reconstructed image of radioactivity distribution. (From Derome, A.E., *Modern NMR Techniques for Chemistry Research*, Pergamon Press, Oxford, U.K., 1987. With permission.)

TABLE 41.6
Positron-Emitting Isotopes Commonly Used in PET

Nuclide	Half-Life (min)	Carrier-Free Specific Activity (Ci/mol)
^{11}C	20.40	9.22×10^3
^{13}N	9.96	1.89×10^4
^{15}O	2.07	9.10×10^4
^{18}F	109.70	1.7×10^3

The production of positron-emitting isotopes is normally carried out using on-site medical cyclotrons immediately prior to use, via the transmutation of stable isotopes; for example, ^{11}C is usually prepared by irradiating nitrogen or boron with accelerated protons, ^{15}O by irradiating nitrogen with deuterons, and ^{13}N by irradiating nitrogen with deuterons or irradiating carbon with deuterons. In the case of the longer-lived ^{18}F, stocks may be obtained from a regional distribution center. At sites remote from an available cyclotron, a radioactive generator system may be used to derive short-lived isotopes from longer-lived radioactive nuclides as they decay.

Although in some cases the positron-emitting isotope can be used directly in its elemental form (e.g., ^{15}O can be

directly used in metabolic studies, and ^{13}N is routinely used for studying lung ventilation), its incorporation into an unlabeled precursor molecule represents a formidable challenge for the imaginative synthetic chemist. In addition to the usual risk of working with body-penetrating radiation, the chemist must design suitable synthetic procedures that will proceed rapidly and in high radiochemical yield, avoid unintentional dilution of the radionuclide by stable carrier, and avoid difficult and time-consuming separation procedures. The number of steps in the synthetic process should be minimal to avoid working with large quantities of radioactivity and to ensure compatibility with the half-life of the isotope.

A description of the successful synthesis of L-[^{13}N]-tyrosine serves to illustrate the previous points [60]. A mixture of [^{13}N]-nitrate and [^{13}N]-nitrite obtained from an on-site cyclotron is converted to [^{13}N]-NH_3 by reduction with Devarda's alloy/NaOH reagent. The product is collected by distillation and immediately reacted with α-ketoglutarate and NADH. The reaction mixture is then passed through a column containing glutamate dehydrogenase immobilized on a solid support to give [^{13}N]-glutamate. Passage of this product down a second column containing immobilized glutamate oxaloacetate transaminase transfers the [^{13}N]-NH_2 group from glutamate to *p*-hydroxyphenylpyruvate to give L-[^{13}N]-tyrosine in radiochemically pure form and 28% overall radiochemical yield. The total synthesis time is 25 min.

Instrument Design

A functional PET unit consists of a data-acquisition system and a data-processing computer. The acquisition system incorporates the radiation detectors, their associated circuitry, and in some designs a mechanical system that imparts a small motion to the detectors to obtain better sampling. Data from the acquisition system are assimilated in a computer, and a display system for immediate viewing and recording of the image is available, with interactive capabilities for image analysis.

Specific Applications

PET is an extremely versatile method for probing fundamental biochemical processes in living systems. Important applications include metabolism, drug disposition and pharmacokinetics, and physiological and neurochemical mechanisms. In the brain, PET has been used to measure regional blood flow [170], glucose utilization, blood volume, oxygen utilization, the oxygen extraction ratio, the permeability–surface area product for water, acid–base chemistry, protein synthesis, and the characterization of dopamine D_2, benzodiazepine, and opiate receptors. PET measurement of regional rates of cerebral glucose metabolism utilizes [^{18}F]-2-fluoro-2-deoxyglucose (FdG) as the tracer component [61,149]. This procedure can quantitatively determine glucose utilization, because FdG, unlike dG, cannot be biotransformed further after its initial conversion to FdG-6-phosphate (FdG-6P) by hexokinase, due to the absence of an hydroxyl group at C2; thus, FdG-6P (and dG-6P) is metabolically trapped within the cell. Regional accumulation of FdG radioactivity is therefore proportional to blood glucose and is a sensitive index of brain function.

Regional blood flow and glucose utilization are established markers of local neuronal activity, and PET measurement of these parameters has been used to identify regions of the brain that are activated during visual, auditory, and somatic sensations; limb and eye movements; speech; semantic associations; and pathologic and normal forms of anxiety. Such parameters are also being used to identify regional brain abnormalities in patients with schizophrenia, panic disorders, epilepsy, Parkinson's and Huntington's diseases, obsessive–compulsive disorder, and clinical depression. An FdG-PET study in patients with acquired immune deficiency syndrome (AIDS) has focused on improving the diagnosis of the AIDS dementia complex (ADC), the major neurological outcome of AIDS that results in behavioral, cognitive, and motor disturbances [60]. In this study, progressive development of ADC correlated well with regional rates of cerebral glucose metabolism, particularly in the basal ganglia and thalamus. PET studies have also indicated that patients with Alzheimer's disease exhibit characteristic patterns of cerebral blood flow and glucose utilization; glucose metabolism in such patients is significantly reduced in the posterior parietal temporal region of the brain, compared to control patients [88]. PET images of cerebral glucose utilization have also been used to localize the epileptogenic foci in patients with intractable partial epilepsy.

The use of PET with radiolabeled synthetic drugs or radiolabeled analogues of naturally occurring neurotransmitters may lead to more reliable diagnoses of neurological disorders, perhaps even presymptomatic diagnosis. *N*-Methyl-[^{18}F]-spiroperidol (NMSP), a neuroleptic drug with high affinity for dopamine-D_2 receptors, has been used to visualize brain receptors in vivo in both normal and schizophrenic patients. The effectiveness of the antiparkinson drug, L-deprenyl, as a monoamine oxidase inhibitor in the brain has been verified from PET studies with ^{11}C-labeled L-deprenyl [150]. Ricaurte et al. [162] employed PET imaging of a ^{11}C-labeled serotonin transporter ligand to assess the long-term neurotoxicity of the recreational drug MDMA (Ecstasy) in humans. Here, reduced serotonergic receptor binding positively correlated with the extent of previous MDMA use.

Other studies [115,116] have shown that [^{18}F]-4′-fluoroclebopride ([^{18}F]-FCP) is a most promising candidate ligand for studying D_2 receptors with PET, and [^{18}F]-(+)-fluorobenzyltrozamicol ([^{18}F]-(+)-FBT) is a suitable ligand for studying cholinergic terminal density with PET via the vesicular acetylcholine transporter [117]. [^{18}F]-FCP has been used in PET studies designed to measure synaptic dopamine levels and D_2 receptor numbers in rhesus monkeys after treatment with psychostimulants such as (–)-cocaine, D-amphetamine, methylphenidate, and D-methamphetamine [115]. In these studies, psychostimulants caused an increase in the rate of washout of [^{18}F]-FCP from the basal ganglia. D-Methamphetamine and D-amphetamine had the greatest effect on washout kinetics of [^{18}F]-FCP relative to (–)-cocaine and methylphenidate, which was consistent with their ability to elevate synaptic dopamine levels. Thus, challenge studies with [^{18}F]-FCP may be a useful technique for

studying the dynamics of interaction between psychostimulant-induced increases in synaptic dopamine and postsynaptic D_2 receptors.

PET and [^{18}F]-FCP analyses have been utilized in chronic stress studies. Chronic stress results in heightened synaptic dopaminergic levels and a concomitant downregulation of D_2 receptors, analogous to the downregulation of dopaminergic receptors found following chronic cocaine exposure. Experiments with [^{18}F]FCP in dominant and subordinate cynomolgus monkeys showed a clearly greater uptake of the ligand in the basal ganglia by the dominant monkeys [60]. The data provide strong evidence that stimuli controlling behavior/physiological consequences (stress) have a neurochemical correlate that can be imaged with PET techniques and suggest that chronic stress results in heightened synaptic dopaminergic levels and a concomitant downregulation of D_2 receptors. Previous PET studies have found downregulation in dopaminergic receptors in human cocaine addicts [204,205] and alcoholics [73]. PET may also be a powerful tool in addressing the etiology of complex behavioral disorders associated with stress.

[^{18}F]-(+)-FBT has high affinity for the vascular acetylcholine transporter and low affinity for σ_1 and σ_2 receptors [119]. Its high uptake in the basal ganglia and reversible binding kinetics in vivo indicate that this tracer can provide quantitative measurements of vesicular acetylcholine transporter function in vivo with PET imaging. The vesicular acetylcholine transporter is a marker for cholinergic nerve terminals and may be directly related to cholinergic neuronal density. An important goal in PET studies is relating cholinergic neuronal density to cognitive performance. Such studies may be able to correlate an individual's change in behavioral performance (i.e., cognition) with a change in (reduction in) cholinergic function.

PET scanning of the torso is routinely employed in the diagnostic evaluation of patients with certain disorders of the heart. Modeling metabolic processes in the heart is more complex than in the brain, because the heart draws energy from several substrate pools, such as fatty acids, carbohydrates, and lactate. FdG-PET studies and blood flow [^{13}N]-ammonia-PET measurements have been used to diagnose coronary artery disease with better than 95% accuracy. Other studies have utilized [^{11}C]-palmitate, which is accumulated homogeneously by the heart muscle, to diagnose cardiovascular abnormalities [52]. Whole-body copper flux following transgastric injection was assessed in Long–Evans Cinnamon (LEC) rats with a defective copper transporter using ^{64}Cu and PET imaging [17]. The authors concluded that increased biliary secretion may not provide sufficient protection against high doses of orally administered copper, whereas the trapping of copper in the upper gastrointestinal tract may be critical to reducing toxicity.

PET images of glucose utilization and amino acid uptake are particularly useful for examining patients with a variety of tumors throughout the body. PET images of labeled estradiol uptake are used to examine patients with primary and metastatic breast tumors.

An established technique in diagnostic medicine, PET is now widely used for neuroscientific, pharmacological, and toxicological studies in vivo. Two major obstacles to more general use of this technique are its cost effectiveness and the availability of appropriate radiotracers. The synthesis of novel positron-emitting drug molecules remains a challenge for the radiochemist. Nevertheless, PET has advantages over other functional imaging techniques such as single-photon emission computed tomography (SPECT). Unlike MRI, it has the potential to measure characteristics of biological compounds, such as neurotransmitters and neuroreceptors, that exist in minute concentrations. Improvements in the methodology for determining anatomical localization, data analysis, and the performance of PET systems are ongoing. This important analytical tool continues to offer unique opportunities for researchers in the study of physiology, biochemistry, pharmacology, and toxicology.

Stable Isotopes

Most elements exist as several isotopes that vary in atomic weight. Some of the heavy isotopes are stable and do not decay with the emission of radiation. These so-called *stable isotopes* typically have a low natural abundance and chemical and physical properties that are nearly identical to those of their more abundant counterparts. Carbon exists naturally as a mixture of isotopes, ^{12}C, ^{13}C, and ^{14}C. ^{12}C is the most abundant isotope of carbon (98.89%); ^{13}C, the stable isotope of carbon, is much less abundant (1.11%); and ^{14}C is a radioisotope of carbon with a natural abundance so low as to be insignificant (except for its special application for carbon dating). Hydrogen also exists as a mixture of isotopes, ^{1}H, ^{2}H, and ^{3}H. ^{1}H (protium) is the most abundant isotope of hydrogen (99.985%); ^{2}H (deuterium), the stable isotope of hydrogen, is much less abundant (0.015%); and ^{3}H (tritium) is a radioactive isotope of hydrogen with insignificant natural abundance. Other stable isotopes that are useful for toxicology research include ^{15}N (0.37%), ^{17}O (0.037%), and ^{18}O (0.204%). A wide variety of chemicals specifically labeled with ^{2}H, ^{13}C, ^{15}N, ^{17}O, and ^{18}O at high atom-% enrichments are commercially available.

Stable isotopes with low natural abundance make ideal tracers for studying mechanism of toxicology. Because they are stable, they pose no significant health risk to the researcher or the experimental subject. No special license is required for the acquisition and use of stable isotopes, and no special precautions are necessary for their safe handling (other than the normal precautions used for handling the unlabeled compound). Because their chemical and physical properties are very similar to those of the more abundant isotope, their behavior in vivo is also quite similar. Compounds enriched in a stable isotope can be studied in the presence of natural abundance compounds with minimal background interference. Finally, a wealth of mechanistic information is available from stable isotope studies through the application of modern magnetic resonance and mass spectral techniques.

Choice and Location of Label

The stable isotope of choice for a toxicology study depends on the nature of the study (ADME, exposure assessment, mechanism of toxicity) and the instrumental method that will be used for analysis. The most common stable isotopes for toxicology research are ^{13}C, ^{2}H, ^{15}N, and ^{18}O. Stable isotopes of heavy metals such as ^{199}Hg and ^{206}Pb have been used to study mechanisms of toxicity and clearance. If NMR is used for analysis, it is best to use a spin 1/2 isotope for maximum sensitivity. For ^{13}C NMR analysis, uniform labeling offers distinct advantages in terms of metabolite characterization as will be discussed further in the following. For mass spectral analyses, the stable isotope that will give the greatest mass difference between natural abundance and labeled analytes is appropriate. Multiple sites of labeling are quite common and provide greater mass discrimination. If GC-MS is used, the optimum mass difference is 2–4 amu. A greater mass difference can lead to altered retention times and introduce uncertainty in quantitative analysis. Unlike radioisotopes where enrichments are quite low, stable isotopic enrichments are typically high, at least 90 atom-%. In general, the factors that govern the position of a radioisotopic label (stability and exchangeability) also govern the position of a stable isotopic label.

Applications: Pharmacokinetics and Metabolism of Isotopically Labeled Nicotine

Due to the widespread use of tobacco products, the pharmacokinetics of nicotine and its metabolites have been the subject of much study. Early studies focused on nicotine and its major metabolites cotinine and nicotine-1′-oxide. Kyerematen et al. [104] reported a pharmacokinetic study of nicotine and 12 of its metabolites in the rat. They administered ^{14}C-labeled nicotine in serial intra-arterial doses and collected blood for up to 30 h and urine up to 120 h. Nicotine and 12 of its metabolites were quantified by HPLC with radiometric detection. One previously unidentified metabolite, collected from the HPLC and analyzed by EI MS, was identified as allohydroxy-demethylcotinine. Plasma nicotine half-life, total body clearance, and apparent volume of distribution and half-lives of urinary excretion of cotinine, cotinine-*N*-oxide, and allohydroxy-demethylcotinine were all determined. Radiolabeled nicotine afforded convenient determination of nicotine and 12 of its metabolites with high sensitivity, demonstrating the power of this method for the study of nicotine pharmacokinetics.

Kyerematen et al. [103] also determined the disposition of radiolabeled nicotine and eight of its metabolites in humans. The use of radiolabeled nicotine enabled detection of plasma and urinary nicotine and metabolites from a very low dose (190 μg/subject), so a comparison of smokers and nonsmokers was possible. Two new urinary metabolites, 3′-hydroxycotinine glucuronide and demethylcotinine $\Delta^{2',3'}$-enamine, were identified by mass spectral analyses.

The ability to distinguish labeled from unlabeled nicotine by GC-MS enabled the study of nicotine pharmacokinetics and bioavailability in humans [15]. After smoking a cigarette, smokers received nicotine labeled with deuterium in both 3′ positions (previously shown to be stable to metabolism). Plasma levels of both labeled and unlabeled nicotine were monitored by GC-MS with SIM. The sensitivity of the analytical method and the specificity of the labeled nicotine that eliminated background interference from unlabeled nicotine gave high-quality pharmacokinetic data.

Metabolomic analysis is also facilitated using these techniques. At the microbial scale, a mixture of uniformly labeled ^{13}C-labeled metabolites was extracted from yeast grown on ^{13}C-labeled substrates [221]. The labeled metabolites were then used as internal standards for the LC-MS analysis of the glycolytic and TCA cycle metabolites produced in yeast grown under varied conditions. The precision and efficiency of cellular extraction, sample processing, and quantification of metabolite recovery for each metabolite were significantly improved when using labeled metabolites as internal standards.

Characterization of Urinary Metabolites

Detection of ^{13}C resonances of natural abundance xenobiotic metabolites in a complex matrix such as urine is often difficult due to interference from endogenous compounds. Appropriate use of ^{13}C-enriched xenobiotics can facilitate the characterization of metabolites in whole urine by ^{13}C NMR spectroscopy with little or no sample cleanup. In higher organisms, the characterization and quantification of dynamic renal xenobiotic metabolism have been extensively explored using ^{13}C-labeled compounds coupled with NMR and other analytical methods [10].

Isotopically labeled ^{13}C-benzene was employed to distinguish benzene metabolites produced from known benzene exposure from background levels typically present in the urine [210]. Human volunteers were exposed to an environmentally relevant dose of benzene, 40 ± 10 ppb, for 2 h. Benzene metabolites, readily discernible from unlabeled background metabolites, were then quantified using GC-MS. The percentage of muconic acid metabolites excreted was higher than that reported after exposure to occupational levels. The authors suggest that such stable isotope studies may be useful for validating toxicokinetic models and occupational exposure risk extrapolation.

Sumner et al. [191] studied the metabolism of [1,2,3-^{13}C]-acrylamide in rats and mice using 1D and 2D ^{13}C NMR spectroscopic analyses of urine. The animals received the labeled xenobiotic orally. Urine was collected for 24 h and then centrifuged, and D_2O was added to a final concentration of 15%. INADEQUATE spectra were used to correlate all carbon signals. HET2DJ spectra revealed the number of protons attached to each carbon for all metabolites. These spectral data, along with calculated chemical shifts of proposed metabolites and spectra of synthetic standards, were used to identify five urinary metabolites of acrylamide. Quantification of the urinary output of acrylamide and the five metabolites was accomplished by integration of the carbons signals in 1D spectra relative to dioxane, an internal standard. Approximately 50% of the administered dose of acrylamide was recovered as the five metabolites, confirming

a previous study wherein 62% of the administered dose of ^{14}C-labeled acrylamide was recovered in urine within 24 h. Stable isotope labeling and ^{13}C NMR analysis enabled the characterization and quantification of xenobiotic metabolites in a complex matrix without the need for tedious sample cleanup or chromatographic separation.

Determination of Toxic Metals Using Stable Isotope Dilution MS

Stable isotopes are widely employed in elemental MS studies of toxic elements. Smith et al. [182,183] used stable lead isotopes to study the effectiveness of a chelating agent (DMSA) in removing lead from skeletal vs. soft tissue and the redistribution of lead in other tissues. Lead has four naturally occurring isotopes: ^{204}Pb (1.4%), ^{206}Pb (24.1%), ^{207}Pb (22.1%), and ^{208}Pb (52.4%). By enriching drinking water given to rats with ^{206}Pb, ingested lead was distinguishable from the endogenous lead already present in the animals. Here, the mass spectrometer used thermal emission to produce ions and measure the isotopic ratios, thereby quantifying the ^{206}Pb dose.

Cadmium is another highly toxic metal. Environmental and industrial cadmium exposure is of great concern. In contrast to the previous thermal emission mass spectrometric method for lead determination, a stable isotope dilution GC-MS method was employed for assessing cadmium exposure. Urinary cadmium was determined by using the chelating agent Li(FDEDTC) to form the chelate $Cd(FDEDTC)_2$. The chelate was then analyzed by GC-MS with EI, where $M^{+\bullet}$ was monitored for quantitation. Precise, accurate cadmium detection in urine at the 10 mg/L level was achieved. This isotope dilution technique eliminated matrix effects, so precision and accuracy were not affected by incomplete recovery.

INTEGRATION OF TECHNIQUES IN TOXICOLOGY

Mechanisms of xenobiotic toxicity are usually complex, involving not only the parent compound but also a broad array of metabolites and tissue-derived endogenous compounds. All of these materials are produced in a dynamic process as the intoxicated organism responds to the toxic insult and attempts to maintain homeostasis. Modern instrumental methods are powerful tools that have their greatest utility when they are used in conjunction with one another in a synergistic fashion to characterize materials in complex mixtures. Numerous examples are available in the literature of the use of multiple instrumental techniques to describe a variety of different chemical entities in complex mixtures.

The forensic toxicologist often conducts hair analysis to assess both acute and chronic exposure to drugs and toxins. Newborn hair analysis may be particularly suited for studying both fetal and maternal exposure to xenobiotics. Tagliaro et al. [192] assayed thyroxine concentration in newborn hair by first extracting the hair, fractioning it with HPLC, and then using a radioimmunoassay to quantify the analyte. The amphetamine and alkaloid drug content in hair samples was similarly assessed by derivatizing the amines in alkaline hair extracts to produce trichloroethyl carbamates, followed by combined GC-MS analysis of the derivatives [55]. Gustafson et al. [65] simultaneously determined plasma levels of THC and two of its metabolites, first by enzymatically cleaving their glucuronide conjugates, followed by SPE, separation, and finally, quantification by GC-MS. Given the large number of biomarkers and dynamic concentration ranges normally present in a metabolomic fingerprint, integrated separation and analytical techniques are commonly employed for mapping these complex mixtures. A method for multicomponent analysis has been developed wherein rat urinary metabolite profiles correlated with toxin exposure may be accurately determined by combining HPLC separation with simultaneous electrochemical redox array and MS analysis to identify intoxicated animals.

In one exemplary study, many of the techniques that have been described in this chapter were used to confirm the identity of many compounds. Wiltshire et al. [216] used diode array UV spectrophotometry, MS, NMR, radiolabeled tracers, open column chromatography, HPLC, and TLC to characterize the complex metabolic pathway of a calcium antagonist (Ro 40-5967) in the rat (Figure 41.35). A classic xenobiotic disposition study was conducted using Ro 40-5967 labeled with ^{14}C. Approximately 80% of the radiolabeled dose was recovered in all excreta including bile, urine, and feces; 37% of the dose was excreted in the bile, so chemical characterization of the biliary radioactivity was carried out. Bile was partially purified by passing it through an open column of Amberlite XAD-2 resin. The fractions collected from the open column were evaporated to dryness and partitioned between ethyl acetate and water. Two of the fractions contained an insoluble gum that was taken up in ethanol. All fractions (aqueous and organic) were taken to dryness and then dissolved

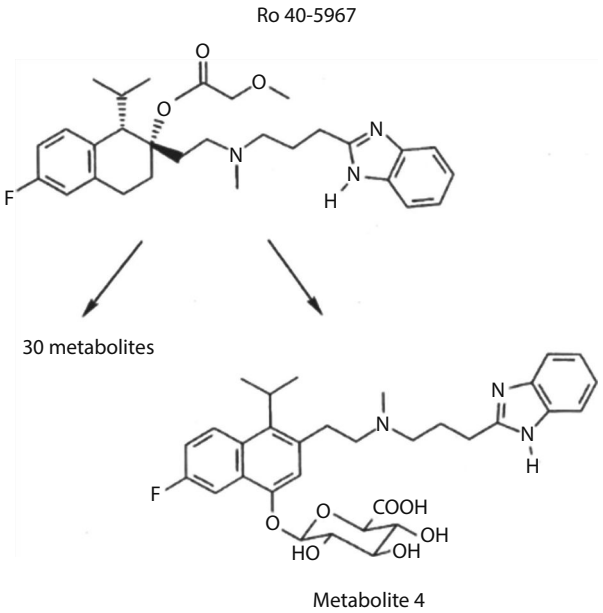

FIGURE 41.35 Metabolism of the calcium antagonist Ro 40-5967 in the rat. Characterization of metabolite 4 exemplifies the integration of modern instrumental techniques to study mechanisms of toxicology.

in aqueous methanol. These fractions were subjected to three sequential reversed-phase HPLC purification steps to obtain material that was suitable for further characterization. It was characteristic of major changes in the parent compound; for example, a λ_{max} at 280 nm is typical of oxygen substitution at the 5 position of the parent compound. It was obvious that approximately four of the metabolites fell in this class.

Further characterization of the metabolites required NMR and MS. Sufficient material from the HPLC purification was obtained from 11 of the XAD fractions. This represented approximately two-thirds of the radioactivity excreted in bile. The proton NMR spectrum of the parent compound possessed a number of characteristic features, and it was possible to see similar features in the spectra of the fractions. A number of the metabolite fractions contained resonances characteristic of glucuronic acid conjugation. Mass spectra were obtained from 45 subfractions of the original XAD fractions by thermospray LC-MS. As with the proton NMR spectra, features in the mass spectra were characteristic of the parent compound; for example, *m/z* 406 was characteristic of the loss of the side chain.

Rather than discussing the entire characterization that involved the description of 31 metabolites, it is instructive to describe the characterization of just one of the metabolites. Metabolite 4 was the major biliary metabolite and equivalent to about 25% of the total biliary radioactivity. It had a class 3 UV spectrum, indicating the presence of a substituted naphthyl group. Remember that the parent compound contains a tetrahydronaphthyl ring system where one of the rings of naphthylene is saturated. The very strong double peak at 240–250 nm is characteristic of a fully aromatic substituted naphthylene.

The proton NMR spectrum of this metabolite showed the presence of glucuronic acid as well as loss of the ester side chain. Evidence that the asymmetric center of the isopropyl side chain had been lost was obtained from a shift in the methyl resonances of the isopropyl group. Aromatization of the saturated ring was obvious by the addition of an aromatic singlet at 7.1 ppm. The metabolite structure was deduced to be an oxyglucuronide derivative of the parent compound in which the alicyclic ring had been aromatized. This was consistent with a mass spectrum containing a protonated molecular ion at *m/z* = 596, loss of the benzimidazole group (*m/z* = 438), and loss of glucuronic acid (*m/z* = 262).

This integrated application of instrumental techniques permitted a thorough characterization of metabolites that varied greatly in chemical structure and polarity. A total of 31 biliary metabolites, representing 67% of the excreted dose of Ro 40-5967, were characterized. Only through the integration of modern instrumental methods could this complex metabolic pathway be thoroughly elucidated.

QUESTIONS

41.1 Name four ways that MS has been used to assess exposure to xenobiotics.

Answer: MS has been used to determine metabolites of xenobiotics in biological fluids, to analyze the metabolomic profile of heavy metal exposure, to determine DNA adducts formed as a result of toxin exposure, and for the rapid identification of intoxicants.

41.2 An unknown compound in an organic extract of urine is sufficiently volatile to pass through a GC-MS system but fragments extensively under EI such that no $M^{+\bullet}$ is observed. Name at least two alternative approaches to determine the molecular weight of this compound using MS.

Answer: (a) A GC-MS system with PICI could be used to form $(M + H)^+$. (b) An HPLC method could be developed, and the material analyzed by LC-MS using a soft ionization method such as ES or APCI. (c) If the compound contained a hydroxyl or amine group, derivatization with a common trimethylsilation reagent could form a compound with a more stable molecular ion.

41.3 An LC-MS method with an ES ionization source runs a binary gradient with mobile phase 1 = 2 mM ammonium acetate and mobile phase 2 = 90% methanol:10% acetonitrile. There is a large background ion in the system at *m/z* 59. CID on this ion produces a fragment at *m/z* 42. What are likely assignments for these ions?

Answer: The *m/z* 42 is protonated acetonitrile. The mass difference of 17 Da (59 – 42) suggests ammonia (NH_3) as a neutral loss; thus, *m/z* 59 is likely an ammonium adduct of acetonitrile, $(CH_3CN)(NH_4)^+$, produced from background mobile-phase components.

41.4 EI mass spectrometers have several ions constantly present in the background mass spectrum at *m/z* 18, 28, 32, 40, and 44. Identify these components.

Answer: These are all components of air with moisture present: *m/z* 18 = H_2O; *m/z* 28 = N_2, *m/z* 32 = O_2, *m/z* 40 = Ar, and *m/z* 44 = CO_2.

41.5 Using positive ES, a large protein is ionized under acidic conditions that create an $(M+4H)^{4+}$ ion in the mass spectrum at *m/z* 712.2. What is the molecular weight of this compound?

Answer: Remember that four protons have been attached to this molecule, so *m/z* = 712.2 = (M + 4H)/4, → *M* = (712.2 × 4) – 4; *M* = 2844.8 Da.

41.6 $CDCl_3$ is a commonly used NMR solvent. Carbon NMR spectra of samples dissolved in $CDCl_3$ contain a residual solvent peak that is split due to ^{13}C–^{2}H coupling.

a. Into how many lines is this signal split?

Answer: ^{2}H has a spin quantum number (*I*) of 1. Applying the $2nI + 1$ rule, the number of lines is (2 × 1 × 1) + 1 = 3.

b. In most cases, carbon spectra are acquired with hydrogen decoupling. Why is not the ^{13}C–^{2}H multiplet reduced to a singlet under the conditions of this decoupling?

Answer: Decoupling is applied at the ^{1}H (protium) frequency. Deuterium (^{2}H) has a very different precessional frequency; therefore, it is not decoupled in the carbon NMR spectrum.

41.7 Why is ^{13}C–^{13}C coupling rarely seen in carbon NMR spectra?

Answer: Coupling is only seen for closely spaced nuclei (fewer than four to five bonds). To see ^{13}C–^{13}C coupling, two ^{13}C atoms would have to be in close proximity. The natural abundance of ^{13}C is only 1%, so the likelihood of two ^{13}C nuclei being in close proximity is very low. Carbon spectra of molecules composed of natural abundance carbon do not show ^{13}C–^{13}C coupling. ^{13}C–^{13}C coupling is only seen with isotopically enriched molecules.

41.8 Sensitivity in NMR depends on what factors?

Answer: (a) Magnetic field strength, (b) magnetogyric ratio, and (c) natural abundance of the spin-active nucleus.

41.9 Describe three approaches for increasing sensitivity for a given nucleus in NMR.

Answer: (a) Using a higher-field-strength instrument (magnet), (b) using a smaller NMR probe or a selectively tuned probe, and (c) signal averaging (FT-NMR).

41.10 Why are chemical shifts expressed in dimensionless units designated δ?

Answer: Chemical shifts vary with the strength of the applied magnetic field. To enable comparisons of chemical shifts from one instrument to the next, chemical shifts are expressed in δ units.

41.11 What factors limit time resolution in online LC-NMR?

Answer: (a) HPLC mobile-phase flow rate, (b) total time required to obtain one transient (scan), and (c) total number of transients acquired per LC peak.

41.12 Why is EPR inherently more sensitive than NMR?

Answer: The magnetic moment of an electron is 700 times greater than that of a proton. This means that the energy difference between spin states is greater for electrons than for protons. This greater energy difference leads to higher sensitivity. It also means that the energy required for an EPR transition is higher than that required for an NMR transition. NMR uses energy in the RF range; EPR uses energy in the microwave range.

41.13 The EPR spectrum of the Fe-DETC complex with ^{15}NO shows doublet hyperfine splitting.

a. Why?

Answer: ^{15}N has a spin quantum number (I) of 1/2. Applying the $2nI + 1$ rule ($n = 1, I = 1/2$), the number of lines is 2 (doublet).

b. How was this used to distinguish between NO produced from ^{15}N-labeled arginine and NO produced from endogenous arginine?

Answer: ^{14}N has a spin quantum number of 1; therefore, it splits electron signals into three lines (triplet hyperfine splitting). Endogenously formed NO is derived from natural abundance arginine. Natural abundance nitrogen is 99.6% ^{14}N, so it leads to triplet hyperfine splitting. Any doublet splitting results from NO produced from added ^{15}N-labeled arginine.

41.14 QWBA is becoming a very popular method for determining xenobiotic tissue distribution.

a. What is the major limitation of this technique?

Answer: QWBA is used to determine total radioactivity. It cannot distinguish between different chemical forms of radioactivity; in other words, it cannot distinguish between xenobiotic metabolites. It determines the sum of parent compound plus metabolites and, therefore, yields no information on xenobiotic metabolism.

b. In practice, how can this limitation be overcome?

Answer: To overcome this limitation, follow-up studies must be performed. QWBA is used to determine the distribution of a xenobiotic. QWBA data reveal the tissues containing xenobiotic-derived radioactivity. The follow-up studies involve administration of the xenobiotic (radiolabeled or natural abundance) and isolation of tissues containing the parent xenobiotic and its metabolites.

Extraction of these tissues provides solutions that can be analyzed for metabolites by suitable separation techniques such as LC-MS or LC-NMR.

41.15 What effect, if any, would you expect to observe in the UV absorbance spectrum of an ethanol solution of each of the following compounds: phenol, aniline, and *trans*-cinnamic acid, after the addition of a drop of 1 N aqueous sodium hydroxide solution to the UV cuvette containing the solution of each compound?

Answer: (a) Phenol—Addition of 1 N NaOH will convert phenol (PhOH) to the phenolate ion (PhO^-), leading to a formal negative charge on the auxochromic group attached to the phenyl ring chromophore. The interaction of these electrons with the conjugated phenyl ring produces a bathochromic–hyperchromic shift; that is, the absorption band of phenol shifts to a longer wavelength, and the extinction coefficient increases in value. (b) Aniline—Addition of 1 N NaOH will have no significant effect on the absorption spectrum of aniline, as the alkaline medium will not alter the structure of the molecule. (c) *trans*-Cinnamic acid—Addition of 1 N NaOH will generate the carboxylate ion of this organic acid ($R–COO^-$). As with phenol, this will result in a bathochromic–hyperchromic shift in the absorption band of *trans*-cinnamic acid.

41.16 Under what circumstances may the UV absorbance of organic molecules deviate from Beer's law?

Answer: The UV absorbance of organic molecules may not obey Beer's law if (a) protonation or deprotonation, (b) tautomerization, (c) dimerization, or (d) complex formation of the analyte occurs in solution.

41.17 Even relatively simple, low-molecular-weight compounds exhibit an abundance of absorption bands in their IR spectrum. What factors influence the number and value of the IR absorption bands in organic molecules?

Answer: The number of fundamental modes of vibrations (i.e., stretching and bending vibrations) is $(3n - 6)$ for nonlinear

polyatomic molecules and $(3n - 5)$ for linear molecules, where n is the number of atoms in the molecule. The factors that influence the value of IR absorption frequencies of chemical bonds in organic molecules are (a) the masses of the two atoms in the bond, (b) the velocity of light, and (c) the force constant of the bond.

41.18 Discuss the usefulness of group frequencies in the IR spectrum of unknown organic molecules.

Answer: Group frequencies are characteristic absorption frequencies of certain functionalities within an organic molecule, which can be used diagnostically to confirm the presence (or absence) of the functionality in an unknown molecular structure. Examples of such functionalities are C═O, C≡N, C≡C, −OH, and $-NH_2$. The frequencies of these structural moieties are usually independent of the structure of the rest of the molecule.

41.19 What is one of the major advantages of using Raman spectroscopy compared to IR spectroscopy for studying biochemical processes?

Answer: One of the major advantages of using Raman spectroscopy in biochemical studies is that spectra can be obtained for molecules in aqueous solution, as water exhibits a weak Raman spectrum that interferes only minimally with the spectrum of the analyte.

41.20 Which general class of nuclides is useful in PET scanning, and which isotopes are commonly utilized?

Answer: Positron-emitting radioactive nuclides, which are neutron deficient, are used in PET scanning. These isotopes are short lived and decay by spontaneous conversion of a proton to a neutron. This process is accompanied by release of a positron, which collides with an electron, resulting in annihilation and release of energy in the form of two tissue-penetrating γ-ray photons. Common positron-emitting isotopes used in PET scanning are ^{11}C, ^{13}N, ^{15}O, and ^{18}F. These nuclides have half-lives ranging from 2 to 110 min.

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42 Methods in Environmental Toxicology

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CONTENTS

Testing of chemicals for their toxic effects traditionally focused on safety and effects in humans using surrogate species. Beginning in the 1960s, the recognition that chemicals in the environment can have effects on nonhuman receptors has led to the emergence of the subdiscipline of ecotoxicology. Subsequently, testing protocols with a wide range of surrogate species have been established to address questions concerning thresholds of toxic effects and mode of action. By expanding the number of species tested in assessing the toxicology of a chemical, we are able to predict and diagnose possible adverse environmental effects and also gain considerable insight into a toxicant's mechanism of action, organ-specific toxicity, and acute and long-term effects. Field protocols emphasize methods and indicators for determining the consequences of chemical exposure on species and their populations. In this chapter, we provide principles and examples of laboratory and field protocols that have been developed to understand the effects of chemicals in the environment.

ENVIRONMENTAL BEHAVIOR OF CHEMICALS

When testing the toxicity of chemicals to organisms in the laboratory, the chemical is usually fed to or applied directly to the animal or incorporated into the medium in which the organism is living (i.e., soil, water, sediment). However, when a chemical is in the environment, it partitions among different media according to specific physical properties and chemical behaviors. This directly influences the amount and form of the chemical to which organisms are exposed. Therefore, to conduct toxicity tests with the relevant chemical form and concentrations, the environmental transport and fate of chemicals must be understood. While a detailed discussion of environmental chemicals is beyond the scope of this chapter, we provide a short review of the most salient topics. Practical methods for estimating environmentally important properties of organic chemicals can be found in Boethling and Mackay [1], while numerous textbooks describe the fundamental concept of chemical fate and transport (see, e.g., Manahan [2]).

Water Solubility and Lipophilicity

The most significant determinant of the transport and fate of a chemical in the environment is its water solubility. Highly soluble chemicals are transported through the hydrologic cycle and thus can be distributed over great distances from their points of introduction into the environment. Conversely, hydrophobic compounds tend to be more static and move little through the hydrologic cycle. Generally, the more water soluble the chemical, the less lipophilic it is, the less it is sorbed to soils and sediments, and the less it accumulates into biota. Water solubility is defined as the maximum amount of a chemical that can be freely dissolved in a given quantity of pure water at a particular temperature. It is important to note that even chemicals described in tables of physical constants as very insoluble may have sufficient water solubility to significantly influence their behavior in the environment. It is particularly important to note that metals and other inorganics with low water solubility may be converted to more water-soluble forms in the environment or introduced as soluble salts. A derivative property of the water solubility and lipophilicity of a chemical is its octanol/water partition coefficient (K_{OW} or P), frequently reported as log K_{OW}. K_{OW} is defined as the ratio of the concentrations of a chemical in the water phase and in the *n*-octanol phase after equilibrating between equal volumes of the two solvents. It is a key property of a chemical for environmental considerations, as it is a predictor of soil and sediment adsorption and subsequent bioaccumulation in organisms and potential biomagnification through trophic transfer up the food chain. In designing studies to assess the toxicity of a chemical in model ecosystems, such as microcosms [3] or mesocosms [4], the octanol/water partition coefficient must be known or experimentally determined, so the behavior of the chemical in the system can be predicted and the system designed appropriately.

Soil Adsorption

The extent of partitioning of a chemical between the solid and solution phases of a water-saturated soil or sediment is described by the soil sorption coefficient (K or K_d) [5,6]. It is determined experimentally using the Freundlich equation:

$$\frac{x}{m} = KC^{1/n}$$

where

x/m is the μg of chemical adsorbed per g of soil
C is the μg of chemical per mL of solution
K and n are constants for a particular soil type

The value of n must be determined experimentally but is frequently assumed to be 1. K_{OC}, the soil sorption constant, is determined from K by dividing by the percent organic carbon in the soil and multiplying the result by 100. This constant is observed to be relatively independent of the type of soil or sediment and is the value most frequently used to describe the adsorption of a chemical to soil or sediment. Consideration of the soil adsorption of a chemical is extremely important for the proper design of ecological test systems.

Atmospheric Particulate Matter

When pollutant-contaminated soils become airborne, chemicals sorbed to the resulting particulate matter must be considered in some testing protocols. Such sorbed chemicals may be bioavailable in biological systems. Soil-derived particulate matter is composed of a mineral fraction and an organic matter fraction. Affinity of organic chemicals for the mineral fraction is believed to be low, but hydrophobic organic chemicals may have a high affinity for the organic portion [1]. Once particulate matter is suspended in the atmosphere, gas/particle partitioning of adsorbed chemicals is a function of the vapor pressure of the chemical. Chemicals with a vapor pressure $<10^{-6}$ atm will be primarily in the particle phase, while those with a vapor pressure >1 atm will partition primarily to the gas phase [1]. Thus, under some testing conditions, the air quality of the test system must be controlled.

Vaporization

The vaporization of a chemical from a solid surface or a solution is an important mass-transfer process. Factors that control volatilization are diffusivity of the chemical, its water solubility, vapor pressure, the Henry's law constant, and temperature. Diffusivity is the rate of diffusion of a chemical through a medium and depends on the nature of the chemical itself and the nature of the medium through which it moves. Vapor pressure is the tendency of a liquid to change from the liquid to the gaseous state and is highly dependent on temperature.

The air/water interface is most important in environmental analyses, so the question of the ability of a chemical to diffuse across that interface is significant when evaluating its environmental behavior. The tendency of a chemical to escape from solution is described by the Henry's law constant. Henry's law states that the solubility of a gas in a liquid is directly proportional to the pressure of the gas above the liquid at equilibrium: $H = P/C$, where C is measured in mol/m^3 and P is in atm; that is, H is the ratio of the saturation vapor pressure and the water solubility of the chemical. Units of H are most often reported as atm-m^3/mol; however, if C is expressed in mol/L and P is expressed in mol/m^3, H is dimensionless. Under these circumstances, the Henry's law constant is sometimes referred to as the air/water partition coefficient.

Movement of chemicals in the environment occurs in the vapor state, as well as in solution or when adsorbed to particulate matter. The most important consideration in evaluating the extent of such movement is the Henry's law constant, because most chemicals will eventually be found in water solution, and their tendency to move into the air will have an impact on their potential for toxic effects in organisms that may be exposed. In the design of toxicology test methods, such considerations must be taken into account. Their importance is illustrated in Figure 42.1, in which the relation of the Henry's law constant of a chemical to its volatility characteristics is depicted.

Bioaccumulation

Certain chemicals accumulate in organisms that are exposed in their environments. Some chemicals biomagnify in the food chain, such that higher trophic-level organisms have higher concentrations of the substance in their tissues than do their prey. There are now many well-known cases of chemicals biomagnifying in food webs to the point that toxic effects are exhibited in organisms that may have had no direct exposure to the chemical itself at its point of application; polychlorinated biphenols (PCBs), organochlorine insecticides, and mercury are a few examples. The tendency of a chemical to be more concentrated in an organism than the concentration in its environment is described by its bioaccumulation factor (BAF) or bioconcentration factor (BCF). The BAF or BCF is calculated by dividing the concentration of the chemical in the organism by the concentration of the chemical in the soil, sediment, or water in which it lives. BAFs assume that exposure has occurred through all potential routes, including direct uptake from the environmental medium and ingestion of contaminated foods. BCFs refer to water exposures and are applied typically only to aquatic organisms; however, BCFs have been used considering pore-water transfer of chemicals to invertebrates in mesic soil environments. BCFs have units of water volume per unit tissue weight (e.g., mL/g) and can be viewed conceptually as the volume of water containing the amount of chemical in 1 g of tissue [7]. The BCF can be estimated from the water solubility of a chemical, K_{OW}, or K_{OC}. A number of empirical equations that are specifically useful for calculating BCFs for particular chemical groups can be found in Lyman et al. [5]. The estimates of BCF should be used in designing toxicity studies to understand the degree to which the test chemical will be taken up and stored in the organisms being used in tests; however, bioaccumulation and bioconcentration assume that the organism is at a steady state with the chemical in its environment and assumes net uptake and so does not account for the ability or rate at which an organism metabolizes the chemical. Therefore, the BCFs or BAFs are often species and site specific, and a lack of consideration of species-specific toxicokinetics may yield misleading conclusions.

Biodegradation

As organic chemicals move in the environment, they are subjected to breakdown, primarily by microorganisms, in a

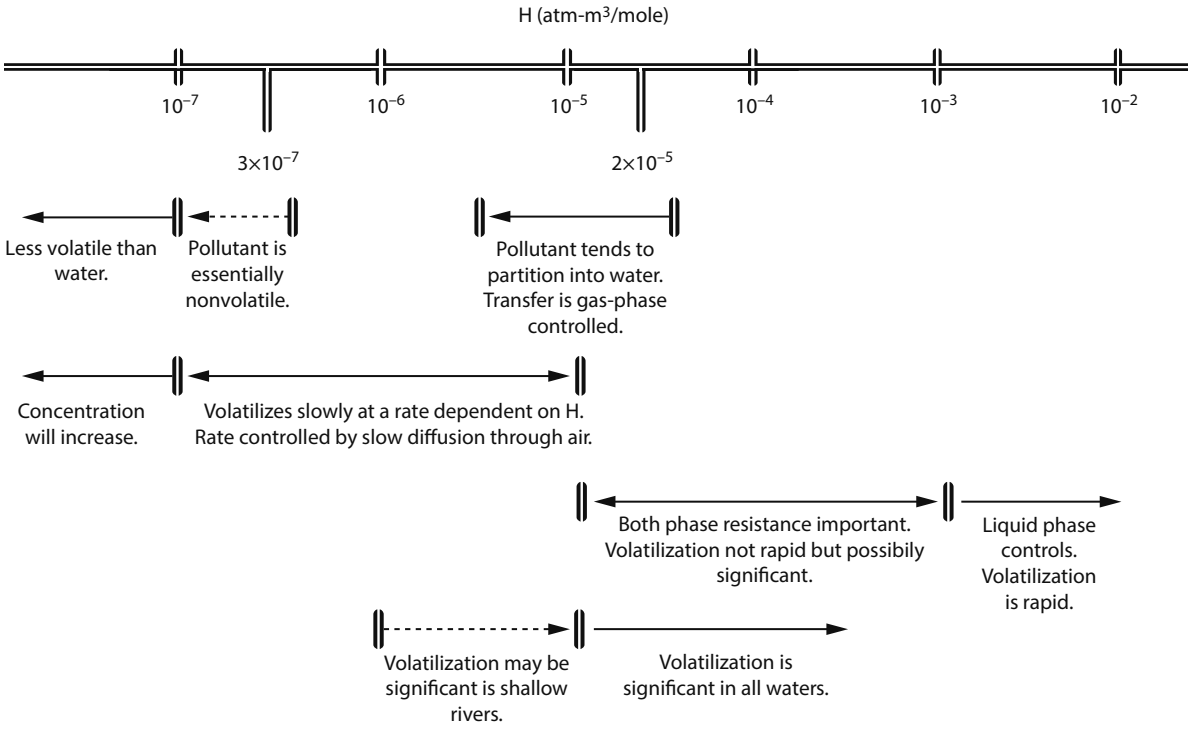

FIGURE 42.1 Volatility characteristics associated with various ranges of Henry's law constant. (From Smith, J.H. et al., *Environ. Sci. Technol*., 14, 1332, 1980; Reproduced in Lyman, W.J. et al., *Handbook of Chemical Property Estimation Methods*, McGraw-Hill, New York, 1982. With permission.)

process known as *biodegradation*. This process represents a significant loss mechanism in soil and sediments that can ultimately lead to mineralization of the compound—that is, degradation to carbon dioxide, water, and the inorganic forms of other elements the chemical may contain. Microorganisms are the primary converters of complex organic chemicals in to inorganic substances, although soil and sediment invertebrates also play a major role in the biodegradation process. In many instances, higher organisms are able to metabolize compounds, but they generally play a less significant role in environmental systems. Photochemical degradation and hydrolysis are other important abiotic degradation factors in the environment.

Almost all degradative reactions in the environment are oxidative, reductive, hydrolytic, or conjugative. Biodegradation can take place in virtually any environmental situation, aerobic or anaerobic; thus, in a test system designed to assess the toxicity of a chemical, the potential for biodegradation must always be considered regardless of the presence or absence of oxygen.

When testing chemicals in anything more complex than a single organism (i.e., the microcosms and mesocosms, discussed later in this chapter), the medium, whether water, sediment, soil, or a combination of these, plays an important role in the behavior of the system. Organic matter in the medium strongly influences its microbial density, and microorganisms may comprise as much as 80% of the biomass of soil. The microbial community in turn determines how stable a xenobiotic chemical will be in the system.

Organic compounds can be divided into four groups according to their biodegradability: (1) usable immediately by an exposed organism as a nutrient or energy source, (2) usable by microorganisms following an acclimation period, and (3) degraded slowly or not at all. In the fourth group, compounds are subject to co-metabolic degradation, wherein a compound that does not provide a nutrient or energy source for the degrading organism is broken down in conjunction with the degradation of other substances. Ideally, when evaluating the effect of xenobiotics on complex systems, one needs to consider all aspects of the biology and chemistry of all of the organisms and chemicals present. The best design of such test systems demands the most complete knowledge possible of all the potential interactions of chemicals and organisms in the system.

Other Considerations

Before presenting and discussing the specific test methods used in ecotoxicology, it is important to note that there are several significant differences between the practice of toxicology using laboratory animals and methods that use wild animals or plants, often located in their usual, or at least simulated, environmental conditions. Most importantly, the goal of ecotoxicology is considerably broader and more varied than that of human toxicological evaluations: while the assessment of a test chemical's impact on the health of an individual human is of primary importance in toxicology, an understanding of chemical ecosystem's impact, as well as its

component parts, is the paramount consideration in ecotoxicology. Furthermore, in ecotoxicology, the test organisms may be the actual targets for a chemical pollutant (such as pesticides), and the test method often investigates the endpoint subjects of concern, rather than a surrogate animal model. Finally, wild organisms live in a variable environment that is subject to a multiplicity of seasonal, physical, and chemical stressors that can alter the toxicity of the chemical [8]. Consequently, extrapolation of toxic responses from laboratory studies to field conditions is difficult and adds uncertainty. Methods for testing chemicals in situ have developed rapidly in recent years, and the methods presented in the following pages provide approaches on which to build the definitive and precise techniques for refining ecotoxicology in the future.

TESTING OF AQUATIC ORGANISMS

Aquatic systems, such as rivers, lakes, wetlands, and estuaries contain biota that may be represented by thousands of species. More complex environments with diverse habitats, such as that of the Gulf of Mexico, can contain tens of thousands of species (Table 42.1). The indigenous flora and fauna may be exposed to a variety of potentially toxic chemicals from human activities, resulting in adverse impacts. The study of these adverse effects of chemicals on freshwater and saltwater biota, and on the ecosystems that contain them, defines the subdiscipline of aquatic toxicology.

Aquatic toxicology differs from mammalian toxicology in several aspects. The primary goal of aquatic toxicology is to assess the effect of toxicants on the many diverse species, populations, and communities of plants and animals that inhabit saltwater and freshwater environments. The biota are usually cold blooded, and the naturally variable physical and chemical characteristics of the aquatic habitat have a considerable effect on their sensitivity to toxicants [8]. The aquatic test species of interest, unlike in studies of human health, can be tested directly. However, the internal dose is generally unknown, and the water or sediment concentration is used as an estimate of exposure. Mechanisms of toxic action may not be well understood, and not all test methods are well standardized.

Various species of aquatic organisms have been used in toxicity experiments for more than 130 years. One of the earliest reported studies was conducted with fish in 1863 [9], and the goldfish was proposed as the first standard test species in 1917 [10]. Toxicity tests have been conducted with increasing frequency since the 1960s due to the numerous environmental regulations that have been enacted in the past half-century. Consequently, the number of standardized test methods continues to increase, with the first couple of them being published in 1960 for animal test species and in 1970 for algae. Hunn [11] provides additional detail on the development of aquatic toxicology as a field of study.

Many toxicity test methods are available [12, Table 42.4]. They differ in their cost, precision, complexity, and the skill needed to conduct them; nevertheless, their objectives are similar. Toxicity tests are conducted to determine the relative potency among chemicals and the relative susceptibility among different species and life stages, as well as to identify environmental variables that influence the overall outcome of exposure. Toxicity tests are conducted frequently to meet regulatory guidelines for the use and discharge of commercial chemicals (including pesticides) and effluents [13–15]. In addition, toxicity results are used to assess water and sediment quality and to support cleanup goals for contaminated sites, as well as for the development of numerical, effects-based water quality standards and sediment quality benchmarks to protect aquatic life.

Aquatic toxicologists do not use all of the available toxicity tests to determine the effects for any single toxicant; instead, a tiered approach is used to provide a systematic and comprehensive process of deriving the toxicity data needed to assess the environmental hazard of a chemical. This approach consists of conducting short-term screening tests prior to using longer-term studies that are more costly and complex. This sequential evaluation provides a more efficient use of resources and reduces unnecessary testing. The criteria used to determine the appropriate level of testing are chosen based on the quality and quantity of data available and the specific regulatory requirements for the test substance of interest. For example, some regulatory programs, such as the U.S. Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), have extensive test data requirements, while other programs, such as the U.S. Toxic Substances Control Act (TSCA), rely heavily on quantitative structure–activity relationship (QSAR) models [16] to predict toxic effects based on chemical structure. The types of toxicity tests used in this tiered approach are discussed briefly in the following text.

TABLE 42.1
Estimated Number of Species of Fauna and Flora in the Gulf of Mexico

Taxa	No. of Species
Algae	30,000
Sea grasses	7
Sponges	800
Cnidarians	340
Crustaceans	2,600
Mollusks	2,500
Annelids	870
Echinoderms	5,200
Fishes	1,500
Reptiles	9
Birds	400
Mammals	30

Source: Felder, D.L. and Camp, D.K., *Gulf of Mexico Origin, Waters and Biota: Vol. 1, Biodiversity*, Texas A&M University Press, College Station, TX, 2009; Gore, R.H., *The Gulf of Mexico*, Pineapple Press, Sarasota, FL, 1992.

TABLE 42.2
Most Commonly Reported Types of Aquatic Toxicity Tests in the Ecotox Knowledgebase[a]

Taxa	Test Type	Number of Records[a]
Fish	Acute	10,566
	Chronic	4,184
	Bioconcentration	796
Arthropods	Acute	8,134
	Chronic	698
Mollusk	Acute	1,722
	Bioconcentration	239
Plant	Acute	933
Algae	Acute	468
	Chronic	571
Amphibian	Acute	393
	Chronic	323
Annelid	Acute	234
	Chronic	121
Rotifer	Acute	210
Echinoderm	Acute	74

[a] 360,566 records searched at http://cfpub.epa.gov/ecotox/ [211]; September 2012 download.

Single-Species Toxicity Tests

Aquatic single-species toxicity tests can be categorized as either acute or chronic studies. Acute toxicity tests are short in duration and assess whole-organism responses to high concentrations of chemicals, with death or immobility as the typical response endpoints. In comparison, chronic tests require longer exposure times and lower concentrations to assess sublethal responses. A large historical database, dominated by acute tests, exists for many chemicals (Table 42.2), including detergent surfactants, trace metals, pesticides, and various other organic compounds [17–20].

Methods

Acute toxicity tests are used most often to quickly screen chemicals for toxicity or to determine the relative sensitivities of different species. Mortality is the effect endpoint measured during the usual test duration of 48 h (invertebrates) or 96 h (fish). In a typical acute toxicity test, 5–10 organisms are exposed under static conditions in glass test beakers to five test concentrations. A control without the test chemical or sample is included. A solvent control is included if a solvent is used to solubilize the test chemical. The test concentrations and control are usually conducted in triplicate. Daily observations are made on survival, and dead organisms are removed. At test termination, the concentration that kills 50% of the test organisms (LC_{50} value) is computed. Cumulative, chronic, and sublethal effects of a chemical are not evaluated in acute toxicity tests, although behavioral changes and lesions caused by a chemical can be reported.

Chronic toxicity tests are more complex and time consuming than acute studies and, for these reasons, are conducted less frequently (Table 42.2). The methods for these tests differ considerably from acute tests, because they are designed for the specific life histories of the various test species. Chronic toxicity tests may be for a full life cycle (egg–egg), partial life cycle (embryo–larvae), and partial life history (e.g., egg–juvenile or adult). Full life-cycle tests are uncommon with fish due to the long durations that are necessary (0.5–2 years), although standard methods are available [21]. Partial life-cycle tests with fish can be as long as 60 days. The early life stage of fish (embryo/larvae) is usually the most sensitive period; consequently, partial life-cycle tests are often used as surrogates for the full life-cycle studies. Chronic tests may be conducted for more than one complete life cycle if algal and invertebrate species are used because of relatively short life cycles compared to fishes. Lethal and sublethal effects monitored daily or weekly in chronic toxicity studies may include changes in growth, reproduction, behavior, and physiology, as well as mortality and histology. Calculated effects thresholds such as the LC_{50} value and the highest no-observable-adverse-effect concentration (NOAEC) and lowest (first)-observable-adverse-effect concentration (LOAEC) and EC_{20} values are determined based on the most sensitive effect parameter of interest. Although toxicity tests have similarities, as discussed in the following text, sensitivities, instrumentation, and methods differ among species and influence the outcomes and utility of the results.

Toxicity tests may be static, continuous flow, recirculating, or static renewal based on the toxicant dosing technique. Static and flow-through procedures are used more widely in toxicity tests conducted with pure chemicals. Chronic toxicity tests conducted with effluents are usually static renewal; those that test toxicity to algae are static (i.e., no renewal of test substance or water). Static tests with no renewal of exposure solution are the simplest and least expensive; however, the toxicant concentrations may decrease due to adsorption, uptake, volatilization, or biodegradation. In a static-renewal test, the test solutions and dilution water are renewed periodically, usually once daily. In a continuous-flow test, the dilution water and test substance are renewed continuously. Because the exposure concentrations remain fairly constant in static-renewal or continuous-flow tests, the dose–response relationship can be better defined.

Various aquatic toxicity test methods have been published for single species, and many have been standardized (Table 42.3). Some are required for approval to produce or release industrial chemicals, pesticides, new drugs, and wastewater (Table 42.4). Test-method development is an ongoing process, however, and test methods are continually being improved or modified to include additional measurement endpoints. For example, the U.S. Environmental Protection Agency (USEPA) currently is developing and validating in vitro and in vivo assays to determine the potential for chemicals to cause endocrine disruption (http://www.epa.gov/endo/pubs/assayvalidation/consider.htm). Endocrine tests will include assays for amphibian metamorphosis, fish screening for estrogenic and androgenic effects, amphibian

TABLE 42.3
Taxa of Aquatic Organisms Used and Toxicity Test Methods Available from Standards-Writing Organizations

Taxa	ASTM[a]	APHA et al.[b]
Phytoplankton	Algal growth potential	Bacterial bioluminescence
	Static tests with microalgae	Ciliated protozoans
	Bioluminescence dinoflagellates	Microalgae
Plants	Freshwater emergent macrophytes	Emergent vascular plants
	Duckweeds	Duckweed
	Seaweed	
Invertebrates	Rotifers	Rotifers
	Amphipods	Annelids
	Polychaete annelids	Mollusks, marine bivalves
	Bivalve mollusks	*Daphnia* spp.
	Daphnia magna	*Ceriodaphnia* spp.
	Ceriodaphnia dubia	Mysids
	Saltwater mysids	Decapods
	Echinoid embryos	Aquatic insects
	Macroinvertebrates	Polychaete
	Copepods	Oligochaetes
		Echinoderms
Vertebrates	Fish	Fish
	Amphibians	Amphibians
Freshwater microcosm	Fish behavior	

[a] ASTM [212].
[b] American Public Health Association, American Water Works Association, Water Pollution Control Federation [12].

development and reproduction, and life-cycle tests with fish and mysid shrimp. Mutagenicity tests in bacterial and cell cultures, cytogenetic tests, and DNA tests for strand breaks and unscheduled synthesis in higher organisms have been used to screen the genotoxic effects of treated wastewaters, pesticides, sediment extracts, and dredged sediments [22,23]. The use of genomic endpoints is an emerging area and provides insight into the potential pathways of toxicity.

Experimental Conditions

In general terms, toxicity tests are conducted in a laboratory where the light cycle (photoperiod) and intensity, as well as air and water temperatures, are controlled. The test solutions containing the test species are typically monitored for pH, temperature, dissolved oxygen, ammonia, and hardness or salinity. The test organisms are exposed for a predetermined duration that varies depending on the type of test and test species.

Test Chambers

The type of test chambers used in toxicity tests depends on the test species. Various sizes of beakers, aquaria, jars, bowls, and petri dishes have been used. The test chambers are usually constructed of materials such as glass, Teflon®, and specific plastics that minimize leaching of potential toxicants such as plasticizers and adsorption of the test substance.

Test Concentrations

The chemical concentrations used in an acute toxicity test are based on results obtained from a pretest or range-finding test. Generally, 5–10 organisms are exposed to several test concentrations that are usually an order of magnitude apart. The dilution water and exposure conditions (i.e., water temperature, salinity, hardness, and pH) in range-finding tests are usually similar to those in the definitive test. The test concentration range for a chronic test is usually based on the results of an acute test conducted prior to the chronic test. The test compound is added to the dilution water, which may be well water, reconstituted water (e.g., deionized water with added salts), dechlorinated tap water, uncontaminated river water, or natural or artificial seawater.

An organic solvent is used to dissolve test compounds that have limited water solubility. Commonly used solvents include triethylene glycol, dimethyl sulfoxide, acetone, and dimethylformamide. The LC_{50} values for these solvents are between 9,000 and 92,500 mg/L [24], meaning they are essentially nontoxic. The concentration of the solvent in the test water should not exceed 0.5 mL/L or should not be greater than 1/1000 of the LC_{50} value of the solvent. A solvent control is included if a solvent is used in the study.

Reference toxicants often are used to determine the health and sensitivity of the test species, particularly in effluent testing. Although there is no universally used reference toxicant, typical ones include sodium dodecyl sulfate (anionic surfactant), sodium chloride, sodium pentachlorophenol, and cadmium chloride.

Toxicant delivery systems are used to deliver, on a once-through basis, the various test concentrations to the test chambers in continuous-flow toxicity tests. The serial proportional diluter (Figure 42.2) is the most common design used to mix the dilution water with the test substance to produce the desired test concentrations. The construction materials in toxicant delivery systems, as for the test chambers, should not be rubber or certain plastics, to prevent leaching of contaminating materials. The test concentrations are confirmed analytically prior to and during chronic toxicity tests. Analyses are performed at least weekly for each test concentration and control for tests of 7-days duration or longer. In tests of shorter duration, analyses are usually conducted on alternate days. Analytical verification of the test concentrations in range-finding and static acute toxicity tests is seldom performed, and the results from these tests are generally based on nominal concentrations of the test substance. However, this practice is being discouraged in favor of measuring all test solutions regardless of the duration of the toxicity tests.

Test Species

Historically, ecotoxicology has centered on determining the effects of anthropogenic chemicals on organisms inhabiting temperate zones. Tropical and polar species have

TABLE 42.4
USEPA and OECD Toxicity Test Guidelines for Aquatic Species

Compartment	USEPA (FIFRA, TSCA)[a]	USEPA (CWA)[b]	OECD[c]
Algae	Algal toxicity	Green alga, growth inhibition test	Freshwater algal and cyanobacteria, growth inhibition test
	Cyanobacteria toxicity		
Aquatic plants	Aquatic plant toxicity, *Lemna* spp.		*Lemna* sp. growth inhibition test
Aquatic invertebrates	Gammarid acute toxicity test	*Ceriodaphnia dubia* acute	*Daphnia* sp. acute immobilization test
	Penaeid acute toxicity test	*Ceriodaphnia dubia*, survival and reproduction	*Daphnia magna* reproduction test
	Mysid acute toxicity test	*Daphnia pulex* and *Daphnia magna* acute	*Chironomus* sp. acute immobilization test
	Mysid chronic toxicity test	Mysid acute	
	Daphnid acute test	Mysid, growth, and fecundity	
	Daphnid chronic toxicity test	Sea urchin, fertilization	
	Bivalve acute toxicity test (embryo–larval)		
	Oyster acute toxicity test		
Fish	Fish acute toxicity test, freshwater and marine	Fathead minnow and Bannerfin shiner acute	Fish, acute toxicity test
	Fish acute toxicity mitigated by humic acid	Fathead minnow larval survival and growth	Fish, juvenile growth test
	Fish early life stage toxicity test	Fathead minnow embryo–larval survival and teratogenicity	Fish, early life stage toxicity test
	Fish life-cycle toxicity test		
		Rainbow trout and brook trout acute	Fish, short-term toxicity test on embryo and sac-fry stages
		Sheepshead minnow acute	Fish sexual development test
		Silverside acute	Fish short-term reproduction assay
		Sheepshead minnow, larval survival and growth	21-day fish assay
		Sheepshead minnow, embryo–larval survival and teratogenicity	Fish, prolonged toxicity test 14-day study
		Inland silverside, larval survival and growth	
Amphibians	Tadpole/sediment subchronic toxicity test		Amphibian metamorphosis assay
Sediment	Whole-sediment acute toxicity invertebrates, freshwater		Sediment–water *Lumbriculus* toxicity test
	Whole-sediment acute toxicity invertebrates, marine		Sediment–water *Chironomus* toxicity
	Chironomus sediment toxicity test		Sediment–water *Chironomus* life-cycle toxicity test
Bioaccumulation	Oyster BCF		
	Fish BCF		Bioconcentration/ flow-through fish test
	Aquatic food chain transfer		
Multiple trophic levels	Generic freshwater microcosm test		
	Site-specific aquatic microcosm test		
	Field testing for aquatic organisms		

[a] USEPA TSCA and USEPA FIFRA [13,14].
[b] USEPA, NPDES [15,31].
[c] OECD [213].

been tested less frequently, but there is a growing database on effects in these species and ecosystems [25,26]. Animal test species have been used more frequently than plant species, and freshwater species more frequently than marine species. This trend can be seen in Table 42.5, where the 10 most common species with test data are all freshwater organisms. Most toxicity tests are conducted with single cultured test species such as those listed in Table 42.6. The more commonly used freshwater species, particularly in tests used for regulatory compliance, are

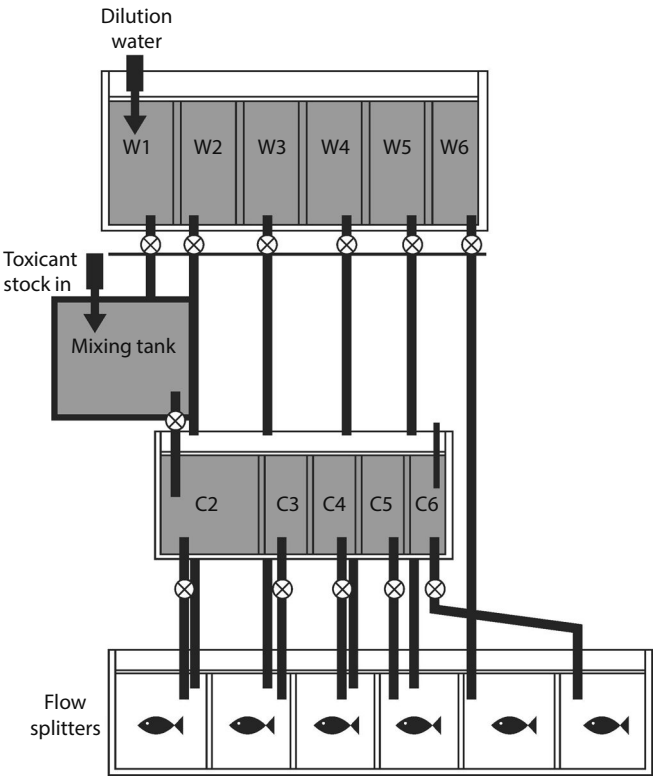

FIGURE 42.2 Diagram of a proportional diluter designed to ensure that five concentrations of a toxicant and a control treatment are delivered to the test species in a toxicity test. (Adapted from Landis, W.G. and Yu, M.H., *Introduction to Environmental Toxicology*, Lewis Publishers, Boca Raton, FL, 1995.)

fathead minnows (*Pimephales promelas*), daphnid species (*Daphnia magna*, *Ceriodaphnia dubia*), and a green alga (*Pseudokirchneriella subcapitata*). Freshwater sediment organisms include the midge (*Chironomus riparius*) and the amphipod (*Hyalella azteca*). Common marine species include sheepshead minnows (*Cyprinodon variegatus*), mysids (*Americamysis bahia*, formerly *Mysidopsis bahia*), and diatoms (*Skeletonema costatum*). The species in Table 42.6 were selected based on several criteria, primarily ease of culture, availability, and reproducible test methods. Sensitivity is also considered when choosing a test species, although the sensitivities of the species in Table 42.6 relative to one another, as well as to indigenous flora and fauna in the ecosystem, are a matter of scientific debate. No single animal or plant test species is consistently most sensitive to all toxicants or most reliable for extrapolation to all other organisms. Frequently, acute toxicity tests are conducted first with a variety of freshwater and marine test species, and the sensitive species are then used in subsequent chronic testing. However, it does not always follow that acutely sensitive species are the most sensitive in chronic studies.

A minimum of 10 animals are exposed to each test concentration and control. The test species are acclimated for a specific time prior to testing to eliminate diseased organisms and to acclimate the organisms to the test conditions. The recommended loading density for the test species is between 0.5 and 0.8 g/L in static tests and 1 and 10 g/L in continuous-flow tests.

TABLE 42.5
Most Commonly Reported Aquatic Test Species Based on Toxicity Records with Endpoint Data in the Ecotox Knowledgebase[a]

Species	Common Name	Percentage of Records[a]
Oncorhynchus mykiss	Rainbow trout	7.7
Daphnia magna	Water flea	6.2
Pimephales promelas	Fathead minnow	5.3
Lepomis macrochirus	Bluegill	2.8
Cyprinus carpio	Common carp	2.0
Danio rerio	Zebra danio	1.8
Oryzias latipes	Japanese medaka	1.4
Ceriodaphnia dubia	Water flea	1.3
Pseudokirchneriella subcapitata	Green algae	1.3
Ictalurus punctatus	Channel catfish	1.2

[a] 279,109 records with test endpoints (e.g., LC_{50}, EC_{50}; NOAEC, LOAEC); http://cfpub.epa.gov/ecotox/; [211] September 2012 download.

Calculations

The results of acute toxicity tests are reported as the LC_{50} value, with its corresponding 95% confidence interval. These calculations are determined using one of several statistical methods that are discussed by Stephan [27], such as probit analysis and moving-average interpolation. Probit analysis [28] is the most commonly used statistical method to determine LC_{50} values. Graphical interpolation can be used also to estimate the LC_{50} value where the proportion of deaths versus the test concentration is plotted for each observation time using probit or logit analysis or graphical interpolation. The NOAEC and LOAEC, as well as effect concentrations (e.g., EC_{20}), are the usual calculations reported from chronic toxicity tests. The NOAEC is the highest concentration in which the measured effect is not statistically different from that of the control. The LOAEC is the lowest concentration at which a statistically significant effect occurred. These concentrations are based on the most sensitive effect parameter, such as hatchability, growth, and reproduction. The statistical procedure for these calculations combines the use of analysis of variance (ANOVA) techniques and multiple comparison tests. In some cases, the maximum acceptable toxic concentration (MATC) is reported from chronic toxicity results. The MATC is a concentration (x) that is between the NOAEC and LOAEC ($NOEC < x < LOEC$) and is expressed as the geometric mean of the two. There is continuing debate in ecotoxicology on the value of NOAC/LOAEC values because of their dependence on experimental design, including number of replicates and data precision [29]. The use of alternative statistically derived effect concentrations based on describing the entire concentration–response relationship (e.g., EC_{20}) has been advocated

TABLE 42.6
Freshwater and Saltwater Test Species Used in Acute and Chronic Toxicity Tests Conducted with Commercial Chemicals and Wastewaters

	Freshwater		Saltwater
Fish	*Salvelinus fontinalis* (brook trout)	Fish	*Cyprinodon variegatus* (sheepshead minnow)
	Oncorhynchus mykiss (rainbow trout)		*Fundulus hetroclitus* (mummichog)
	Carassius auratus (goldfish)		*Menidia* sp. (silverside)
	Pimephales promelas (fathead minnow)		*Gasterosteus aculeatus* (three-spine stickleback)
	Lepomis macrochirus (bluegill)		*Pleuronectes vetulus* (English sole)
Invertebrates	*Brachydanio rerio* (zebrafish)	Invertebrates	*Acartia tonsa* (copepod)
	Daphnia magna (daphnid)		*Penaeus aztecus* (shrimp)
	Daphnia pulex (daphnid)		*Palaemonetes pugio* (shrimp)
	Ceriodaphnia dubia (daphnid)		*Crangdon migricauda* (shrimp)
	Gammarus lacustris (amphipod)		*Uca* sp. (fiddler crab)
	Gammarus fasciatus (amphipod)		*Callinectes sapidus* (blue crab)
	Ephemerella sp. (mayfly)		*Crassostrea virginica* (oyster)
	Chironomus tentans (midge)		*Capitella capitata* (polychaete)
	Physa integra (snail)		*Arbacia punctulata* (sea urchin)
	Brachionus calyciflorus (rotifer)		*Americamysis bahia* (mysid)
Microalgae	*Pseudokirchneriella subcapitata* (green algae)	Microalgae	*Skeletonema costatum* (diatom)
	Microcystis aeruginosa (blue-green alga)		*Thalassiosira pseudonana* (diatom)
	Navicula pelliculosa (diatom)		*Dunaliella tertiolecta* (flagellate)
Vascular plants	*Lemma minor* (duckweed)	Macroalga	*Champia parvula* (red macroalga)
	Lemna gibba (duckweed)		
	Myriophyllum spicatum (water milfoil)		
	Ceratophyllum demersum (coontail)		

for sublethal and chronic toxicity testing, but regulatory guidance still prescribes the use of NOAECs and LOAECs [29].

Variability/Precision

Laboratory toxicity tests conducted with freshwater and saltwater species are considered relatively precise and reliable based on current information from inter- and intralaboratory comparisons of toxicity results. Generally, the LC_{50} values from acute toxicity tests conducted under similar experimental conditions with the same toxicant vary less than threefold. This has been observed for metals, effluents, reference toxicants, and different organic compounds [15,20,30,31]. Statistical analyses of a large database of acute toxicity have shown the standard deviation of test variability to be about three [32]. Anderson and Norberg-King [33] reported the CV values for a variety of freshwater and marine species and found minimal variation. They concluded that biological tests can be conducted with a precision similar to that for chemical-specific measurements.

Multispecies Toxicity Tests

The results of the standard acute and chronic single-species toxicity tests conducted in the laboratory cannot be used alone to predict effects of chemicals on natural populations, communities, and ecosystems. First, the cultured species in laboratory tests are often different from those found inhabiting most ecosystems and conditions, and the size of the test species, its life stage, and nutritional state, among other factors, can have an effect on toxicity. Second, the laboratory tests conducted under controlled conditions cannot duplicate the complex interacting physical and chemical conditions of ecosystems, such as seasonal changes in water temperature, dissolved oxygen, salinity, and suspended solids. Third, aquatic species are usually exposed simultaneously to numerous potential toxicants (mixtures). Although the toxicities of binary and ternary mixtures have been evaluated for some chemicals in laboratory toxicity tests, the resultant information has predictive limitations.

Because of the deficiencies of single-species toxicity tests, multispecies toxicity tests have been developed to address ecosystem structure and functional processes [3,34,35]. As described in the following text, these tests include the use of laboratory microcosms and mesocosms such as outdoor ponds, experimental streams, and enclosures (Figure 42.3). Standardized procedures for these tests, with the exception of a freshwater microcosm [3], do not exist. They can be conducted indoors and outdoors, with plant and animal species obtained from laboratory cultures or collected from natural sources. The toxic effects, in addition to those determined in single-species tests, are determined for structural parameters such as community similarity, diversity, and density, and functional parameters such as community respiration and photosynthesis. Effects on these parameters are reported usually as the NOAEC and LOAEC. A brief description of the major types of multispecies toxicity tests follows, and

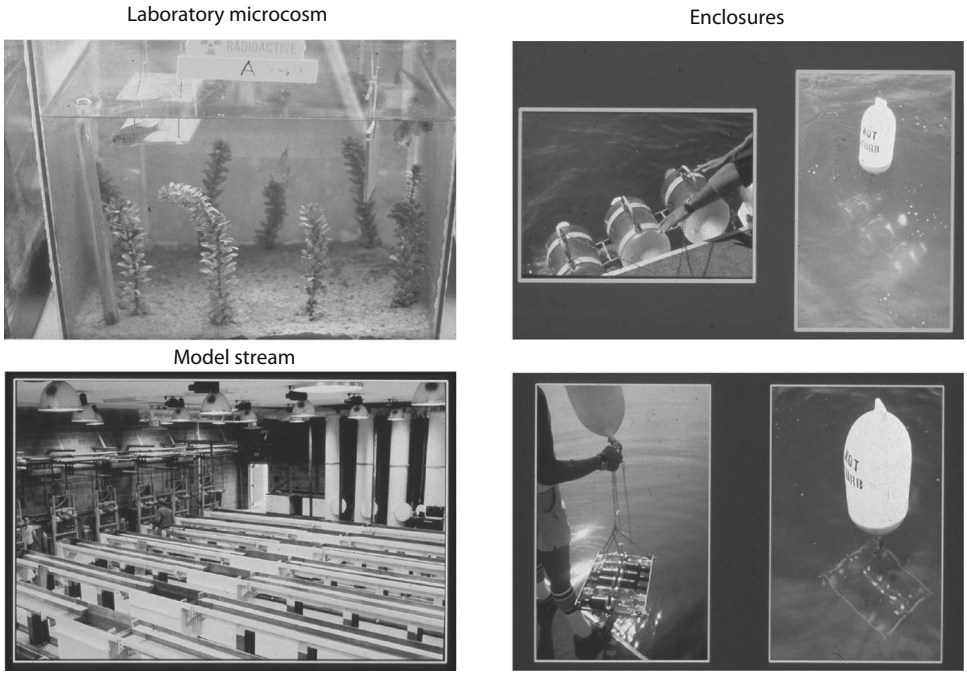

FIGURE 42.3 Examples of experimental systems used to expose either multiple test species simultaneously or natural biotic assemblages to toxicants. Test durations ranged from 3 h to 21 days.

TABLE 42.7
Advantages and Disadvantages of Single-Species and Multispecies Toxicity Tests

	Advantages	Disadvantages
Single species	Simple, rapid, reproducible	Responses of only individual determined
	Relatively low cost	Use nonindigenous species
	Standard protocols available	Ecological relevance uncertain
	Existing databases	Fate processes ignored
		Recovery rate not considered
		Cumulative effects not studied
		Ecological interactions not considered
Multispecies	Conducted under more realistic conditions	Costly
	Can simultaneously study different trophic levels	Standard protocols limited
	Can use indigenous species	Limited replication
	Can study the effect of environmental modification	Adaptation not considered
	Can be site specific	Stability of exposure concentration uncertain
	Can study species interactions	Data interpretation more difficult

their advantages and disadvantages relative to single-species studies are summarized in Table 42.7. More detail concerning specific test conditions, replicability, and regulatory usefulness is available from a number of reviews [34–37].

Laboratory Microcosms

A laboratory microcosm is a small model or component of an ecosystem contained in a test chamber. The use of microcosms provides an opportunity to study the effect of contaminants on a biotic community in a controlled environment. Microcosms are assumed to be functionally similar to the ecosystem they represent, but they may differ in origin and structure. The biotic component can be constructed from several cultured single species (artificial microcosms), or it may represent a sample of a natural ecosystem collected and placed in the test chambers. For example, a microcosm may contain sediment, water, and indigenous flora and fauna collected from a river, lake, or pond, or it may contain reconstituted water, artificial sediment, and a predetermined number of protozoa, plants, invertebrates, and fish obtained from laboratory cultures. The standardized aquatic microcosm (SAM) method has a set density of 15 species [3]. In this method, 24 3 L microcosms are used, and they can be aquaria. They contain a well-defined medium of trace metals and vitamins and 200 g of sand (sediment), to which chitin and cellulose are added. The tests are conducted in the laboratory at 22°C with a 12 h period of light at 80 $\mu E/m^2/s$. Ten species of freshwater algae and five species of freshwater invertebrates are added

to the microcosms, which are intended to represent a new pond ecosystem. The test species are exposed to three test concentrations of the contaminant for 63 days, during which observations are made on a variety of parameters, including algal biovolume, species diversity and density, and dissolved oxygen concentrations. The toxicant concentrations are renewed periodically during the study, and biological and chemical measurements are taken weekly or biweekly, depending on the parameter.

Outdoor Ponds

Outdoor ponds have been used primarily to investigate the fate and effects of pesticides on aquatic life [38]. There is no universally accepted test design, and various types of plant and animal life have been used in these systems. Ponds of various sizes and shapes have been used, with depths usually 1 m or less, and volumes ranging from 10 to 650 m^3.

Experimental Streams

Experimental streams have been used to assess the effects of thermal effluents, nutrients, metals, insecticides, and municipal and industrial effluents on natural biota in studies ranging in duration from several days to several years. Experimental streams may be flow through or circulating, and may be located indoors or outdoors. The streams range in length from 1 to 1000 m and have been constructed from Plexiglas®, concrete or aluminum troughs, or plastic-lined wood flumes. The depth of most streams is usually less than 1 m. The streams are colonized with organisms prior to use. Sources of organisms (periphyton, invertebrates, and fish) can be laboratory cultures or natural ecosystems. Time of colonization has varied from a month to over a year. See Kosinski [39] for additional detail.

Enclosures

Enclosures isolate a portion of an ecosystem that can be dosed with the toxicant in such a way that significant contamination beyond the limits of the enclosure will not occur. The size, shape, and volume of enclosures used have varied considerably. In freshwater experiments, the volumes have ranged from 8 to 3,000,000 L. Plastic tubes, carboys, plastic bags, and limnocorrals have been used to enclose the biota [40–42]. The effects of oil, mercury, phenolic compounds, acidification, and pesticides, among other toxicants, have been investigated in studies ranging up to 3 months [40–42].

Single Species to Multispecies Toxicity Comparisons

The results of multispecies tests have been compared to those of single-species tests conducted with the same toxicant; for example, the results from the experimental stream study of Hansen and Garton [43] were compared to those for single-species chronic toxicity tests conducted with fish, daphnids, algae, and snails. These single, cultured species were more than an order of magnitude more sensitive than the stream communities to the insecticide diflubenzuron. Other comparisons of this type are discussed by DeLaender et al. [44], Hose and Van den Brink [45], and Cairns and coworkers [34,35]. In most cases, the outcomes of these comparisons have been species and compound specific; thus, the use of toxicity test results for single species to predict effects on natural biotic assemblages without supporting data requires careful consideration. The validation of laboratory-derived toxicity data continues to receive high priority in aquatic toxicology.

Predictive Toxicology

The large number of chemicals—combined with the many different regulations, the cost of conducting test batteries, and the number of species to be protected—limits the full use of standard toxicity tests to only a relatively few chemicals and species. In the absence of empirical data, methods have been developed to predict chemical toxicities. These methods include the use of in silico or computer modeling, or predicting of toxicity based on similar chemical analogs or moieties using QSARs [16], interspecies extrapolation modeling of the acute toxicity of chemicals from one species to others [46,47], and prediction of chronic toxicity from acute toxicity data [48]. In aquatic toxicology, QSAR models have been developed based on the single chemical property of hydrophobicity (i.e., log octanol/water partition coefficient) for neutral or polar organics causing narcosis [49]. QSAR models have been limited to a few standard freshwater test species such as green algae, daphnids, and fathead minnows [49]. The USEPA has developed an Internet-based interspecies correlation estimation tool, Web-ICE, to allow prediction of toxicity to a broader range of species [47]. Web-ICE uses the known toxicity of an aquatic or wildlife species (the surrogate) as model input to predict toxicity to another species, genus, or family [47]. The acute-to-chronic ratio (ACR) is commonly used to predict chronic toxicity when only acute toxicity data are available for a chemical. ACR values have been reported for a variety of chemicals, particularly metals and pesticides, and most are 25 or less [50].

Computational toxicology is an emerging field in ecotoxicology that offers an alternative to traditional animal-intensive approaches [51]. It uses bioinformatics, in vitro screening, and computer models and combines techniques from computational chemistry, genomics, and systems biology to improve prioritization of data requirements and risk assessments for chemicals. The value of computational toxicology as a predictive tool, and its utility in ecological risk assessment, is still being determined.

The results of single-species toxicity tests are often used to predict effects in natural ecosystems, with little being known about the accuracy of the prediction. In some cases, multispecies toxicity test results are available and can be used with more confidence. In other cases, conservative correction factors are used to compensate for the limitations of the toxicity data. A variety of these correction techniques have been reported [52] and their usefulness reviewed [37,53]. One of the more simple and common methods is the use of numerical safety or uncertainty factors to estimate safe, concern, or risk concentrations [54,55]. Laboratory-derived chronic toxicity results (usually the NOAEC) can be divided by factors of

100 or 10, depending on the level of uncertainty or protection desired, to determine the concentration that, if exceeded in the ecosystem, may represent an ecological risk. The magnitude of the factor used depends on the quantity and quality of the toxicity data available for the toxicant of interest. The greater the quality and quantity of data, the smaller the safety factor applied. The technical validity and magnitude of these factors are largely unproven, although they are in general use; consequently, this issue continues to be a subject of considerable debate within the scientific and regulatory communities [56].

An additional tool related to differences in interspecific sensitivities that has received considerable attention is the use of species sensitivity distributions (SSDs). An SSD is a cumulative frequency distribution (usually a lognormal or Weibull function) of the sensitivities of a set of species to a chemical of interest. The available toxicity data (e.g., NOAEC or LC_{50} values) are assumed to be a representative sample of the entire variation of sensitivity of all species to the chemical. A concentration of the chemical of concern that will be protective of a desired percentage of all species can be calculated from the frequency distribution in a manner similar to determination of a benchmark dose. Generally, the value selected is the lower confidence interval of the 5th or 10th percentile of the distribution. A modification of this approach is used for development of water quality criteria, and SSDs are used frequently in ecological risk assessments to extrapolate individual species toxicity values to an assemblage of species. A detailed description of SSDs and their applications and limitations is available in Posthuma et al. [57] and Maltby et al. [58].

Sediment Toxicity Tests

Toxicity tests have been conducted primarily with water-column or planktonic organisms, with the objective of controlling water pollution. However, sediments act as reservoirs for chemicals that can adversely affect benthic, or bottom-dwelling, aquatic organisms and, at times, also affect organisms in the water column [59]. This concern has led to the development of a variety of assessment techniques [60], including the use of sediment quality guidelines to protect benthic organisms [61]. Test methods have been developed to support the derivation of these guidelines and other related regulatory activities, such as the disposal of dredged materials [62]. Methods have also been developed for identifying which chemicals are the cause of observed toxicity in environmental samples of sediments. The sediment toxicity identification evaluation (TIE) consists of comparative toxicity testing and chemical fractionation techniques similar to those used for effluents [63].

Sediment toxicity tests may be conducted in the laboratory with a variety of single species of freshwater and marine benthic organisms, either with clean sediments spiked with known amounts of chemicals to derive toxicity thresholds or with samples collected from the environment to determine the amount of in situ toxicity. Methods were reviewed by Traunspurger and Drews [64]. Several toxicity tests and methods for the collection and preparation of sediments used in toxicity tests have been standardized (Table 42.8).

TABLE 42.8
Examples of Standardized Sediment Toxicity Test Methods

USEPA [104]	Whole-sediment acute toxicity invertebrates
	Chironomid sediment toxicity test
	Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates
	Methods for assessing the chronic toxicity of marine and estuarine sediment-associated contaminants with the amphipod, *Leptocheirus plumulosus*
ASTM [212]	Standard guide for conduction of 10-day static sediment toxicity tests with marine and estuarine amphipods
	Standard guide for collection, storage, characterization, and manipulation of sediments for toxicological testing
	Standard guide for designing biological test with sediments
	Standard test methods for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates
	Standard guide for conduction of sediment toxicity tests with marine and estuarine polychaetous annelids
	Standard guide for determination of bioaccumulation of sediment-associated contaminants by benthic invertebrates
APHA et al. [12]	Test procedures using the marine polychaete *Neanthes arenaceodentata*
	Test procedures using the freshwater and marine oligochaetes *Pristina leidyi*, *Tubifex tubifex*, and *Lumbriculus variegatus*
	Sediment test procedures for marine bivalves
	Sediment pore-water toxicity

Additional test guidelines for marine sediment [65] and freshwater sediment [66,67] have also been developed. In situ methods for assessing the toxicity of sediment contaminants in aquatic environments have also been reviewed [68]. Most tests conducted to date are acute and are of 10-day duration or less. Toxicity tests usually are conducted with the whole sediment (solid phase) or pore water [69]. An example of a whole-sediment acute toxicity test is shown in Figure 42.4. In this static test, a burrowing amphipod or an epibenthic mysid is exposed to undiluted field-collected contaminated sediment, and mortality is recorded after 10 days of exposure. The mortality of amphipods is made obvious by the reduction in their burrowing entrances (Figure 42.5).

The results of a chronic toxicity test conducted with amphipods exposed to different concentrations of a contaminated sediment are shown in Table 42.9. Survival, growth, and reproductive effects of the burrowing amphipod *Leptocheirus plumulosus* were determined at the end of the 28-day toxicity test. Significant effects on the test species were noted at contaminated sediment concentrations of 10% (young production, fertility), 50% (survival), and 100% (length).

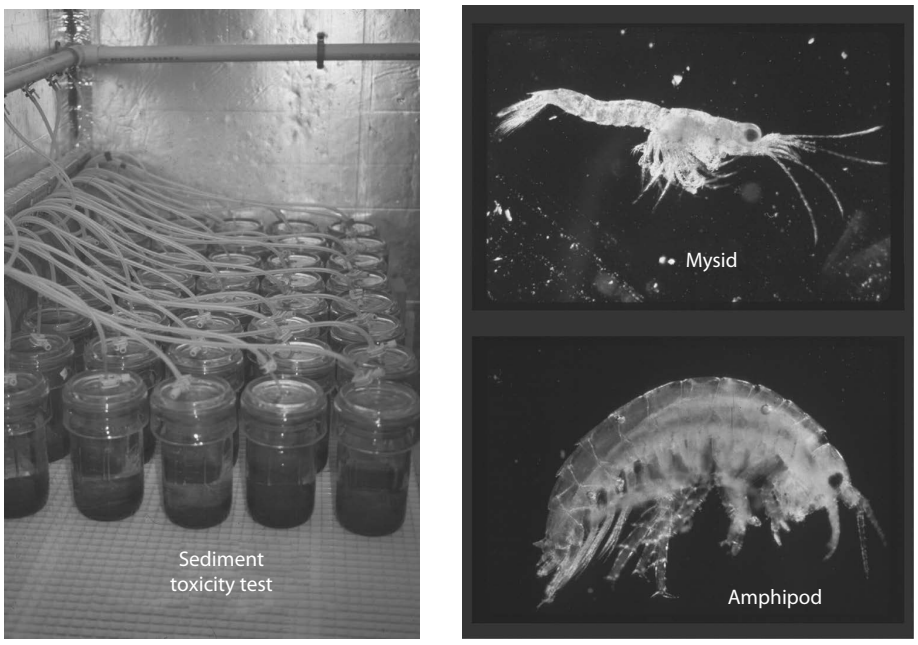

FIGURE 42.4 Example of a whole-sediment acute toxicity test conducted for 10 days with epibenthic mysid and burrowing amphipod test species.

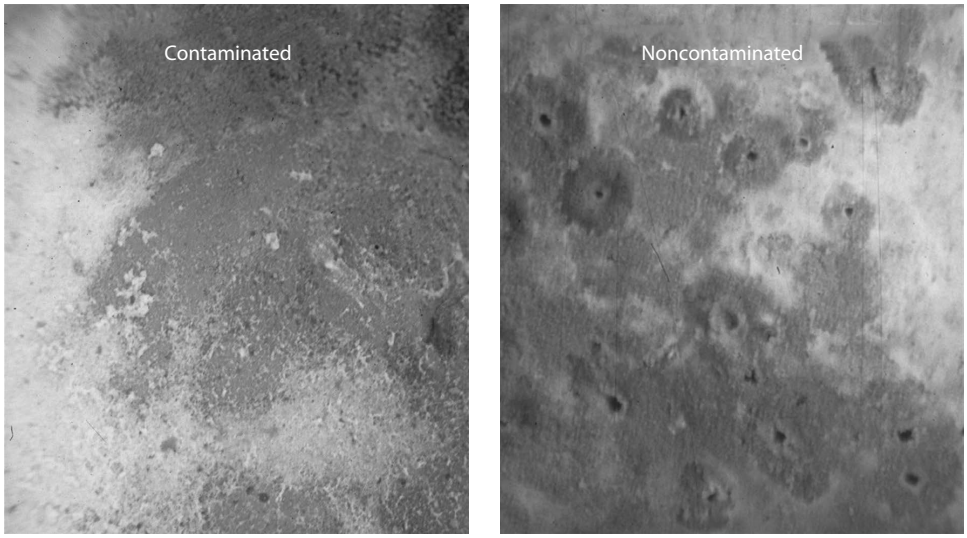

FIGURE 42.5 Burrowing entrances of infaunal amphipods exposed for 10 days in an acute toxicity test to contaminated and noncontaminated estuarine sediments. Lack of entrances indicates mortality.

TABLE 42.9
Example Results of a 28-Day Chronic Sediment Toxicity Test Conducted with the Marine Amphipod *Leptocheirus plumulosus*

Test Concentration[a]	Mean Survival (%)	Young Production (Number of Juveniles)	Fertility	Mean Length (mm)
Control	97	91	59	5.83
10	93	32	31	5.31
25	92	30	19	5.45
50	83	8	6	4.95
100	9	0	0	3.06

[a] Contaminated sediment was diluted with a noncontaminated reference sediment to obtain test concentrations.

Effluent Toxicity Tests

Toxicity tests are used in the U.S. National Pollutant Discharge Elimination System (NPDES) permitting process to determine the toxicities of municipal and industrial effluents prior to discharge into aquatic life [15,31,70]. A summary of the experimental conditions for test methods using freshwater species appears in Table 42.10. The methods for assessing the toxicity of wastewater discharge samples differ slightly from those used for pure chemicals. For example, the choice of the dilution water and the technique used to collect the effluent are important considerations. In most cases, water collected from the receiving water above the outfall is used for dilution, and composite samples of treated effluent are used. The test species (algae, invertebrates, or fish) are exposed to five effluent dilutions for 4–7 days. The tests are static renewal, except those for algae, which are static. The calculation results reported are the LC_{50} value, the NOAEC, and the LOAEC, expressed as percent effluent. The specific causes of toxicity in the effluent can be identified by using a TIE that consists of comparative toxicity testing and chemical fractionation techniques [71].

Phytotoxicity to Aquatic Plants

The majority of aquatic toxicity tests have been conducted with animal test species, because they were once thought to be more sensitive than plants. This generalization is not true for several chemical classes [72], and recently, phytotoxicity tests have been more common, although with a limited number of algal species and even fewer species of vascular plants. Example test data are listed in Table 42.11.

Standardized methods are available to determine the phytotoxic effects of chemicals and effluents (Table 42.12). Species of microalgae have been used more frequently than vascular species. The freshwater algal species used most frequently is the green microalga *Pseudokirchneriella subcapitata*, for which a relatively large database exists. Marine species include the diatom *Skeletonema costatum*, the flagellate *Dunaliella tertiolecta*, and the red macroalga *Champia parvula* [73].

Most toxicity tests conducted with algae are chronic tests, despite their short duration, because growth of the test population is measured over a significant portion of the algae's life span. These tests typically are 3–4 days in duration, although exposures can be less than 1 day if effects on photosynthesis are measured. The static exposures occur in a liquid nutrient-enriched medium under conditions of controlled pH, temperature, and light (Figure 42.6). Inhibitory and stimulatory effects on population growth are monitored during the exponential growth phase. Five test concentrations and a control are included in each study. The most common calculation reported is the 96 h IC_{50} value (concentration that reduces the parameter of interest by 50%), but algistatic (completely stops growth) and algicidal (lethal) concentrations have also been reported. In addition, the SC_{20} (stimulatory concentration) is reported if growth stimulation is observed. The SC_{20} value represents that concentration that increases algal growth 20% above that of the algal population in the control. Additional information on the use of algae in toxicity tests is available in Thursby et al. [74] and Nyholm and Källqvist [75].

Freshwater floating and rooted vascular plants have been used more frequently in toxicity tests than marine species.

TABLE 42.10
Example Experimental Conditions for Acute Toxicity Tests Conducted with Effluents and Freshwater Fish, Invertebrates, and Algae

Experimental Condition	Fish (*Pimephales promelas*)	Invertebrate (*Ceriodaphnia dubia*)	Alga (*Pseudokirchneriella subcapitata*)
Test type	Static nonrenewal, static renewal, or flow-through	Static nonrenewal, static renewal, or flow-through	Static nonrenewal
Test duration	24, 48, or 96 h	24, 48, or 96 h	96 h
Temperature	20°C ± 1°C or 25°C ± 1°C	20°C ± 1°C or 25°C ± 1°C	25°C ± 1°C
Light intensity	10–20 μE/m²/s (50–100 ft-c)	10–20 μE/m²/s (50–100 ft-c)	86 ± 8.6 μE/m²/s
Photoperiod	16 h light, 8 h darkness	16 h light, 8 h darkness	Continuous illumination
Test chamber size	250 mL	30 mL	125 or 250 mL
Test solution volume	200 mL	15 mL	50 or 100 mL
Renewal of test solutions	After 48 h	After 48 h	None
Age of test organisms	1–14 days; ≤24 h range in age	Less than 24 h old	4–7 days
No. of organisms per test chamber	10 for effluent and receiving water tests	5 for effluent and receiving water tests	1×10^4 cells/mL
No. of replicate chambers per concentration	2 for effluent tests, 4 for receiving water tests	4 for effluent and receiving water tests	3–4
Test concentrations	5 and a control	5 and a control	5 and a control
Endpoint	Mortality	Mortality	Growth (cell counts, biomass, chlorophyll fluorescence)

Source: USEPA, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, 5th edn., EPA/821/R-02/012, Office of Water, U.S. Environmental Protection Agency, Washington, DC, 2002.

TABLE 42.11
Response of a Diatom (*Dunaliella tertiolecta*), a Fish (*Cyprinodon variegatus*), and an Invertebrate (*Mysidopsis bahia*) Exposed in Laboratory Toxicity Tests to Treated Municipal Wastewater (Test Durations 4–7 Days)

		Effect Parameters					
Species	Wastewater Concentration (%)	Growth (% of Control)					
Diatom	Control	100					
	6	160					
	12	144					
	25	222					
	50	178					
	100	756					
	Wastewater Concentration (%)	Mortality (%)	Mean Weight (mg)				
Fish	Control	0	1.00				
	6	0	1.19				
	12	0	0.84				
	25	2	1.01				
	50	2	0.90				
	100	2	0.90				
				Reproductive Maturity			
	Wastewater Concentration (%)	Mortality (%)	Mean Weight (mg)	♀ Eggs	♀ No Eggs	♂ Immature	♂ Mature
Invertebrate	Control	2	0.21	8	7	17	7
	6	4	0.30	8	7	18	6
	12	12	0.27	3	9	14	9
	25	8	0.32	6	7	17	7
	50	20	0.19	2	3	13	10
	100	63	0.25	1	5	9	19

Source: USEPA, *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms*, EPA/821/R-02/014, Office of Water, U.S. Environmental Protection Agency, Washington, DC, 2002.

The duckweeds (*Lemna* spp.) are the most commonly used freshwater species due to their small size and rapid growth. Published methods are available for *L. minor* and *L. gibba* [76,77]. Tests with these species are usually 4–14 days in duration, during which effects on frond number and chlorophyll content are monitored. The results are expressed as an EC_{50} value and the NOAEC. As for algae, the tests are conducted in a nutrient-enriched medium. The test chambers can be jars, plastic cups, test tubes, or Erlenmeyer flasks.

The use of rooted vascular plants as test species in toxicity tests has been limited due to their large size, slow growth, and, until recently, lack of standardized methods. Standardized tests are now available for a few species (Table 42.12). In addition, other experimental techniques are available [77,78]. Several of these describe the use of seeds and seedlings of macrophytic vegetation to assess the toxicities of chemicals and effluents (Figure 42.6). In conclusion, the use of rooted macrophytes and their seeds in toxicity tests will increase in the future as the development of sediment quality criteria to protect aquatic life and protection of wetland vegetation increase in regulatory importance. However, for this to occur, identification of sensitive species and response parameters and validation of the laboratory-derived results is required [26,79].

Bioaccumulation Testing

A bioaccumulation study is conducted to derive information on the potential for a chemical to concentrate a toxicant in tissues of aquatic organisms [80]. This uptake and accumulation can be hazardous to the organism or can form an exposure pathway to other aquatic life, wildlife, or humans through trophic transfer in the food web. Bioconcentration tests assess the potential accumulation of a waterborne chemical, whereas bioaccumulation tests can assess multiple pathways, including diet and sediment exposures, as well as water. These tests are conducted with single chemicals and single species of algae, fish, and bivalve mollusks. A variety of fishes have been used, including the fathead minnow, bluegill, rainbow trout, and sheepshead minnow, and several invertebrate species, including oysters, scallops, and mussels. Several test methods are available to estimate the bioaccumulation potential of a compound (Table 42.13). In a typical bioconcentration test, the aquatic organism is exposed to a low level of toxicant during an uptake and depuration or clearance phase. A control is included in which the test species is not exposed to the toxicant. The uptake phase is usually for 28 days or until an apparent steady state is attained. The depuration period should continue until the concentration in

TABLE 42.12
Examples of Experimental Conditions in Several Phytotoxicity Tests Conducted with Micro- and Macroalgae, Duckweed, and Emergent Vascular Plants

Test Type	Duration (Days)	No. of Test Concentrations	Test Species	No. of Replicates	Temperature (°C)	Light Intensity
Algae						
Static	3	5	*Pseudokirchneriella subcapitata* (F) *Scenedesmus quadricauda* (F) *Chlorella vulgaris* (F)	3	21–25	120 μE/m²/s
Static	4	5	*Scenedesmus subspicatus* (F) *Microcystis aeruginosa* (F) *Anabaena flos-aquae* (F) *Navicula pelliculosa* (F) *Skeletonema costatum* (M) *Dunaliella tertiolecta* (M)	3	20–24	30–90 μmol/m²/s
Duckweed						
Static	4	5	*Lemna gibba* (F)	4	25 ± 2	2150–4300 lx
Static	7	3–5	*Lemna minor* (F)	3	25 ± 2	6200–6700 lx
Emergent/submersed vascular plants						
Static renewal	14	5	*Spartina pectinata* (E) *Scirpus acutus* (E)	5	20–30	150–200 μmol/m²/s
Static	14	5	*Myriophyllum sibiricum* (F)	5	20–25	100–150 μmol/m²/s

Source: Methods are from APHA et al., *Standard Methods for the Examination of Water and Wastewater*, 21st edn., American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC, 2005; ASTM, E1415-91: *Standard Guide for Conducting Static Toxicity Tests with Lemna gibba G3*, Vol. 11.06, American Society for Testing and Materials, Philadelphia, PA, 2004, 10pp; OECD, *OECD Guidelines for the Testing of Chemicals*, Organization for Economic Cooperation and Development, Paris, France, 1993.

Notes: F, freshwater; M, marine; E, estuarine.

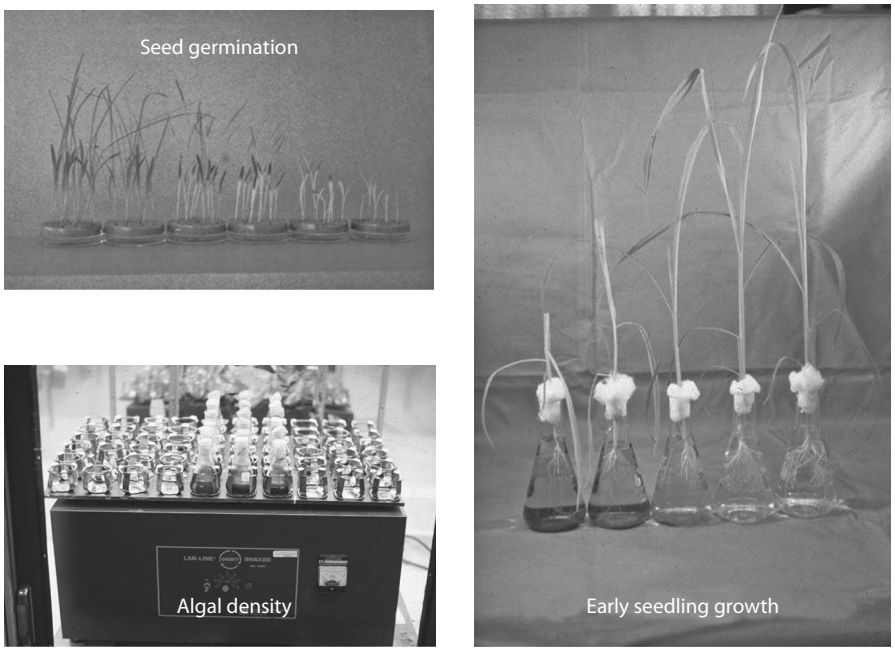

FIGURE 42.6 Examples of toxicity tests conducted in the laboratory for 4–28 days with microalgae and rooted vascular plants. Toxicants include commercial chemicals, effluents, and contaminated sediments.

TABLE 42.13
Examples of Bioaccumulation Tests

Test	Reference
Bioaccumulation tests with whole sediments (dredged materials)	USEPA/U.S. Army Corps of Engineers [62]
Oyster bioconcentration factor	USEPA [104]
Fish bioconcentration factor	USEPA [104]
Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates	
Bioconcentration: flow-through fish test	OECD [213]
Standard guide for conducting bioconcentration tests with fishes and saltwater bivalve mollusks (E1022-94)	ASTM [215]
Standard guide for determination of the bioaccumulation of sediment-associated contaminants by benthic invertebrates (E1688-10)	ASTM [212]

the test species is 10% of the steady-state concentration in the tissue. During both phases, the test water and test species are analyzed daily for the test chemical. All results from a bioconcentration study are based on measured concentrations. The uptake rate, the depuration rate, and the BCF (ratio of fish to water concentration at equilibrium) are computed and reported [80]. The relevance of tissue-concentrated chemicals to the survival, growth, and reproduction of the organism and its population dynamics is a continuing area of research [81–83]. Databases have been developed for interpreting tissue residue effects on aquatic organisms, and the literature has been reviewed by McEloy et al. [82].

TERRESTRIAL SYSTEMS

Standardization of toxicity testing for environmental problems in terrestrial systems has matured during the past decade. Risk-based soil quality guidelines have been developed by the Canadian Council of Ministers of the Environment [84], selected Canadian provinces [85], and several European countries [86]. The USEPA has developed ecological soil screening levels (Eco-SSLs) to standardize the approach for contaminated site assessments [87], although the supporting documentation specifically states that these are not to be used as cleanup values. Several of the USEPA regions have developed soil preliminary remediation goals (PRGs) for contaminated site cleanup for the protection of human health [88] that provide information and some guidance to assessing risks to wild plants and animals as well.

Because of the lack of applicable site-specific soil quality criteria, and the difficulty in applying such criteria to specific sites within the heterogeneous soil ecosystem, terrestrial environmental toxicology still relies on standardized toxicity tests for assessing the toxicity of new and existing chemicals to plants, wildlife, and soil ecosystem functions. These tests fall into one of two major categories: (1) a priori toxicity tests of single chemicals proposed for use or potential release in terrestrial systems and (2) site-specific assessments of extant (post hoc) contamination. Both approaches use the same suite of tests for determining plant, wildlife, and soil toxicity, and to some extent, they rely on data generated for human toxicology studies, although they differ significantly in their risk assessment approach [89]. Many of the tests discussed in detail have standard operating procedures published by the USEPA, the American Society for Testing and Materials (ASTM), the European Union (EU), or the Organization for Economic Co-operation and Development (OECD). A subset of these tests are under discussion for revision to incorporate up-to-date statistical designs and measurement of potential disruption of the endocrine system [90]; however, at the time of this writing, no revisions have been formally accepted to the protocols as they are presented here. Table 42.14 lists all the terrestrial bioassays for which detailed protocols currently are available. Additional tests are being developed, and where appropriate, references are provided to direct the reader who desires further information.

One major challenge remaining in terrestrial toxicology is extrapolation of results from test species to species of concern that have not been tested. Given the large number of terrestrial biota (mammals, birds, reptiles, amphibians, invertebrates, and soil microbes), it will never be possible to test all substances using all species, so protection levels must be established based on some method of extrapolation from the data at hand. Several methods currently are in use: (1) Calculate a fifth percentile of the distribution of species mean toxicity values [57], (2) use a fixed extrapolation factor on a calculated threshold for adverse effect based on the toxicity endpoint of interest, or (3) use the interspecies correlation estimation tool, Web-ICE, described earlier in this chapter [47]. If toxicity endpoints (e.g., LD_{50} values) are available for fewer than five species, the method of Luttik and Aldenberg [92] can be used to estimate the fifth percentile from the available data by applying the extrapolation factors derived for small samples (Table 42.15) to the geometric mean of the species tested. This method is based on pooled lognormal standard deviations. If more than one toxicity value is available for a species, the geometric mean of all available values is used as the input for the species. Further information on statistical analysis of ecotoxicity data is available in OECD [93].

Risk to wildlife species can be ascertained using methods similar to those used in estimating risks to humans, wherein dietary exposures are estimated and compared with a safe level of exposure using a hazard quotient paradigm. The safe level is often referred to as the toxicity reference value (TRV) and can be derived using various methods [94,95]. In screening approaches, TRVs have been developed using no-observed-adverse-effect-levels (NOAELs) and LOAELs and extrapolating between species using assessment factors [95]. Other approaches have used, as described, SSDs [46]; however, these often assumed minor influences in response are realized from differences in study design. Other methods use dose–response functions as a point of departure to capture the threshold of toxicity for species extrapolation (e.g., benchmark dose) [38]. This curve-fitting algorithm is

TABLE 42.14
Standardized Ecotoxicological Effect Tests for Terrestrial Organisms

Species	Guideline Number				Year[e]	Title
	OECD[a]	EU[b]	OPPTS[c]	Env Can[d]		
Terrestrial vertebrates						
Birds	233	—	850.21		1984	Avian acute oral toxicity
	205	—	850.22		1984	Avian short-term dietary toxicity
	206	—	850.23		1984	Avian reproduction
	—	—	850.24		1982	Wild mammal acute toxicity
	—	—	850.25		1996	Field testing for terrestrial wildlife
Terrestrial invertebrates						
Bees	213	C.16	850.302		1998	Honeybees, acute oral toxicity test
	214	C.17	850.303		1998	Honeybees, acute contact toxicity test
	—	—	850.304		1982	Field testing for pollinators
Others	ISO 11267[f]	—	—	EPS 1/RM/47	1999	Collembola (springtail) reproduction test
	207	—	850.62	EPS 1/RM/43	1984	Earthworm (sub)acute lab test
	222	—	—	EPS 1/RM/43	2004	Earthworm reproduction test
	220	—	—		2004	Enchytraeid (potworm) reproduction test
Terrestrial plants						
	—	—	850.41	EPS 1/RM/46	1982	Seedling emergence, tier I
	—	—	850.4225		1982	Seedling emergence, tier II
	208	—	850.423	EPS 1/RM/46	1984	Early seedling growth toxicity test
	227	—	850.415		1996	Vegetative vigor test
	—	—	850.42		1982	Seed germination/root elongation toxicity test
	—	—	850.245		1996	Terrestrial (soil-core) microcosm test
	—	—	850.43		1982	Terrestrial plants field study
Soil microorganisms						
	216	C.21	—		2000	Nitrogen transformation test
	217	C.22	—		2000	Carbon transformation test

[a] OECD, Organization for Economic Co-operation and Development.
[b] EU, European Union.
[c] OPPTS, USEPA's Office of Pollution Prevention and Toxics.
[e] Earliest year published by the OECD, EU, OPPTS, or Environment Canada.
[f] ISO, International Organization for Standardization.

TABLE 42.15
Extrapolation Factors for the Median and One-Sided 95% Left Confidence Limit for the Log-Logistic Distribution of Bird and Mammal Toxicity Values

Sample Size	Median Estimate		One-Sided Left Confidence Limits	
	Birds	Mammals	Birds	Mammals
1	5.7	3.8	32.9	14.9
2	5.7	3.8	19.6	10.0
3	5.7	3.8	15.6	8.4
4	5.7	3.8	13.7	7.6
5	5.7	3.8	12.4	7.0

Source: Luttik, R. and Aldenberg, T., *Environ. Toxicol. Chem.*, 16, 1785, 1997. With permission.

more robust than the straight-line calculation on an EC value. However, optimal study design should include at least five treatments, inclusive of a control, to best fit models used in the benchmark dose model [M.S. Johnson, unpub. data]. Few data exist for wildlife from dermal exposures, although for some substances and species, the dermal route could be significant [96]. At the present time, insufficient data exist to quantify risks for dermal exposures for most nonmammalian species. Values have been developed for inhalation exposures using allometric and route-to-route extrapolations, as for burrowing mammals in functionally confined spaces where soil vapor levels are a concern [97]. Few inhalation data are available for wildlife of other classes.

Other concerns include the extrapolation of controlled testing designs to realistic environmental conditions. The use of *ad libitum* feeding in toxicity tests is contrary to what many wildlife species typically encounter and has been suggested to result in skewing of the effect levels to lower concentrations in

rodent studies. It has been shown that limited food resources maintain healthier individuals that are able to cope with low-dose exposures [98]. This point, however, applies to gavage dosing studies and cannot be applied to feeding studies using contaminated feed. Constant temperature and humidity additionally are not typically experienced in many wild populations, and the relevance of this condition influencing the response to toxicants is poorly understood. However, given the incidence of nonspecific opportunistic infections experienced in laboratory amphibian studies, it is typically beneficial to have much cooler laboratory temperatures when housing those species (8°C–17°C) than is typical for studies using endothermic species.

Avian Tests

The USEPA requires tests with northern bobwhite (*Colinus virginianus*) and mallards (*Anas platyrhynchos*), at a minimum, for pesticide registration, so the largest database of toxicity information and testing experience for such compounds is available for these species. Relatively large databases also exist for Japanese quail (*Coturnix coturnix japonica*) (required by the EU for pesticide registration) and ring-necked pheasant (*Pashianus colchicus*) [99,100]. The test methods, however, are equally applicable to all other avian species, provided that appropriate adjustments are made in caging and feeding regimens [101,102]. Single-chemical laboratory tests for acute, subacute, subchronic, and reproductive toxicity have accepted standard methods.

Acute Toxicity Test

The acute toxicity test (LD_{50}) is required when determining effects of ingesting pesticide granules, seed treatments, or baits [13] or for comparative toxicology [101,102]. A modified version of this test has been developed for testing potential toxicity of alternative nontoxic shot pellets developed for waterfowl hunting [103]. The LD_{50} test [104] requires the use of adult birds (e.g., ≥16 weeks old for bobwhite and mallards; >6 weeks old for Japanese quail). Within a given test, all birds should be from the same hatch, unless the test is being conducted with wild birds. All birds should be of uniform weight and size, and absent of obvious signs of disease. Birds should be housed under acceptable animal husbandry practices, with a 10:14 light/dark (L/D) cycle to avoid inducing a reproductive state. Animals should be acclimated to the test environment for a minimum of 2 weeks prior to starting the test. For pesticide testing, a minimum of six birds per dose level is required, with the doses arranged by geometric progression, such that at least one dose will be below the estimated LD_{50}, one dose above, and one control group (administered carrier only). For shot toxicity studies, 10 birds of each sex are dosed with eight no. 4 shot pellets, and a similar negative control (steel shot) and positive control (lead shot) also are dosed. Birds should be fasted for 15 h prior to exposure; at all other times, they should be allowed *ad libitum* access to food and water. Chemical or shot should be administered through oral intubation into the crop or proventriculus, preferably in an encapsulated form, as birds can regurgitate liquid formulations or small pellets. The test animals should be observed for a minimum of 14 (pesticide) or 30 (shot) days after exposure, recording all signs of intoxication and number and time of mortality. For pesticide studies, if more than one bird from the control group dies during the 14-day period, the test may be considered invalid. Necropsies and histopathological studies of all birds that die during the test are encouraged. For studies of alternative shot, measurement of hematocrit, hemoglobin concentration, and other specified blood chemistries is required on days 15 and 30 after exposure, and histopathological analysis is required of liver and kidney at test termination. Additionally, analytical chemistry analysis of these or other tissues may be required to ascertain residue concentrations. The LD_{50} value is calculated, along with the slope and the 95% confidence interval, using probit analysis [28] or another acceptable statistical method.

In 2010, the OECD approved a new avian test guideline that significantly reduces the number of birds required for LD_{50} determination [105]. This approach provides for a limit test to approximate toxicity when a substance is suspected to be nontoxic (LD_{50} >2000 mg/kg-bw), using 5 or 10 birds tested at the limit dose, in addition to a control group. It also uses 24 or 34 birds, in addition to a control group, in 3–4 repeated stages of testing to approximate the LD_{50} and then refines this estimate to within acceptable limits of statistical error. For those instances where the slope of the dose–response curve is needed, as well as the LD_{50}, the design provides for additional stages and requires the use of more animals. The test requires gross examination of the internal organs of a subset of birds from all dose groups.

Other methods that are not prescribed for pesticide registration can be used to determine acute toxicity, including the stagewise probit design [106–108]. This design typically uses fewer animals and is flexible to adjust treatment exposures given results of previous stages. Furthermore, it can provide an LD_{50}, 95% confidence interval, and slope where stopping rules may preclude the development of criteria using the up/down method. Initially, four individuals of the same sex are each given different oral doses, typically at one-quarter log intervals above and below 750 mg/kg bw (assuming there is no other information on potential toxicity) [106]. After a period of observation, the next stage allows for 10 animals to be placed in treatment at dose levels set to intervals surrounding the expected partial response level. Following observation, the final stage uses another 10 animals that are again placed at treatment doses surrounding the partial-effect level. A greater number of animals should be placed in treatments that approximate the partial-effect level. All animals are observed for a period of 14 days and evaluated as mentioned previously.

Subacute Toxicity Test

The subacute avian dietary toxicity test (also known as the dietary test or LC_{50} assay) is conducted using 10- to 14-day-old bobwhite or 5- to 10-day-old mallards, although the test is not limited to these species [104,109–111]. Birds used in the test should be either wild birds or pen-reared birds that are

phenotypically indistinguishable from wild birds, preferably from colonies with known breeding history. If possible, all test birds should be from a single hatch, and only birds free of obvious injury and disease should be used. Birds must be kept in brooders of appropriate dimensions and temperature (about 35°C for bobwhite and mallards). The standard protocol does not require a certain lighting regimen; however, because the length of the photoperiod influences daily food consumption, it is recommended that a 10:14 or 12:12 L/D cycle be maintained. Water should be available *ad libitum*. A standard commercial game bird diet in mash or crumble should be used for mallard and bobwhite tests; appropriate formulations should be developed as needed for other species. If possible, the test material should be added to the diet without the use of a vehicle. If a vehicle is needed, water is preferred, but reagent-grade evaporative material (acetone, methylene chloride) can be used if necessary and completely evaporated at room temperature prior to feeding. Other acceptable vehicles include table-grade corn oil, propylene glycol, carboxymethylcellulose, and gum arabic.

In the dietary test, the toxicant is added to feed in a ratio of two parts solution to 98 parts feed by weight, and subsampling should be done to confirm uniformity of mixing. Large batches can be mixed in mechanical food mixers or similar devices. It is encouraged that feed batches be analyzed, and actual concentrations be reported along with the nominal value. The chemical should be administered in at least four concentrations spaced geometrically to produce mortality ranging from 10% to 90%. A concurrent control group and a vehicle control group (if appropriate) are required. A minimum of 10 birds is required for each concentration and control group. Birds should be acclimated to the testing conditions and fed prior to presentation of treated feed. Birds are exposed to treated feed for 5 days, followed by an observation period on clean feed for at least 3 days or until mortality ceases. There must be at least 72 consecutive hours without treatment-related mortality, and control mortality cannot exceed 10% during this period for the test to be considered valid. Throughout the test, all signs of intoxication should be recorded, including time of onset and duration. Times of all mortalities must be recorded. Average food consumption must be estimated for each pen of birds; therefore, feed must be weighed at the beginning and end of the pretreatment, treatment, and observation periods. Provisions for minimizing spillage should be reported. Necropsies and histopathological examination of all dead birds are suggested. The LC_{50} and 95% confidence interval must be reported, along with the method used to determine these statistics.

The avian dietary test can also be used as an indication of the chemical's repellency (the inherent property of a test substance that causes a reduction in feed ingestion) [112]. Frequently, sufficiently severe reduction in food consumption by the chicks occurs due to inherent properties of the chemical (e.g., bad taste) to induce starvation by the end of the 5-day treatment period, significantly confounding the results of the dietary test. Thus, the LC_{50} should be calculated based on the measured concentration of chemical in the feed and the actual amount of food consumed (the daily dietary dose [DDD]), or alternative test designs should be employed [93]. Repellency is calculated as the percent reduction in food consumption, or the highest dose where no food reduction is observed. A standard guidance document on testing for avoidance behavior has been proposed [113] that uses adult birds presented with two dietary choices (treated vs. nontreated), to avoid confounding the data with starvation-induced mortality and to present a more ecologically realistic scenario. This approach, however, remains controversial, with several experts arguing that birds can detect chemicals in feed, so more than two feed treatments are needed to generate realistic worst-case scenarios for exposure potential.

Subacute avian tests can also be performed using oral gavage procedures [86,114,115]. This method benefits from a more precise method of compound delivery and is easy to accomplish using a conventional gavage needle and syringe. Test substance and vehicle are delivered into the crop, providing a simulated slow release. Gavage methods avoid the uncertainty inherent in calculating dose based on food ingestion, given the hydroscopicity of the feed, spillage, defecation in the feed, and feed avoidance. It also avoids drastic changes in body mass that can occur as a result of food avoidance [116]. Studies have been conducted in 14- and 60-day repeated-dose designs using northern bobwhite, Japanese quail, zebra finch (*Taeniopygia guttata*), and pigeon (*Columba livia*), although the latter can produce an inefficient emetic response [114]. In such cases, investigators are encouraged to sample emesis for analytical determination of actual dose.

Reproductive Toxicity Test

Avian reproductive effects and chronic toxicity endpoints are determined according to methods described by the USEPA [104,117] and OECD [118]. The U.S. Fish and Wildlife Service [103] has modified these protocols for the testing of alternative shot to include a cold stress (ambient temperatures of 6.6°C–4.4°C) and dietary stress (a diet for mallards consisting of whole-kernel corn). Both these tests are one-generation studies. A two-generation avian reproduction test guideline using Japanese quail is currently in draft form, in support of second-tier testing of potential endocrine-disrupting chemicals [119,120]. Changes to the one-generation protocol also have been under discussion, to reduce the length of the study and increase the statistical power of the test [90].

Birds in the dietary avian reproduction study should be pen reared and phenotypically indistinguishable from wild birds. Additionally, it is recommended that the birds come from a colony that has maintained breeding records and that all birds be free of obvious disease or injury. Bobwhites should be at least 16 weeks old at the beginning of the test period, and mallards should be at least 7 months of age. Ages of other bird species should correspond to known times of reproductive maturity. Birds should be acquired in a quiescent reproductive state, so that the onset of egg laying occurs in the test facility. Birds should be acclimated to the test environment for 2–6 weeks prior to the presentation of treated feed. Birds must be maintained in pens or rooms

that conform to good husbandry practices [121], with minimum space being defined as the ability to stand upright and stretch the wings to their full extent. Birds can be housed as one pair per pen or in groups (one male and two females per pen for bobwhites or two males and five females for mallards). Generally, tests are conducted indoors; however, for some birds (e.g., American kestrels [*Falco sempervirens*] and eastern screech owls [*Megascops asio*]), it is more appropriate to house them outside, and the alternative shot protocol allows for this practice if the study is conducted in a cold-weather environment. The photoperiod must be controlled rigorously, because the onset and maintenance of reproductive activity are determined by the length of the light period. During the acclimation period and for the first 8 weeks of a test conducted indoors, the photoperiod should be set at 7–8 h of light per day. The photoperiod should then be increased to 16 h of light per day, preferably by gradually increasing the day length over a 2-week period, and should be maintained at this level for the remainder of the test. Lights should have an intensity of approximately 65 lx (6 fc) at each cage. For outdoor test environments, the tests should be initiated according to the phenology of the species tested, such that presentation of treated feed begins approximately 10 weeks prior to the anticipated onset of egg laying.

Feed preparation should follow the same guidelines discussed previously for the subacute test, with a minimum of one control and two test doses. Both test doses should be at sublethal concentrations and frequently represent known environmental concentrations. If a NOAEL determination is required, then three concentrations arranged in a geometric progression should be used in addition to the control group. The test chemical should be administered for at least 10 weeks prior to the onset of egg laying, and administration should continue until all control pens have produced 25 eggs or 6 weeks after 50% of the control hens have laid one egg. Studies designed to test alternative shot use oral gavage with one no. 4 lead pellet (positive control) or eight no. 4 pellets of either the test shot or steel shot (negative control), with a minimum of four pairs in the lead treatment group and 20 pairs in the other treatment groups. Shot is administered on 0, 30, 60, and 90 days after the start of the study.

Food and water should be presented *ad libitum* for the entire test period, and food consumption should be recorded at least biweekly throughout the study. Eggs should be collected daily, marked according to the pen from which they were collected, and stored at 16°C and 65% relative humidity. All eggs should be set in incubators once a week at a temperature and humidity suitable for the species being tested. Parental incubation and rearing of chicks may be used if suitable artificial husbandry parameters cannot be determined. Eggs should be candled on day 0 to look for cracks and on days 11 and 18 (bobwhite) or 14 and 21 (mallard) to determine fertility and embryo survival. Eggs collected on 1 day of weeks 1, 3, 5, 7, and 9 of the egg-laying period should be opened at the equator, the contents washed out, and the shells dried and measured for the thickness of the shell plus the membranes to the nearest 0.01 mm. Eggs should be transferred to a hatcher 1 day before expected pipping (day 21 for bobwhite and 23 for mallard). Hatchability for each egg batch is recorded, and chicks are placed in brooders for 14 days and fed a control diet *ad libitum*. Survival at the end of 14 days is recorded. All birds should be weighed weekly prior to the onset of egg laying and at the termination of the experiment; handling should be minimized during the egg-laying period to reduce disturbance. In addition to weight changes, reported endpoints include daily egg production per pen; type and frequency of abnormal eggs (including cracks and other gross defects); the number of incubated eggs that are fertile (fertility); the number of fertile eggs that produce hatchlings that completely free themselves from the shell (hatchability); the number of normal young surviving to 14 days of age; weight of young at 1 day and 14 days of age; eggshell thickness; and chemical residue in tissues of adults, chicks, and eggs. Alternative shot studies also require measures of hematocrit, hemoglobin concentration, and appropriate blood chemistries. Standard appropriate statistical analyses should be conducted to determine whether treated birds differ from controls or to ascertain the dose–response relationship.

Field Tests

Specific methods for examining effects of toxic substances on birds under field conditions were developed initially by the USEPA's Office of Pesticide Programs for use in obtaining pesticide registration data, but have since been discontinued for this purpose. Similar methods are being used in post hoc evaluations of contaminated sites. In pesticide evaluations, field studies were used to determine effects of specific toxic substances if preliminary laboratory studies indicated a potential hazard (e.g., high acute toxicity) and were used primarily for pesticide registration testing. These techniques have been adopted for use in determining individual-level and population-level effects of existing environmental pollution, such as at hazardous waste sites [122], although methods for field surveys of bird density, community composition, nesting success, and other ecologically relevant endpoints have a long history of use in biological assessments [123]. Integration of specific ecological methods is beyond the scope of this chapter and will not be discussed further here; the standardized controlled field studies that evaluate toxicity and/or evaluate exposure are described as follows.

The European starling (*Sturnus vulgaris*) nest-box study [124] is one means of introducing standardized testing procedures into a field study and has been adapted for other cavity-nesting passerines, because of concerns regarding the sensitivity of starlings [102]. Nest boxes encourage birds to nest within a study site and establish a large local population, with readily accessible nests from which chemical-induced reproductive, behavioral, and biochemical perturbations can be documented. The full-scale test is a 3-year procedure. The first field season serves as a pilot study (and allows birds to locate and colonize the nest boxes) in which 30 nest boxes are erected on each of 12 fields, and occupancy and reproduction are documented. Pesticide application occurs in the definitive study during years 2 and 3 in a paired block design (i.e., each

treated field is paired with a similar control field). The type of fields selected depends on the pesticide (or other chemical) to be applied and the bird species of interest; for example, starlings and bluebirds prefer grasslands (e.g., hay fields), which should be selected in preference over other field types when using these birds as a representative species. Study fields are typically 16 ha in size and should be located next to similarly cropped fields to encourage foraging and enhance exposure to test sites. Study sites must be located at least 3 km from each other to reduce the possibility that birds will leave their initial study site and forage in another study area.

Nest boxes are constructed from utility-grade lumber and measure 29 × 23 × 38 cm ($l \times w \times h$) (dimensions vary, depending on the study species). Study designs are available to reduce probability for nest-box predation [125]. The back portion of the box that attaches to the post is 58 cm long, centered so that 10 cm extends above and below the box. The box is attached to the top of a 10 × 10 cm × 3.67 m ($l \times w \times h$) post, with aluminum sheet metal tubing placed around the post starting immediately below the box and extending down about 75 cm to reduce predation. Nest boxes must be placed in the fields so that crop culture can occur with minimal disturbance; therefore, boxes are placed in a single row down the middle of the field in the same orientation as the crop rows. Boxes are separated by 10 m and are erected at least 2 months before the beginning of the breeding season.

Following the placement of the nest boxes, a period of 7 days is allowed to pass before first observations are made. This allows time for the birds to locate and use the nest boxes without disturbance. During the initial nest-building stage (by males), observations are made every 4 days. When the females arrive and during the mating period, observations are made every 3 days. When eggs have been observed in the nest, observations are made every other day. Daily observations are made from the time that hatching begins until fledging occurs. During all monitoring periods, observations are made between 11:30 a.m. and 3:00 p.m., the time when the birds are least active. The following is the reproductive information observed or calculated from nest-box observations:

- Date of nest-box selection (based on the presence of some nesting material)
- Notes on the development of the nest (e.g., amount of nest-building material, quality of the nest, presence of a nest cup)
- Date the first egg is laid (the previous day is designated the date of nest completion)
- Interval of egg laying
- Clutch size
- Number of eggs missing or broken
- Number of eggs that hatch
- Date of egg hatch
- Number of missing or dead nestlings
- Weight of 16-day-old nestlings (g)
- Number of fledglings on each day
- Date of fledging

Additional information collected includes weather data (maximum and minimum daily temperature, precipitation events, wind speed), observations of adult birds' behavior, and presence of predators in the study area. The study continues until all eggs and nestlings from the second clutch (starlings generally produce two clutches of eggs per breeding season) have hatched and fledged or died, and at least 2 weeks have passed without any more eggs being laid. If none of the eggs hatch but they remain in the nest box, the study is terminated when the last box occupied prior to the chemical application has been monitored for 12 days after the laying of the final egg in the clutch. All nestlings that are found dead in the box are analyzed for brain cholinesterase activity, liver enzyme induction, and tissue residues to verify the cause of death (choice of assay depends on the chemical being studied).

If the study being conducted is a pesticide safety study, the application rate of the pesticide should be the highest concentration proposed for registration. The timing of application depends on the age class of starling nestlings. The initial (or single) application to the fields should be performed when there is the greatest number of 1- to 14-day-old nestlings in the boxes. Pesticide application rates are verified through analysis of vegetation, spray cards, and subsampling of the pesticide holding tanks. For granular formulations, the deposition rate of the granules from the dispensing apparatus is measured. About 50 samples per field are required for an accurate representation of application rate.

The reproductive success of the nesting pairs (as summarized by the number of fledglings per field) should be analyzed initially using a two-way ANOVA randomized block design, with the number of fledglings per field as the dependent variable, to determine differences among treatment groups. It is advisable to transform data using a square-root transformation prior to analysis. If this initial test shows significant differences, then inferential statistics should be used to identify which parameters were affected.

Guidance for designing and performing avian field studies for toxicity determinations is provided by Fite et al. [126]. General guidelines are given for two kinds of studies: a general screening study to detect acute toxic effects resulting in mortality or obvious behavioral changes and a detailed, definitive study to quantify the magnitude of acute mortality, determine and measure reproductive impairment, and integrate indirect effects (e.g., food reduction) that may influence the long-term survival of the subpopulation under study. The species and location for the study are determined primarily by the known or proposed location of the pollutant and the population densities and species diversity of the bird communities. The number of replicate study plots (both reference control and treatment) required to make a determination of effect is based on the expected probability of occurrence of effect and the known variability of the system. True replicates are required, and the tendency toward pseudoreplication must be avoided [127]. Many statistical techniques are available for determining the minimum number of replicates required [128].

Information on population density, age, and sex structure, as well as survival data, can be acquired through a range of methods, including the use of mark–recapture techniques, radiotelemetry [129], line transects (for carcass searches), captures per unit effort, or counts of animal signs [110]. Various models are available that use these types of data to determine the probability of survival per unit time and intrinsic population growth rates [130,131]. Reproductive success (from time of initiation of egg laying to fledging of young) is determined through visual observations of nests (egg and fledging counts), radiotelemetry of young, and behavioral observations [129,132].

Studies can also be conducted on contaminated sites to investigate effects on open-cup nesting birds. Nests are found through spot mapping singing males on territory for many passerine species [125,133]. Mist netting can be used to age and color mark individuals for long-term assessment and observation. For persistent, bioaccumulative compounds, residues in food sources from the lower portion of the food web for species of concern can be measured to determine site-specific BAFs. Hatchlings, typically greater than 10 days old, can be used to gather food items for species-specific dietary assessments using the throat ligature technique [134]. Combined with samples for analysis of collocated soil or sediment concentrations, site- and species-specific BAFs can be determined. The throat ligature is a small wire tie that can be fitted snugly around the base of the throat. Nests are observed for evidence of parental feeding, and immediately following feeding events, food is removed from the mouths of fledglings with forceps and subsequently analyzed for chemical content. The wire tie is removed harmlessly using small diagonal cutters. Because bioaccumulation is not a linear function of exposure concentration for most chemicals, and because the process attenuates at higher doses, BAFs should be calculated for multiple areas within a contaminated site to establish the appropriate uptake function.

Additional data that should be collected when assessing effects of contaminated soils include the effects of the chemical on the food source, competitors, or antipredator behaviors of the species of concern. Information on the abundance and composition of the predator community should be gathered to ascertain whether indirect effects of food reduction or changes in community composition may be affecting the endpoint species in addition to (or instead of) direct toxicological effects. These data must be interpreted in the context of what is known about the natural history and normal phenology of the species being studied [135].

Exposure through specific (geophagy) and inadvertent soil ingestion can also be determined for species exposed to contaminants in soil through an evaluation of silica content in feces [136]. These data are particularly useful in exposure characterization for substances that do not biomagnify in food items, and can be used to refine exposure estimates and remedial criteria.

In addition to documenting direct toxic effects on the species of concern, information also is gathered during a field study about the distribution and persistence of the chemicals in the environment. Relating harmful effects on animals to the degree of environmental contamination depends on establishing that a toxic dose has reached either the animal or another species upon which its survival depends. Such information is collected by determining the tissue residues of the chemicals or their metabolites. Biosamples (e.g., blood) can be used to gather information on biomarkers of exposure or effect [94,133]—for example, suppression of brain or blood cholinesterase activity following exposure to organophosphorus or carbamate insecticides or inhibition of δ-aminolevulinic acid dehydratase (ALA-D) due to lead exposure. Both ALA-D and cholinesterase inhibition are legally acceptable indicators of harmful effects of exposure to environmental pollutants [137]. For some chemicals, the concentration of the chemical in the blood or a specific organ can be related to adverse effects, such as blood lead levels, liver lead concentrations, or concentrations of DDT or mercury in the brain [138]. These critical tissue residues can be used as highly specific risk threshold values [94,115]. Other biomarker responses have been developed and used in wildlife exposure studies for risk assessment purposes (e.g., cytochrome P450 induction, corticosteroid levels) but are not yet standardized or have questionable relevance in estimating risk [139].

Mammalian Tests

In environmental toxicology, mammalian-effects data generally are gathered as part of human health impact studies. Both the USEPA [104] and ASTM [106,140] have published general guidelines for conducting acute and subacute toxicity studies with wild mammals and reference the avian and laboratory mammal protocols for more detailed approaches. For large or relatively scarce species, a single-dose regimen may be used where a group of three or more test animals is exposed to the chemical at a dose thought to be representative of expected environmental exposures and observed for at least 10 days for signs of intoxication. Additional doses may be tested sequentially if needed to develop a dose–response curve. This follows the method described for estimating acute oral toxicity in rats by the up–down or the stagewise probit method discussed previously [106–108].

Efficacy testing of anticoagulants in rodents (particularly rats and mice) is well developed, because these animals are the target species [141]. The tests present the animals with both contaminated feed and control feed simultaneously for a free-choice scenario, using a single concentration of chemical in the feed. Exposure times vary from 8 to 20 days, depending on the species being tested, with a 7-day postexposure observation period. The higher the mortality, the greater the efficacy of the product.

Detailed protocols have also been developed for mink (*Mustela vison*) and the European ferret (*Mustela putorius furo*) as representative carnivores, due to concerns about compounds that biomagnify in the aquatic or terrestrial food chain, respectively, and therefore might be expected to cause secondary poisoning due to high concentrations in

the tissues of the target species. Both of these animals can be propagated successfully in the laboratory, and stocks of known genetic origin are available. Additionally, the mink has been shown to be extremely sensitive to polychlorinated biphenyls [142], polybrominated biphenyls [143], hexachlorobenzene [1], aflatoxins [144], and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [145]. Three protocols are available: dietary LC_{50}, reproduction, and secondary toxicity [146]. For all three tests, animals should be of mature body weight (about 18–20 weeks of age). Either sex can be used in the subacute dietary and secondary toxicity tests; however, the two sexes must be treated as separate subgroups due to significant size differences. Test animals can be obtained from commercial sources or reared in the laboratory and should be free from obvious disease or injury. It is recommended that mink be vaccinated against canine distemper, virus enteritis, infectious pneumonia, and botulism and that ferrets be vaccinated against canine distemper and botulism. Although space requirements for most carnivores have not been standardized, adherence to the guidelines of the Fur Farm Animal Welfare Coalition [147] should provide adequate husbandry guidance. Cages measuring 61 × 76 × 46 cm ($l \times w \times h$) have proven adequate for housing individual mink or ferrets. Solid dividers should be used between adjoining cages to reduce aggression, and a nest box containing straw, shredded wood, or marsh hay must be provided for females in reproduction tests prior to parturition. No specific photoperiod is required, but the day length should not be altered from that in which the animals have been reared. A minimum of 7 days of acclimation is required, during which time food consumption should be measured and an initial body weight determined. Diets must be formulated to meet the requirements of the test species. Suggested composition of mink diets is provided by the National Research Council [148] and by Ringer et al. [146]. Fresh food and water must be provided daily *ad libitum*. Test diets are prepared by mixing the chemical directly into the feed or by dissolving or suspending in a solvent or carrier prior to mixing in the feed. If a solvent or carrier is used, it must also be added to the control-group diet. If a volatile solvent is used (acetone or hexane), the diet should be air-dried to evaporate the solvent prior to feeding. Sufficient diet should be mixed to provide feed for the entire exposure period and frozen in aliquots sufficient for 1–2 day feeding. All diets should be analyzed after mixing to determine actual concentrations and homogeneity of the test substance in the diet.

Subacute Dietary Toxicity Protocol for Ferrets or Mink

The doses for a definitive LC_{50} study [146] should be arranged in a geometric progression, with the highest dose set so an animal will consume the equivalent of an LD_{50} dose in 1-day feed. The test concentrations should then be spaced to achieve at least two concentrations, yielding between 10% and 90% mortality. This generally can be achieved with four to six different concentrations. A minimum of eight animals per dose is required. If an LD_{50} value is not available, one can be determined from a range-finding procedure. A geometrically spaced series of doses is administered by gavage to two animals per dose, and the LD_{50} is determined to be the dose at which one or two animals die during a 1-week observation period. Animals should be treated by gavage with a 3-in., 14-gauge, curved, stainless-steel animal feeding needle. If a lethal dose is not found, the highest dietary concentration should be set at 5000 mg/kg. It is recommended that the highest concentration to be tested be fed to two to four animals for several days prior to the start of the test, to be sure that it is palatable. If it is not, the highest concentration should be reduced to a level that will be eaten. The test diet is then presented for 28 days. A withdrawal period when clean feed is given should be included if animals are still exhibiting signs of intoxication at the end of the exposure period but should not exceed 14 days. Body weights should be recorded at the beginning of the treatment period and weekly thereafter. Feed consumption should also be measured weekly. Mortality, behavioral abnormalities, and other signs of toxicity should be recorded daily. A test is considered invalid if more than 12.5% of the control animals die. It is recommended that a dietary concentration group be removed from the test when food consumption values indicate that feed consumption has dropped to less than 10% of control values after the first 2 weeks of measurements or if animals lose 30% of their initial body weight. It is recommended that necropsies be performed on all animals at the time of death and on all test animals euthanized at the end of the observation period [149]. Gross and histopathological examinations of all major organs should be conducted, including measurement of organ weight. Comparison of body- and organ-weight changes and feed consumption between control and treatment groups should be made by ANOVA procedures with a posterior comparison of groups conducted by Dunnett's method [150]. The LC_{50} value is calculated by probit analysis or another standard method.

Reproduction Test Protocol for Ferrets or Mink

This test [145] primarily measures female reproductive effects, because the male is used only for the period of time needed for insemination of the female. Proven breeders should be used if possible. A minimum of 12 females per treatment group is required. One male is needed for every three females in each treatment group; males should not service females in more than one treatment group. If proven breeders are used, the number of females per group can be reduced to eight, and the number of males to two. Males are left with the females only for the duration of breeding. A minimum of two test concentrations and one control group is required. The highest test concentration must (1) produce an effect, (2) contain at least 1000 mg/kg, or (3) be at least 100 times higher than the known or expected environmental concentration. Animals are fed test diets for 8 weeks prior to breeding; during breeding, gestation, and parturition; and for 3 weeks of lactation (approximately 23 weeks in total). The test will be longer for mink than for ferrets, because mink exhibit a variable delay in implantation of fertilized ova, while ferrets do not. The gestation period for mink can range

from 42 to 60 days, whereas the ferret has a more constant gestation period of 42 days. Under natural conditions, mating attempts begin at the first of March for mink and at the end of April for ferrets.

To breed mink, a female is presented to a male (in his cage) and, if receptive, is allowed to mate. If not receptive, the female is removed and a mating attempt is tried again in 4 days. Successful mating is verified by the presence of viable spermatozoa in a vaginal aspiration taken after copulation. Following a successful mating, the female is given a second opportunity to mate to the same male either the next day or (preferably) 8 days later. In breeding ferrets, females are presented to males when in estrus (determined by extent of vulvar swelling) and left with the male overnight. They are not given the opportunity for additional matings.

When the breeding period is over, animals should be left undisturbed except for daily feedings. Body weights should be measured weekly only during the 8 weeks prior to breeding. Feed consumption should be measured weekly throughout the duration of the test. Observations of behavioral changes, mortality, or other signs of toxicity should be recorded daily. During parturition (up to 3 weeks in length), females should be checked daily for newborn. Number, weight, and sex of all newborns are recorded within 24 h of birth. It may be necessary to remove the female from the nest box to check for newborns. Offspring should be weighed again at 3 weeks of age. After this time, they will begin eating the adult diet and should be fully weaned at 6 weeks of age, at which time the test is terminated. At the end of the test, all males and at least an equal number of females are euthanized [148], and necropsies are performed for gross and histopathological examinations. A test is considered invalid if more than 20% of the control animals die during the test. The following reproductive parameters must be reported:

- *Length of gestation*—Time, in days, from last confirmed mating until parturition.
- *Number whelped, not whelped*—Number of females giving birth or not giving birth in a treatment group per number of females with confirmed matings; number whelped includes those females that die during parturition.
- *Live newborns per females whelped*—Average number of live newborns produced by all females that give birth in a treatment group; this does not include females that die during whelping.
- *Average birth weight*—Average weight within 24 h of birth of all live newborns within a treatment group.
- *Average litter weight*—Average weight of all litters (live newborns only) within each treatment group.
- *Percent newborn survival to 3 weeks*—Number of live newborns in a treatment group surviving to 21 days of age, expressed as a percentage of all live births in the treatment group.
- *Average body weight at 3 weeks of age*—Average weight of all live newborns in a treatment group on the 21st day after birth.
- *Total newborns per female whelped*—Average number of all newborn (alive and dead) produced by all females that give birth in a treatment group (including those females that die during whelping).
- *Percent newborn survival to 6 weeks*—Identical to 21-day survival but extended to 42 days.
- *Average 6-week body weight*—Identical to 21-day weights but measured at 42 days of age.

Number whelped and percent survival should be analyzed for differences among treatment groups using contingency tables and Bonferroni's chi-square test or similar statistical procedures. The remaining variables can be compared among treatment groups by ANOVA techniques with Dunnett's method [149] for comparison to controls.

Secondary Toxicity Protocol for Ferrets or Mink

This protocol [145] is used to determine the comparative toxicity of both a parent compound and its metabolites administered in the diet, such as might be ingested when consuming contaminated prey. The toxicity of the parent compound is determined following the procedures detailed in the preceding text for subacute dietary toxicity testing (LC_{50}). Secondary toxicity testing is conducted using the same protocol, with the exception that the contaminated feed consists of mink and ferret prey items that have themselves been fed the test substance. Prey animals can be any species that is readily consumed by the test animals, including fish (salmon, perch, alewife, sucker, carp, and bloater chubs), birds (chickens), and mammals (beef, nutria, rabbits, voles, pocket gophers, rats, and mice). Prey animals may be contaminated by dietary, inhalation, or dermal routes and should be exposed to the same test substance (same source and lot number) as fed to the mink or ferrets in the primary toxicity test. The concentration of the test substance to which the prey are exposed should be sufficient to generate tissue residues of parent compound or metabolite at levels known to cause 10%–90% mortality of the mink or ferret. This may need to be determined through a range-finder test with the prey species. The final body burden in the prey should allow for dilution of the tissues by the remainder of the dietary ingredients provided and for dilution by noncontaminated portions of the prey; for example, if 10 mg/kg causes a 50% lethality in the mink or ferret, and a diet consisting of 40% prey tissue is being fed, then a prey body burden of 25 mg/kg is required to yield the final dietary concentration of 10 mg/kg. Prey animals that are not killed by the test substance should be euthanized in a manner that will not interfere with the test results. The gastrointestinal (GI) tract can be removed from the carcass if the test substance is known to accumulate in tissues. This will reduce the probability of direct poisoning from consumption of undigested material in the upper GI tract; however, some compounds do not readily accumulate in tissues (e.g., organophosphorus insecticides), and poisoning of carnivore species occurs primarily from ingestion of material in the GI tract. In these instances, the GI tract can be left intact. All prey-animal carcasses should be frozen until being fed to the mink or ferret.

Other Acute, Subacute, and Subchronic Mammalian Models

Other species have been used in various study designs intended for the derivation of TRVs. *Peromyscus* spp. (e.g., white-footed and deer mice) are native to eastern and western North America, respectively, and are available from several captive-bred facilities, including genetic variants [151]. Typically, these species are not amenable to repeated-dose gavage studies, because they are high strung and difficult to handle on a daily basis; however, acute gavage studies using the up–down or stagewise probit methods can be accomplished. Repetitive exposures are best accomplished using dietary studies, although care is needed to use feed receptacles that reduce spillage and do not allow the animals to either nest or urinate/defecate in their food. Spilled feed should be weighed daily to account for unconsumed diet. Traditional polycarbonate mouse cages can be used with additional housing material for enrichment, because these species are crepuscular and nocturnal. Other small-mammal models include voles (*Microtus* ssp.) and shrews (*Sorex* ssp.), although these species require additional care in handling due to an increased tendency to bite. Cotton rats (*Sigmodon* ssp.) should not be handled by the tail, because they can shed the outer layer of skin and can also inflict a painful bite. The use of mail gloves is advised when handling any wild rodent.

Honeybees

The European honeybee (*Apis mellifera*) is important economically, due to its use for pollination of many crops (e.g., fruit trees). Methods have been developed to evaluate the effects of pesticides on honeybees [14,152–154], and these tests have been adapted for use in assessing environmental pollution in situ [155,156]. Toxicity to bees of residues on foliage is determined using individual worker bees of uniform age [14,155]. The compound of concern is applied to alfalfa foliage in the field and allowed to weather under natural conditions. Worker bees—honeybees, the alfalfa leafcutting bee (*Megachile rotundata*), or the alkali bee (*Nomia melanderi*)—are collected from the frames of established colonies, and 50–100 individuals are introduced into each test cage. Cages are constructed by cutting wire screen into 5 cm strips 46 cm long and stapling the ends to form a cylinder. Tops and bottoms of 150 × 15 mm plastic petri plates serve as the tops and bottoms of the cages. Honeybees (50–100), leafcutting bees (20–40), or alkali bees (15–30) should be placed in each cage. Ambient temperature during the 24 h test period should be 24°C–26°C (honeybees) or 29°C–31°C (leafcutting bees and alkali bees). Bees are fed during the test by providing cotton squares (5 × 5 cm) soaked with 50% sugar syrup and placed under the treated foliage.

The test compound is applied to 0.01-acre alfalfa plots in a randomized block design at applications that simulate known environmental concentrations or proposed use patterns. The test can be designed to evaluate a single dose; preferably, a geometrically spaced range of doses is used to determine an LC_{50} value. Foliage is harvested at 3, 8, and 24 h after application. If greater than 25% mortality of bees occurs when exposed to 24 h foliage, sampling should continue at 24 h intervals until mortality resulting from exposure to treated foliage is not significantly different from control groups. Foliage is chopped and mixed, and 500 mL is introduced to each cage. Mortality is determined after 24 h of exposure to the treated foliage. At least three cages of bees must be used per replicate, and each treatment, including controls, must be replicated at least three times.

The honeybee acute contact LD_{50} test is based on the protocol developed by Atkins et al. [151] for screening pesticide dust and follows the same husbandry procedures detailed previously. Worker bees of uniform age are exposed to the test substance through a dusting procedure. Twenty bees are transferred to the dusting cage and dusted for a period of 15 s. The dusted bees are then put in holding cages and observed for mortality over the next 24 h.

The honeybee subacute test [13] is designed to test the effects of a chemical on the colony as a unit. The study is intended to identify those chemicals that may cause adverse reproductive, behavioral, or other subacute effects that can be brought back to the hive by exposed foragers. This test is less developed than the tests described in the preceding text for individual bee toxicity, and consequently, it will not be detailed here, but it was discussed at a recent workshop on risk assessment for pollinators [154]. The general approach involves exposure of intact bee colonies to the test substance through feeding in pollen or sugar syrup (there is no consensus as to which component of the diet should contain the test compound). Through caging or location, the colonies are restricted to feeding only on the treated food provided. Periodic observations are made for 42 days to 4 months (42 days is the approximate time needed to complete two brood cycles). Observations include the following:

- Colony strength
- Weight of the hive
- Pollen, honey, and nectar stores
- Mortality at the hive (via the use of dead-bee traps or collecting sheets)
- Mortality of drones and pupae
- Mortality in the crop
- Presence of the same queen
- Foraging activity in the crop
- Returning foraging bees (can be counted automatically)
- Behavioral abnormalities
- Residues in pollen, nectar, pollen pellets, wax, bee bread, and dead bees
- Assessment of the brood, including an estimate of adults
- The area containing cells, eggs, larvae, and capped cells
- Disease or pest levels (as a measure of resistance)

Analysis of pollen content can also be useful in understanding exposure to soil contamination.

Soil Invertebrates

Direct measures of chemical contamination of the soil can be, and frequently are, made following traditional analytical chemistry methods; however, these methods are costly and time consuming and do not provide information about the toxicity of the soil to terrestrial organisms, particularly because synergistic, antagonistic, and additive effects of the complex mix of compounds found in contaminated soils can occur. Furthermore, chemicals in soil become bound to soil particles during the aging process and become progressively less bioavailable [157]. Chemical assays that use harsh extraction procedures to extract all the chemical from the soil do not provide a realistic estimate of exposure to soil organisms. Models are being developed to calculate porewater concentrations for varying soil types (especially with reference to pH, organic matter, and clay content) to more closely estimate actual exposure regimes. Even these methods cannot account for biological adaptations, behaviors, and microenvironment alterations; therefore, bioassays have been developed to provide direct measures of toxicity.

Plant germination and growth tests are the most commonly used methods for determining soil toxicity and are described later in this chapter. The amphibian bioassay (FETAX) described in the following text can be conducted using eluates from contaminated soils. A solid-phase version of the aquatic MICROTOX test has been developed and is used occasionally. Soil microbial function assays are available for nitrogen and carbon transformation processes [158–160] and are described briefly in the following text. Analysis of the soil transcriptome (i.e., transfer RNA) is being studied as a sensitive and more realistic in situ method for measuring functional changes in the microbial community.

Soil invertebrates play a pivotal role in the terrestrial ecosystem, providing functions such as decomposition of organic matter, as well as providing a prey base for many higher-order organisms. A well-developed, but infrequently applied, test determines an LC_{50} in soil for harvester ants (*Pogonomyrmex owyheei*) [161]. Crickets (*Acheta domesticus*), isopods (e.g., *Porcellio scaber*), millipedes (*Brachydesmus superus*), centipedes (*Lithobius mutabilis*), staphylinids (e.g., *Philonthus cognatus*), oribatid mites (e.g., *Platynothrus peltifer*), and soil nematodes also have been studied as possible toxicity test organisms for soil toxicity determinations [162,163], but none of these protocols has yet been accepted by the OECD, EU, or United States for regulatory purposes. The soil invertebrates most frequently used for toxicity testing are the oligochaetes: the potworm (Enchytraeids, such as *Cognettia sphagnetorum*) and earthworms (e.g., Lumbricidae, such as *Eisenia foetida* and *Eisenia andrei*); the Collembola (*Folsomia candida*, *Folsomia fimetaria*, and *Orthonychiurus folsomi*) also are used. Standard assays are available for earthworm survival, reproduction, and avoidance behaviors [164–166]. Both of these types of worms are found in nearly every soil type (except for xeric soils) and play an important role in soil decomposition [167]. Furthermore, they are composed of a high percentage of lipids and readily bioaccumulate contaminants from soils; a standard test is now available for bioaccumulation in oligochaetes [168], which are a significant food item for many vertebrates and, therefore, are often an important first step in the movement of soil contaminants into the aboveground food web. Standard test protocols have also been adopted by the OECD and Canada for Enchytraeid, Collembola, and predatory mite *(Hypoaspis aculeifer)* reproduction assays [169–172].

Earthworm Tests

The earthworm survival test [165,173,174] uses adult (>60 days old, 300–500 mg, with clitellum) *E. foetida* or *E. andrei* grown in a single culture chamber. Worms can be purchased from a commercial source or collected outdoors. The identification must be verified [175] to ensure that the species is *E. foetida* or *E. andrei*. Test soils are first homogenized using a blender and then mixed with artificial soil (10% 2.36 mm screened sphagnum peat, 20% colloidal kaolinite clay, and 70%-grade 70 silica sand) to prepare 700 g each of a geometric series of test soil concentrations (e.g., 100%, 50%, 25%, 12.5%, 6.25%, 3.13% w/w) plus a 100% artificial soil control. The total amount for each concentration is blended—to ensure even mixing—prior to dividing into aliquots for the test. After mixing, the soils are hydrated to 75% water-holding capacity (WHC). Standard 1-pint canning jars with screw-top lids and rings are used as test chambers. Three replicate chambers are filled with 200 g (dry weight) of soil for each dilution. Ten earthworms are placed on the soil surface, the jars are capped, and they are incubated at 20°C ± 2°C under continuous light (540–1080 lx) for 14 days. Worms are not fed during the test. Soil pH is measured at the beginning and end of the test, and the temperature of the environmental control chamber is monitored continuously. The total organic carbon of the test soils should be measured in one test jar for each test concentration and the control. Jars are examined daily for dead worms (worms are considered dead when they do not respond to a gentle touch on their front end). At the end of the 14-day period, the soil is emptied into a tray and the jar is thoroughly searched for worms. Dead worms decay very rapidly, so all 10 worms in a container may not be accounted for during the test, and it is assumed that all missing worms have died. The percent mortality for each concentration is determined, and the EC_{50} for mortality is calculated by probit analysis. Mortality is defined as a lack of response to a gentle mechanical stimulus (e.g., a touch with a small glass rod). Loss of earthworm biomass and behavioral and morphological endpoints such as coiling, segmental swellings or constrictions, lesions, rigidness, and flaccidness also can be used as toxicity endpoints.

Growth and reproduction are biological endpoints in earthworm tests of longer duration, generally 140 days [3,173,174]. Test conditions are the same as described for the 14-day study, although worms must be fed for any study with duration greater than 28 days. Control of pH, temperature, and soil moisture content is very important, because variations in these environmental parameters have been shown to significantly affect the outcome of earthworm reproduction

studies. Mortality and other sublethal observations are made at least weekly. At the conclusion of the test, the containers are emptied, and the number of adult worms remaining is tabulated. The number of cocoons formed, cocoon mass, number and growth of young worms, and rate of clitellum development are measured as reproductive endpoints.

Enchytraeid Tests

The potworm, *C. sphagnetorum*, is readily obtained from the field and is easy to culture in the laboratory on a 75 vol% sphagnum peat substrate mixed into 25 vol% LUFA 2.2 soil (LUFA is a natural soil type found in Europe that contains ~3.9% organic matter and 3.5% clay and has a pH of 5.8) [176]. Animals of similar size (i.e., with the same number of segments) are used in each study. A single study is conducted to determine percent mortality and effects on growth rate of adults and fragments. Potworms reproduce asexually, resulting in the production of three fragments: head, middle, and tail. The middle and tail fragments develop a functional mouth within 2–3 weeks, and all three fragments continue to add new segments. After another 2 weeks of adding segments, the new worms are indistinguishable from older specimens. Worms that have >30 segments are considered to be adult. Growth is defined as the increase in number of segments over the test period, reproductive success is quantified as the mean number of fragments that survive and add segments over the test period, and the fragmentation rate is defined as the average number of fragments produced per day. The chemicals of interest are dissolved in water or an organic solvent and mixed with the soil substrate, and the soil is wetted to 80% WHC 24 h before introducing the worms. For chemicals dissolved in organic solvent, the chemical is first mixed with a small amount of sand to allow the solvent to evaporate and then mixed into the final batch of soil. A food source is required due to the long duration of the test, so 1% algae (*Pleurococcus* spp.) or 0.2% baker's yeast is added to the soil substrate prior to putting ~2 g of soil into each test tube. Animals are maintained one per test tube at 15°C ± 1°C, with a relative humidity in the test chamber of 75% under constant light, after first counting the number of segments present. At weekly intervals for 10 weeks, the tubes are examined for the number and size of fragments and number and size of unfragmented worms. Animals are transferred to vials with fresh soil and food (one worm or fragment per vial) prior to returning them to the incubation chamber. Copper chloride is used as a positive control (reference); a test is considered valid if the growth rate in control animals is >1 segment/week and there is <10% mortality of controls.

Springtail Tests

Springtails, including *F. candida, F. fimetaria*, and *O. folsomi*, are common soil organisms that occupy a position in the soil food web, being consumers of fungi, detritus, nematodes, and bacteria, and are an important prey group for other invertebrate predators. Historically, *F. candida* has been the preferred species for studying the effects of contaminated soils, and an International Organization for Standardization (ISO) protocol was published in 1999 but is no longer available; Environment Canada published a more comprehensive protocol in 2007 [169]. Laboratory-cultured springtails should be used as the source of the test organism, making sure that the animals are not excessively inbred. All springtails used in a test must be derived from the same population and be of the known developmental state and the same age and size; information about the laboratory stock should be provided. Test vessels consisting of 100–125 mL wide-mouthed glass jars or beakers with plastic or metal lids are thoroughly cleaned and rinsed and filled with 20 g (wet weight) of soil. Test soils can be either negative control soils spiked with chemical or soil collected from contaminated sites. Negative control soils, in turn, can be either natural soils or artificial soils; regardless, physicochemical characteristics should be described (e.g., pH, particle size, texture, conductivity). Positive control soils consisting of soils spiked with a known toxicant should also be included. For field studies, reference soils (i.e., similar soils collected from sites known to not be contaminated with the chemicals of interest) can be included as well. Concentrations of chemicals in soils should be analyzed at the beginning and end of the test. Soils should be at 70% WHC, and any field collected should have debris and indigenous macroorganisms removed and press-sieved through mesh size 4–6 mm and thoroughly mixed. Test vessels are seeded with 10 female and 5 male organisms at 28–31 days of age for *O. folsomi*, 10- to 12-day-old juveniles for *F. candida*, and 23- to 26-day-old organisms for *F. fimetaria*. Test duration is 28 days for *O. folsomi* and *F. candida* and 21 days for *F. fimetaria*. Air temperature should be maintained at 20°C ± 2°C, with light intensity at 400–800 lx and a photoperiod of 16 h L:8 h D. Animals are fed granulated dry yeast on days 0, 7, 14, and 21 by sprinkling onto the soil surface in each test vessel. Vessels should be opened briefly at least once per week to aerate and should be rehydrated as necessary. Measurements made during the test include daily (or continuous) air temperature, percent moisture, and pH of soil in each vessel at start and end; excessive growth of fungi; feeding activity; and presence and quantity of uneaten food. Endpoints measured are number alive (percent survival) and total number of live progeny at the end of the test. Depending on the test design, concentration–response functions can be derived or differences between test soils and reference soils calculated. Tests are considered invalid if mean survival of adults in negative control soil is <70%.

Soil Microorganism Tests

Soil microbes (i.e., fungi, bacteria, actinomycetes, and protozoa) make up 80% of the living matter in soil and contribute significantly to essential soil function, such as decomposition of organic matter, nutrient cycle, and soil aggregation [177]. Because of the diversity of species and the importance of microbial functions, tests for the effects of chemicals on soil microbes use functional endpoints rather than effects on individuals or population structure. Two standardized tests are available—nitrogen transformation [159] and carbon

transformation [160]—but significant controversy remains over the relevance of these and other soil function assays. The nitrogen transformation test adds a nitrogenous substrate to reference or contaminated soils collected in the field (or clean soils spiked with chemical). Soils are incubated for 28 days and then analyzed for the production of mineralized nitrogen (i.e., amount of ammonium, nitrite, and nitrate production). The carbon transformation test is similar and measures the soil respiration rate (carbon dioxide production). A dilution series of five soil concentrations of the chemicals of concern is preferred, and at least a 20% difference between reference and treated soils is required to specify that a significant effect has occurred.

Amphibians

The frog embryo teratogenesis assay *Xenopus* (FETAX) is a standardized bioassay developed for obtaining data on the developmental toxicity of test materials. Amphibians usually are associated with wetlands, many of which are affected by chemical pollution due to their distribution in low-lying areas of the landscape and their connection to the surface and subsurface hydrology [71]. FETAX is used primarily as a measure of toxicity to amphibians of environmental samples (water, sediments, and soil eluates); however, results from this bioassay have an 85% correspondence with results from mammalian developmental toxicity tests, so the information can be extrapolated to other classes of animals with a high degree of certainty. The standard protocol, as described in the following text, is a laboratory assay. A field application (by putting the egg masses in porous containers submerged in the water at the study site, and following all other standard procedures) also has been used [178].

In FETAX [179], a range-finding and three replicate tests are performed. Each test includes both a negative control (no test material added) and a positive control (6-aminonicotinamide). The 96 h LC_{50} and 96 h EC_{50} (malformation) are determined, and the teratogenic index (TI) is calculated by dividing the LC_{50} by the EC_{50}. The FETAX protocol is designed to use embryos of the South African clawed frog, *Xenopus laevis* (Daudin), which is a tetraploid (4N) species. Other North American species can be used and are listed in the appendix of the protocol, although breeding times and methods would have to be adjusted to produce an appropriate egg mass for the test. For *Xenopus*, adult frogs that are proven breeders should be purchased and maintained in single pairs. The frogs should be bred in the same water in which the test is to be conducted (natural, nonchlorinated water known to be free of contaminants) and fed ground adult beef liver three times per week. Temperature should be kept at 23°C ± 3°C on a 12:12 L/D photoperiod. Breeding is induced in the males and females by injection into the dorsal lymph sac of 250–500 and 500–1000 IU, respectively, of human chorionic gonadotropin. Egg deposition usually occurs 9–12 h later. The eggs should be inspected for fertility, which must be >75% for the egg mass to be used in a test. Eggs are then separated from the jelly coat by gently swirling them for 1–3 min in a 2% w/v L-cysteine solution prepared in FETAX solution (625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4{\cdot}2H_2O$, and 75 mg $MgSO_4$ per liter of deionized or distilled water; final pH 7.6–7.9). Eggs should be removed from the dejellying solution and rinsed with clean water as soon as dejellying is completed or survival will be reduced. Only normally cleaving embryos should be selected for use in the test. The *Atlas of Abnormalities* [180] should be consulted to determine abnormalities during the assay. Each test must use embryos derived from a single mated pair.

FETAX is a 96 h test. Each test consists of at least five concentrations arranged in a geometric progression, three of which should fall within the 16%–84% effect range on the mortality and malformation dose–response curves. A range-finding test should be conducted first, consisting of at least seven concentrations that differ by factors of 10. Each test concentration and the positive control should have two dishes, each containing 25 embryos and 10 mL of test solution. The negative control groups must have four dishes of 25 embryos each. The positive control (6-aminonicotinamide) consists of two dishes exposed to 2500 mg 6-aminonicotinamide per liter and two dishes exposed to 5.5 mg/L. Dishes are placed in an incubator to maintain the temperature at 24°C ± 2°C. The pH of the test solutions should be 7.7 (range, 6.5–9.0). The test material is renewed every 24 h during the test. Renewal should be done by removing the old test solution with a Pasteur pipette, with the orifice enlarged and fire-polished to accommodate embryos without damage if they are picked up accidentally. Fresh solution is added quickly to minimize embryo desiccation.

Dead embryos are removed at the end of each 24 h period during the 96 h test (at the time the solutions are changed). Death at 24 h is ascertained by the embryo's skin pigmentation, structural integrity, and irritability. At 48, 72, and 96 h, a lack of heartbeat indicates death. The total number of dead embryos and numbers and types of malformations occurring at the end of 96 h are reported. The ability of the test material to inhibit growth is determined at 96 h by recording head-to-tail length. If the embryo is curved or kinked, the measurement should be made as if the embryo were straight (i.e., following the contour of the embryo). The minimum concentration of test material that significantly inhibits growth (MCIG) should be determined from these data using the *t*-test to determine significance at $p = 0.05$. The LC_{50} and EC_{50} (malformation) should be determined using probit analysis.

Postfertilization protocols using native amphibian eggs have been developed to evaluate sediment and water contamination [141]. Exposures can be extended to metamorphosis. Effects of compounds that inhibit thyroid hormone production can be particularly evident as delayed or inhibited metamorphosis [181,182]. Moreover, tests involving native species can reveal a greater sensitivity than *Xenopus*, yet their overall sensitivity to environmental contaminants has been questioned [183].

Soil contamination effects have been tested using a plethodontid terrestrial salamander species, the red-backed

salamander (*Plethodon cinereus*) [110,184–186]. Individuals are wild-caught and exposed to treated or contaminated soils in glass petri dishes. Salamanders can be housed for long exposure periods, lasting several months. Small amounts of sphagnum moss are wetted and used for humidity control and for environmental enrichment. Individuals can be maintained on mutant (flightless) *Drosophila melanogaster*, although attempts to integrate compounds within food items have not been productive due to direct toxicity to live food (Johnson, unpublished data). Animals are particularly sensitive to irritants in soil and are considered conservative models in estimating risk to compounds that are likely to penetrate via dermal routes. Excess mucus production is an early indicator of stress. Animals should be euthanized in a buffered MSS-222 solution at the end of the test exposure period and decapitated to obtain 10–20 µL of plasma obtained for determination of packed-cell volume and red and white blood cell counts. Whole animals should be fixed in formalin and embedded in paraffin, and cross sections should be cut and stained with hematoxylin and eosin for histopathological evaluation.

Ambystomid salamander species also have been tested under controlled laboratory conditions, though with variable results [96,187,188]. Wild-caught *Ambystoma maculatum* and *Ambystoma tigrinum* are fed tainted live earthworms (*Lumbricus* ssp.), although shipping-related stress can cause sepsis (red leg), which can be difficult to treat. Salamanders reared from egg masses are more successful in toxicity tests, although larval stages require specialized feed (e.g., copepods).

Plant Tests

A standard guide for conducting terrestrial plant toxicity tests has been completed and published by the ASTM [189]. Included with the guide are standard protocols for seedling emergence, root elongation, woody plant assays, and a *Brassica* life-cycle test. Additionally, a seed germination test is available [169,190–192], as is a test for vegetative vigor [164,190,193]. These bioassays were developed initially to determine hazards due to soil contamination at hazardous waste sites or in sludge disposal areas [194]; however, they also can be applied to a priori safety testing requirements. A comprehensive review of plant toxicity tests is available in Kapustka [195].

Seed Germination

The seed germination test is a 120 h static test and measures seed survival, germination, and seedling emergence. Butter crunch lettuce (*Lactuca sativa*) is the most commonly used test species, although more than 30 species have been accepted by regulatory agencies. Butter crunch lettuce seeds and many other domestic plants can be purchased from a commercial vendor. Only one lot should be used for each test, and information on the germination percentage should be provided by the seed source. Alternatively, native seeds may be field collected. Regardless of source, only untreated seeds (no applications of fungicide, repellents, etc.) should be used. The seed lot should be examined to discard trash, empty hulls, and damaged seed. Seeds are sized by stacking four wire-mesh sizing screens on top of each other with a collection pan underneath; the largest mesh screen should be on top. The seeds are poured in, and the nest of screens is shaken until no more seeds fall through the screens. The size class containing the most seed is selected and used for all of the tests. Seeds can be stored in a desiccator at 4°C in airtight, waterproof containers until used.

Test soils are first homogenized using a blender and then mixed with artificial soil (commercially available, 20-mesh, washed silica sand) to prepare 400 g each of a geometric series of test soil concentrations (e.g., 100%, 50%, 25%, 12.5%, 6.25%, 3.13% w/w) plus a 100% artificial soil control. The ASTM method [189] requires the use of boric acid as a positive control. The total amount for each concentration is blended to ensure even mixing, prior to dividing into aliquots for the test. The soils are air-dried, and 100 g of each concentration is placed in each of three replicate 150 mm plastic petri dishes labeled with the appropriate soil dilution. The petri dishes are then randomized, and 40 seeds are placed in each dish at least 1.25 cm from the edge. The seeds are pressed into the test soil with a glass rod or beaker bottom. Soils are hydrated with deionized water to 85% of WHC. Cover sand (90 g commercially available, 16-mesh sand passed through a 20-mesh screen to remove fines) is poured over the top of the soil and leveled with a ruler. The dishes are then placed in an incubator for the duration of the test. They should be incubated at 24°C ± 2°C in the dark for 48 h, followed by 16 h of light and 8 h of dark, until termination of the test at 120 h. Light intensity should be 4300 ± 430 lx. Soil pH should be measured at the beginning and end of the test (and should be between 4 and 10), and soil temperature should be measured every 24 h in one replicate of each concentration and the control. At 120 h, the number of germinated seeds in each dish is determined by counting each seedling that protrudes above the surface. The LC_{50} and its 95% confidence limits are determined by probit analysis.

Seedling Emergence

The seedling emergence test is very similar to the seed germination test. Seeds are placed in pots, rather than petri dishes, and need not be stored in the dark for the first 48 h. Soil is hydrated to WHC throughout the test. Test duration is twice the amount of time required to achieve acceptable germination for whatever test species is being used. In addition to counting the number of emergent seedlings at the end of the test, optional measures include shoot and root growth. Shoot measurements are made from the transition point between hypocotyl and root to the tallest point on the shoot. Roots are measured from the same transition point to the tip of the root. If sufficient growth is present, dry-weight measurements can be obtained. Material is harvested and placed in a preweighed drying vessel and dried at 70°C until constant weight is achieved (approximately 24 h). Weights are taken to the nearest 0.001 g.

Root elongation is a key component of the early stages of plant growth and development. The root elongation bioassay tests the toxicity of water or soil eluates (i.e., the water-soluble constituents) to seed germination [192]. It generally is used as a bioassay of hazards due to soil or water contamination at hazardous waste sites or in sludge disposal areas; however, as with the seed germination test, it also can be applied to a priori safety testing requirements. As a general rule, root elongation is more sensitive than seed germination. The test is a 120 h static test that measures seedling root growth. Butter crunch lettuce is the most commonly used and is the most sensitive species, but other plants have been used as well (e.g., cucumber, wheat, alfalfa, radish, red clover, rape). Seeds are purchased, sorted, and sized as described for the seed germination assay. Soil eluates are prepared by mixing 4 mL of deionized water per gram (dry weight) of soil. The slurry is mixed in total darkness for 48 h at 20°C ± 2°C. After mixing, the eluate is centrifuged and filtered through a 0.45 μm cellulose acetate or glass fiber filter. Water samples (or soil eluates) are diluted in deionized water to provide a geometrically spaced range of concentrations, generally using a 0.3 or 0.5 dilution factor and five concentrations. As with the seed germination study, boric acid should be used as a positive control. A sheet of Whatman No. 3 filter paper is placed in each of three replicate 100 mm plastic petri dishes, and 4 mL of the test solution is poured over the paper. Five seeds are placed in a circle on the filter paper, equidistant from the edge to the center and equally spaced from each other. The lid is placed on each petri dish, and the dishes are then set in layers in a black 33-gal plastic garbage bag within a cardboard box. Moist paper towels are placed between layers of dishes to keep the humidity level elevated. The bag and box are sealed and placed in a controlled environmental chamber at 24°C ± 2°C for 120 h. The pH and hardness of the test solutions are measured at the beginning of the test, and temperature is recorded every 24 h. At the end of the 120 h period, root lengths are measured to the nearest millimeter by placing the seedling on a glass work surface and measuring the distance from the transition point between the hypocotyl and root to the tip of the root. Swellings or deformities at the transition area should be noted. Roots can be harvested, dried to a constant weight, and weighed to the nearest 0.001 g as an optional endpoint. The percent inhibition of the treated seeds as compared to the controls is calculated, and an EC_{50} (the concentration that inhibits root growth to 50% of the control length) is calculated.

Whole-Plant Toxicity

Whole-plant toxicity is evaluated using a 5-day screening test (TOXSCREEN) conducted in hydroponic solutions [196] or a longer full life-cycle test using vascular plants in soils, such as *Brassica* [189].

Hydroponic Tests

TOXSCREEN uses whole plants such as soybean (*Glycine max*), barley (*Hordeum vulgare*), and woody perennials that have been grown in hydroponic culture for at least 28 days. Alternatively, known-age plants may be purchased commercially and adapted for hydroponic exposures [197]. Regardless, exposures occur hydroponically when young plants are transferred from nursery containers to containers filled with exposure medium. Geometrically spaced dilutions of the exposure medium or chemical concentrations within the exposure medium are used to develop a dose–response curve. Soil eluates may be used to fill exposure containers. Tests are conducted under a 16:8 L/D photoperiod (light intensity of 350 μmol/m²/s at the top of the canopy) at 25°C/21°C ± 2°C (light/dark) and relative humidity of 50%–70% for 5 days. Following the exposure period, survival, root and shoot growth, and total biomass of the plants are measured. Probit analysis is used to calculate LC_{50} values.

Woody Plant Tests

An alternative procedure to TOXSCREEN is available in the woody plant test protocol [189]. This is similar to the TOXCREEN test, but woody plants are grown in silica sand, a formulated soil, or a contaminated soil, rather than in a hydroponic solution. Plants are obtained from the field or a horticulture source, and the roots are washed gently by dipping the root mass into deionized water. Plants are kept moist and sorted according to size and stage of development, to begin the study with uniform-sized plants in similar stages of root and shoot growth. The starting conditions for all endpoints are measured and recorded. Plants are sorted randomly into treatment groups and placed in pots of sufficient dimension to contain test medium to a depth of approximately two times the root length of plants. Each replicate container should be planted with one test replicate; five replicates of each soil sample, sample dilution, and positive and negative control are required. Medium should be added to cover roots completely and to bring the level within 1–2 cm of the top of the container. Medium can be gently packed by hand but should not be compacted. Deionized water is used to bring the pots to WHC. A regular water application schedule should be followed thereafter for the duration of the test. Test duration should be approximately twice the length of time required to achieve amounts of shoot and root growth acceptable for statistical characterization. Tests are conducted under a 16:8 L/D photoperiod (light intensity of 100–200 μmol/m²/s) at 20°C–30°C and relative humidity of >50%. Measured endpoints include wet and dry shoot mass, number of new shoots or leaves, number of root initiation points, and changes in total plant weight. Additional observations on general plant condition and leaf or root malformations are also noted. Plants can be harvested, dried at 85°C until constant weight (approximately 24 h), and weighed to the nearest 0.01 g.

Full Life-Cycle Plant Test

The full life-cycle plant test evaluates the effect of test materials on germination, growth of shoots and roots, photosynthetic systems, flower development, and reproductive capabilities of plants. It is conducted using either *Arabidopsis thaliana* [195] or *Brassica rapa* [189,196] exposed to the toxic

substance for 25–36 or 36–44 days, respectively. Hydroponic exposures [198,199] occur in double-pot, static-replacement systems, in which a vermiculite-filled growth container (for *Arabidopsis*) or greenhouse potting soil (for *Brassica*) is nested above a second, larger pot that serves as a nutrient solution reservoir. Nutrients, toxics, and water move from the reservoir to the vermiculite or potting soil via polyester wicks that are draped between the two pots. Alternatively [189], *B. rapa* seeds can be planted directly into test soil, as described in the preceding text for root elongation or woody plant tests. Dilutions and eluates of test substances are made as described previously. Seeds are planted uniformly on the surface of the vermiculite or potting soil and incubated in the conditions described for TOXSCREEN and the woody plant test. Plants will germinate, grow, mature, and set seeds. Exposure to the test substance occurs from the time that seeds are planted and continues until the mature plant drops its own seeds. Observations include leaf and flower structure, total biomass, vegetative and reproductive biomass (e.g., stems and leaves vs. seeds and reproductive structures), foliar height, initial flowering date, time to flowering, stunting, chlorosis, and survival. Probit analysis is used to calculate EC_{50} values for each endpoint. ANOVA techniques can be used to determine which test concentrations produce effects significantly different from control values.

Soil-Core Microcosms

Soil-core microcosms [200] can be used to develop site-specific or regional information on the probable chemical fate and ecological effects in a soil system resulting from release or spillage of chemicals into the environment. This test is most useful in the assessment process after preliminary knowledge of the chemical properties and biological activity of the compound of interest has been obtained. The test is designed to determine impacts in agricultural or natural field soil ecosystems and may not be applicable to forest soils. Specifically, the test will determine the effect of a chemical on growth and reproduction of either natural grassland vegetation or crops, nutrient uptake and cycling, potential for bioaccumulation in the plant tissues, and potential for and rate of transport of the chemical through soil to groundwater.

Soil cores are extracted from the site or region of interest with a specially designed steel extraction tube. The steel tube surrounds an ultrahigh-molecular-weight, high-density, nonplasticized polyethylene pipe to prevent the tube from warping or splitting during extraction. The tube is 60 cm deep and 20 cm in diameter. In agricultural systems, the plowed topsoil is moved aside prior to coring and backfilled into the upper 20 cm of the tube after collection. For the natural grassland system, the vegetation is clipped before the core is extracted. The polyethylene tube containing the soil core is removed from the steel coring device and placed within a specially designed wheeled cart that holds six to eight tubes packed within insulating Styrofoam® beads. The tube sits on a Buchner funnel that is covered by a thin layer of glass wool and connected by polyvinylchloride (PVC) tubing to a flask for collection of leachates. For the natural ecosystem, the natural plant cover collected with the soil core is suitable for the test. For agricultural systems, a mixture of grasses and broadleaves (e.g., legumes) that are typically grown together in the region of interest should be planted in the soil cores. The seed application rate should duplicate standard farming practices. Microcosms should be watered with purified water on a predetermined regimen, usually established on the basis of site history. Microcosms are leached once before dosing and once every 2 or 3 weeks after dosing. Microcosms that take longer than 2 days to produce 100 mL of leachate are not used in the test. The microcosms in their insulated carts are kept in a greenhouse or environmental chamber where temperatures and photoperiods are set to simulate outdoor conditions during a typical growing season in the region of interest.

If information is available, only one concentration of the test chemical above that known to cause at least 50% change in plant growth or 50% change in bacterial growth or respiration needs to be tested. Generally, three concentrations are used, choosing one that produced a 20%–25% change in productivity. In any case, the lowest treatment level should not be less than 10 times greater than the analytical limits of detectability of the parent compound. A range-finding test using concentrations of 0.1, 1.0, 10, 100, and 1000 µg/g in the upper 20 cm of topsoil can be used to determine the appropriate dose for the definitive test. The range-finding test should last a minimum of 4 weeks, while the definitive test lasts for 12 weeks. For aqueous compounds, the test solution is mixed with water and applied as single or multiple (daily, weekly) exposures in sufficient volume to bring the microcosm to field capacity. In no case should the exposure volume be sufficient to cause leaching in any of the microcosms. Frequency of application should reflect the situation encountered in the region of interest. The use of carriers should be avoided. If the test substance does not mix with water, it should be applied evenly to the top of the unplanted microcosm and mixed into the topsoil prior to planting. If the test substance is normally sprayed on growing plants, then it should be sprayed on the plants with a nebulizer at the seedling stage. The number of replicate microcosms per dose is determined by the desired power of the statistical test and the variability between microcosms (determined in the range-finding test). Individual microcosms should be assigned to treatment groups in a randomized block design, with the cart as a blocking variable.

The parent compound should be radiolabeled with ^{14}C, either in an appropriate aromatic, cyclic carbon group or in a linear chain, to follow uptake and degradation rates. The following parameters are measured:

- Primary productivity, the total yield or yield of harvestable portion (e.g., grain), reported as oven-dried weight
- Physical appearance and abnormalities of plants
- Amounts of nutrients in leachate (e.g., calcium, potassium, nitrate–nitrogen, ammonium–nitrogen, *ortho*-phosphate, dissolved organic carbon)

- Amounts of parent compound in all plant parts, soil horizons, and leachate (by measuring radioactivity of parent compound)
- Amounts of chemical degradation products in plant material, soil, and leachate
- Soil properties, including pedologic identity (according to the USDA Seventh Approximation Soil Classification System), percent organic matter, hydraulic characteristics, cation exchange capacity, bulk density, macro- and micronutrient content, organic matter content, inorganic mineral contents, exchange capacity, particle-size distribution, and hydraulic characteristics

Data analysis should follow standard statistical techniques as appropriate, such as ANOVA for comparing biomass data or regression analysis on sequential measures of productivity. The 5% level should be considered as the level of significance with a power of 0.90 or 0.95.

AIR POLLUTION

Air pollution has the potential to seriously injure plants and animals. A good example of air-pollution effects is the change in the forest vegetation of the San Bernadino Mountains in southern California [201]. There, prolonged exposure to photochemical oxidants (smog) resulted in a shift from ozone-sensitive pine trees to more ozone-tolerant oaks and shrubs. Ozone injury in pines results in decreased photosynthesis due to foliar injury and premature needle fall, reduced nutrient retention in needles, and decreased growth. In general, gaseous pollutants have the potential to disrupt plant-leaf biochemical processes through absorption by stomata or cuticle, while trace metals and organochlorine compounds tend to accumulate in humus and organic matter. Animals are affected either by uptake through the food chain or direct exposure via inhalation [202]; however, there has been little direct measurement of toxicity to wildlife from air pollutants other than studies of the effects of smelter emissions and a documented reduction in small-mammal diversity in the ozone-impacted San Bernardino Mountains. Many species of freshwater animals have been affected by wet deposition of sulfur dioxides, also known as "acid rain." In general, fish are most sensitive to acidification, followed by invertebrates, algae, and microbes [202].

Certainly, the potential impact on biodiversity from global climate change is significant. The amount of production or the type of plant that will grow efficiently may change in response to increasing CO_2, changes in the amount and timing of precipitation, or to the amount of available solar radiation. CO_2 enrichment alone would increase the rate of plant growth, while higher temperatures would increase the rate of microbial decomposition of organic matter, adversely affecting soil fertility [203]. On the other hand, higher temperatures generally hasten plant maturity in annual species, shortening the growth stages of some plants. At the same time, midlatitude summer dryness is likely to reduce yields by 10%–30% [204].

Air-pollution levels generally are measured mechanically, through the use of biosensors or air-sampling devices in situ; however, as with chemical analysis of soils and other media, this type of monitoring does not provide information about the toxicity of air pollutants to terrestrial organisms. Lichens are particularly sensitive to low levels of some forms of air pollution. Some are killed by pollutant levels that are too low to cause visible injury to other plants; consequently, lichens are now used as qualitative indicators of air quality in parts of the United States and Europe, in particular for sulfur dioxide, hydrogen fluoride, acidic precipitation, ozone, nitrogen dioxide, heavy metals, and radioactive compounds [91]. Lichen species in the area of study are identified, and species diversity, density, and frequency are recorded, as is percent cover. Plants also are observed for obvious signs of damage. These parameters are compared with historical accounts of lichen communities to determine whether airborne pollutants have affected the plants. Additionally, some compounds (e.g., heavy metals, organochlorines) are bioaccumulated by lichens without visible signs of damage to the plants. Residue analysis of lichens has been used to map the location and distribution of these pollutants.

Studies conducted with terrestrial vertebrates suggest that some species may also constitute useful sentinels of air pollution. The common pigeon (*C. livia*) is abundant in many urban environments and has been used as a bioindicator of pollution [4,156]. Blood samples can be obtained from the birds with relative ease through jugular and brachial venipuncture, and the bird released unharmed. The blood sample subsequently is analyzed for the presence of airborne pollutants (e.g., lead). If the bird is euthanized, other more invasive measures yield more specific information (e.g., chemical concentrations in target organs).

ECOLOGICAL RISK ASSESSMENT

The toxicity tests described in this chapter ultimately become a component of the process of establishing the likelihood that adverse ecological effects are occurring or may occur as a result of exposure to one or more chemicals or other stressors. The process of ecological risk assessment has evolved rapidly in the 20 years since the USEPA issued their *Framework for Ecological Risk Assessment* in 1992 [205] and the supporting *Guidelines for Ecological Risk Assessment* in 1998 [131]. Many other countries in Europe and Australasia have adopted the same general approach for improving the quality, consistency, and applicability of assessments of the impact of environmental stressors on components of an ecosystem [176]. In 2007, the Registration, Evaluation, and Authorisation and Restriction of Chemicals (REACH) came into force in the EU, requiring a base data set on aquatic toxicity for all chemicals in commerce. This resulted in a significant increase in the amount of ecotoxicity testing being conducted, which has resulted in a large amount of new toxicological information.

Ecological risk assessment involves three stages in a continuous process: (1) problem formulation, (2) analysis of exposure and effects, and (3) risk characterization. Because

ecological risk assessment must consider effects at the population, community, and ecosystem levels, as well as to the individual species, and relevant assessment endpoints are not universally accepted, the process is generally more complex and protracted than are most human health risk assessments. Furthermore, ecological risk assessment frequently must consider the effect of mixtures of chemicals that interact in a complex chemical and physical environment that typically includes other environmental stressors. Many examples of comprehensive ecological risk assessments can be found in the scientific literature (see, e.g., [205]).

The ecological risk assessment process is illustrated in Figure 42.7. The first, and most critical, phase of the process is problem formulation. It is during this phase that the affected environmental resource and the stressors of concern must be considered within the context of the overall situation that is to be evaluated. It is frequently useful at this stage to involve risk assessors, risk managers, and stakeholders in discussions about how to proceed so the results of the assessment will be most useful to those who will have to use them. It is also worthwhile to spend some time modeling the problem, at least in a conceptual sense, so that assessment endpoints will be reasonable and achievable, and a sampling and analysis plan with appropriate measurement endpoints can be established. Here, pathways of exposure are determined for each receptor or guild, and a conceptual site model is developed. A quality assurance project plan should also be established at this stage.

During the analysis phase of the risk assessment, the exposure level or concentration of the stressor of concern on the environmental resource must be established, and the effects assessed. The effects assessment should include both potential toxicological effects, as described in this chapter, and ecological effects. Whereas toxicological effects are usually measured on individual organisms using a variety of standardized tests and are relatively easily quantified, ecological effects involve predictions about changes in populations and communities of organisms in longer-term studies, which are more difficult to conduct and interpret. Studies in microcosms, mesocosms, small ponds, and streams, as described earlier, would be most useful in this regard, but are not used routinely due to their complexity and cost, and the difficulty of interpreting the results. Food-web relationships may have to be elucidated and bioaccumulation effects determined. Finally, indirect effects of contaminants on the organisms of interest must be considered, such as changes in food availability, habitat structure, or predator abundance. The analysis of exposure and effects also must include consideration of

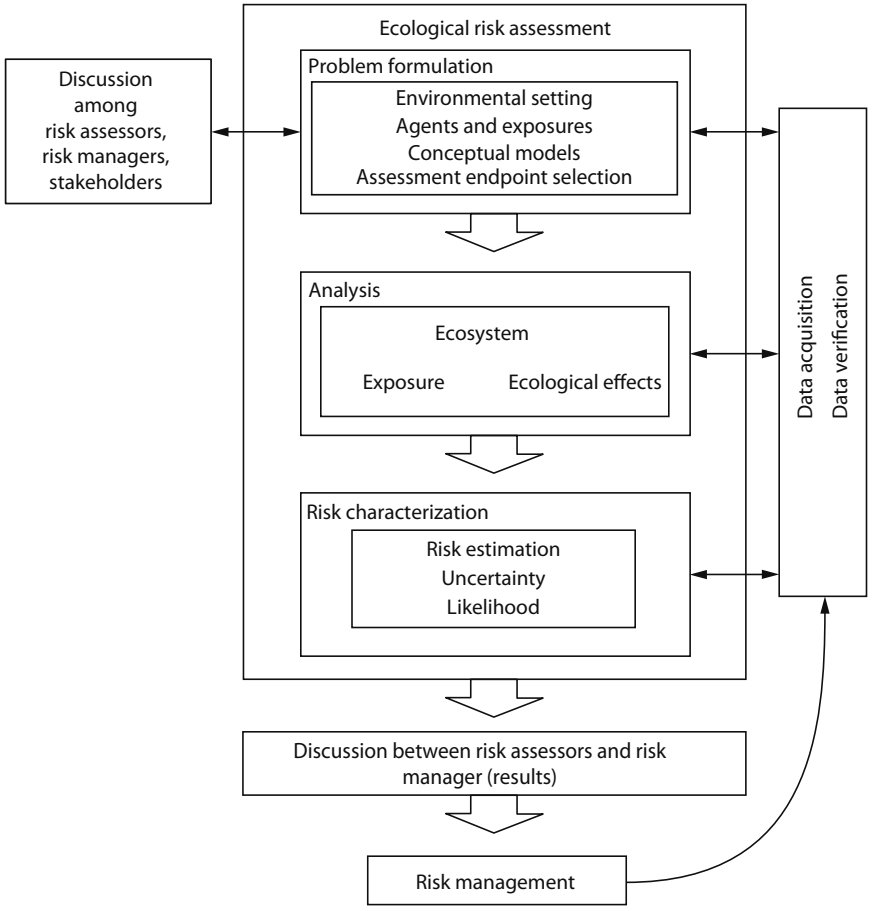

FIGURE 42.7 The ecological risk assessment process. (From USEPA, *Framework for Ecological Risk Assessment. EPA/630/R-92/001. Risk Assessment Forum,* Environmental Protection Agency, Washington, DC, 2002.)

fate and transport of the stressors. As described earlier, exposure is dependent on the bioavailability of the stressor, which depends on its chemical, physical, and environmental characteristics, and effects are entirely dependent on exposure; thus, knowledge of the physical and chemical behavior of stressors in the ecosystem is critical.

The final step in the risk assessment process is to integrate the exposure of the resource to the stressors with the observed or predicted effects within the context of the problem formulation, to estimate the degree of risk and the probability of adverse environmental changes actually being observed. Very simple systems in which statistical analyses can establish the confidence of the predicted result are rare in ecological risk assessment. The more usual cases involve a high degree of uncertainty, which in turn, requires the risk manager to establish the parameters within which regulation must be effected, frequently using a worst-case scenario. Obviously, the more quantitative and precise the assessment results presented to the risk manager, the more effective will be the management of the ecosystem. New tools that consider the heterogeneous nature of contaminated sites and the manner in which individual animals move about the area and come into contact with contaminated portions of their ranges provide a more accurate estimate of exposure and risk within a probabilistic framework [176,206]. In reality, as indicated in Figure 42.7, ecological risk assessment is a continuous, iterative process in which risk managers and risk assessors must interact in concert with stakeholders to strive for continuous improvement in understanding the ecosystem and providing for optimum functionality [13]. More detailed explanation of applications of the ecological risk assessment process can be found in Suter [207].

CONCLUSION

Clearly, toxicity testing under realistic environmental conditions is a much more complex enterprise than determining the effects of single chemicals on single species under controlled laboratory conditions; however, the information obtained in a well-designed, well-executed environmental study provides a valuable supplement to the toxicity data obtained in more traditional laboratory studies. Understanding the impacts of a pollutant within the environment, including the organisms that live in it, requires more complex evaluation and study. Interactions of organisms with each other and with their environment can be altered by the introduction of foreign chemicals. The impacts of such chemicals must be assessed in terms of these interactions, as well as the effect on individuals if the overall health of an ecosystem is to be properly evaluated and understood. This is the essential difference between ecotoxicology and the discipline of toxicology as it relates to human health. Toxicologists have developed rigorous methods for assessing the effects of chemicals on single species under controlled conditions; however, effects on ecosystems in which many species are involved are much more difficult to identify and measure quantitatively. Subtle changes in population-level effects are often variable and not discernible in the short term. Species diversity and functional redundancy provide resiliency within the community and greater ecosystem. Great strides have been made in the development of controlled ecosystems, in the form of microcosms and mesocosms, for this purpose. There is still much work to be done, however, to perfect the methodology to the extent that effects can truly be assessed at the ecosystem level. The truism that "the whole is greater than the sum of its parts" definitely applies.

Much progress has been made by the various environmental regulatory authorities in codifying procedures and requirements for the use of field-derived toxicology assessments for populations and communities, to evaluate the impacts of chemicals on the environment [208]. The guidelines include not only community-level toxicological data but also the requirement to understand the spatial and temporal scales of stress and the natural variability in biotic community dynamics. Ultimately, the extra costs in terms of time and money for conducting environmental toxicology evaluations and ecological risk assessments will be returned manyfold in our wiser use of natural resources and the protection of the environment in which we live.

QUESTIONS

42.1 What are the differences in experimental considerations and methods used to assess the toxicities of contaminants in surface water and sediment?

42.2 What predictive methods are available to compensate for the limitations in the chemical toxicity databases for aquatic flora and fauna? What are the advantages and disadvantages in the use of these methods?

42.3 What environmental factors affect the toxicity of anthropogenic chemicals to aquatic life? How do these physical and chemical factors differ between freshwater and saltwater ecosystems?

42.4 What methods are available for extrapolating toxicity thresholds from tested to nontested species?

42.5 What are the differences between acute, subacute, subchronic, and chronic avian toxicity studies?

42.6 What are the advantages or disadvantages in conducting field studies as compared to laboratory toxicity bioassays?

42.7 What is the real effect of ad libitum testing regimes for animals that are often food limited?

42.8 Why are no standardized rodent protocols described for ecotoxicity testing?

42.9 What is the importance of honeybee studies?

42.10 What ecological role do soil invertebrates play, and what are standard test species?

42.11 How do soil microbial assays differ from single-species toxicity tests?

42.12 Many amphibians have both an aquatic and a terrestrial life phase. What tests are available to determine the effects of soil and water contaminants on these species?

42.13 How do terrestrial plant bioassays differ from aquatic plant tests?

42.14 What are soil-core microcosms, and what is the advantage in using these types of studies?

42.15 How are terrestrial ecosystem effects of air pollution measured (including climate-induced changes from elevated CO_2)?

KEYWORDS

Ecotoxicology, Environmental, Aquatic toxicology, Terrestrial toxicology

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Glossary

50th percentile: The number in a distribution such that half the values in the distribution are greater than the number and half the values are less. The 50th percentile is equivalent to the median.

95th percentile: The number in a distribution such that 95% of the values in the distribution are less than or equal to the number and 5% are greater.

95% upper confidence limit (95% UCL) for mean: The 95% UCL for a mean is defined as a value that, when repeatedly calculated for randomly drawn subsets of size *n*, equals or exceeds the true population mean 95% of the time. Although the 95% UCL provides a conservative estimate of the mean, it should not be confused with a 95th percentile. As the sample size increases, the difference between the UCL for the mean and the true mean decreases while the 95th percentile of the distribution remains relatively unchanged at the upper end of the distribution. The Environmental Protection Agency's (EPA) Superfund program has traditionally used the 95% UCL for the mean as the concentration term in point estimates of the reasonable maximum exposure (RME) for human health risk assessment.

Absolute white blood cell and reticulocyte counts: Counts expressed as cell concentration (i.e., cells/volume of blood). Absolute counts, as opposed to relative counts (%), are preferred for interpretation and reporting.

Absorbed dose: Energy imparted to matter when radiation passes through; measured in grays or rads.

Absorption: Uptake of the chemical from the site of administration into the general circulation. Absorption may involve a number of stages (e.g., dissolution) and diffusion through membranes. Chemicals may be changed during absorption due to metabolism or degradation, such that it is possible to have complete absorption and low bioavailability.

Absorption barrier: Any of the exchange barriers of the body that allow differential diffusion of various substances across a boundary. Examples of absorption barriers are the skin, lung tissue, and gastrointestinal tract wall.

ACB: Accelerated cancer bioassay.

Acceptable daily intake (ADI): Daily intake of a chemical (e.g., food additive, pesticide) that, during the entire lifetime, appears to be without appreciable risk (affects 1 in 1 million people or less) on the basis of all known facts at the time.

Accessory cells: Cells that support T or B cells in the induction of an immune response. These cells usually express major histocompatibility complex (MHC) class II molecules.

Accuracy: A measure of the extent to which the mean estimate of a quantity approaches its true value: (1) the closeness of agreement between a test method result and an accepted reference value and (2) the proportion of correct outcomes of a test method. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *concordance* (see *Two-by-two table*). Accuracy is highly dependent on the prevalence of positives in the population being examined in the validation study.

Acid–base balance: Maintenance of pH; kidneys function in acid–base balance by the regulation of H^+, HCO_3^-, and NH_4^+ ions.

ACTH: Adrenocortical tropic hormone.

Action level: Level of unavoidable contaminants in foods and feeds considered as the upper limit of safety but which are not subjected to regulatory control.

Active ingredient: Chemical or substance component of a pesticide product that can kill, repel, attract, mitigate, or control a pest.

Acute: Characterized by a time period of short duration; commonly used to describe single-dose exposure in toxicity studies.

Acute toxicity: The adverse effects occurring within a short time of administration of a single dose of a substance or multiple doses given within 24 h.

Acute toxicity study: Usually, a single-dose study in which animals are observed for a 2-week period postdose to determine overt signs of toxicity, normally including some form of assessment of the lethal dose.

Acute-to-chronic ratio (ACR): A ratio determined experimentally or mathematically for a chemical that is used to predict chronic toxicity when only acute toxicity data are available.

Ad libitum: Available with unrestricted access; this term is commonly used in toxicity studies to describe free access by animals to feed or water.

Adenohypophysis: The anterior lobe of the pituitary gland.

ADH: Antidiuretic hormone or vasopressin; an octapeptide.

Adjunct test: A test that provides information that adds to or helps interpret the results of other tests and provides information useful for the risk assessment process.

Adjuvant: A material that enhances an immune response, it traditionally refers to a mixture of oil and mycobacterial cell fragments.

ADME: Absorption, distribution, metabolism, and excretion—the processes that determine the disposition and fate of an administered molecule.

Administered dose: The amount of a substance given to a test subject (human or animal) in determining dose–response relationships, especially through ingestion or inhalation. In exposure assessment, because exposure to chemicals is usually inadvertent, this quantity is called *potential dose*.

Advance access: The online publication of papers in manuscript form soon after they have been peer reviewed and deemed final; they appear considerably sooner than as print publications.

Adverse effect: A test compound–related effect (e.g., morphological, biochemical, developmental) that alters the function of an organ or system or alters the ability to respond to additional environmental challenges.

AEGL: Acute exposure guideline levels are values intended to provide estimates of concentrations and exposure durations (minutes to hours) above which one could reasonably anticipate observing adverse health effects. Compare to emergency response planning guidelines (ERPGs).

Aerodynamic equivalent diameter: The diameter of a spherical particle of unit density (1 g/mL) that has the same terminal settling velocity as the particle in question.

Aerosol: A suspension of either microscopic liquid or solid particles dispensed in a gas, the particles of which have a negligible falling velocity; also used to characterize a product or chemical form that contains particles that can enter the respiratory tract.

Aglycone: The xenobiotic substrate that is conjugated by glucuronosyltransferases.

Air elutriation: A process in which particles are separated on the basis of size by pitting their settling velocity against the velocity of a current of air with which they move.

Air shower: A device that uses high-velocity, ultrafiltered air to remove particulates from the surfaces of the clothing worn by personnel.

AL: Ad libitum.

Alkaloids: Nitrogenous heterocyclic compounds that protect plants from attack by microorganisms, pests, and herbivores. Any of a large, heterogeneous group of alkaline, bitter tasting, biologically active, usually water-insoluble, nitrogenous organic compounds produced by plants.

Allergic contact dermatitis: Chemically induced immunologic (delayed hypersensitivity) dermatitis.

Allergy: A state of altered immunity in which contact with an antigen (allergen) results in a hypersensitivity response.

Allogeneic: From a different genetic background. In the context of immunotoxicology, it generally refers to the use of genetically dissimilar cells in vitro assays to elicit a cell-mediated immune response.

Allometry: The study of the relationship between body size and various biological and physiological parameters, such as organ sizes, blood flow rates, and metabolic rates.

Alloxan: A chemical that can cause destruction to the pancreatic beta cell.

Alpha particle: Nucleus of a helium atom emitted by certain radioisotopes upon disintegration. Contains two protons and two neutrons.

Alveolar macrophage: A motile, phagocytic cell of the pulmonary region of the lung, essential to removal of particulate matter from the alveoli and sterility of alveolar surfaces.

Alveolus: The smallest functional gas-exchange unit of the pulmonary region of the lung. The alveolus contains a very thin epithelial and endothelial surface that allows the rapid exchange of oxygen and carbon dioxide, as well as a portal for the absorption and elimination of volatile substances from the vasculature.

Amatoxin: One of two groups of thermostable toxins isolated from poisonous species of *Amanita*; they are extremely toxic, bicyclic octapeptides that act on the RNA polymerase II system of eukaryotic cells.

American Conference of Government Industrial Hygienists (ACGIH): A professional organization made up of individuals in the industrial hygiene and occupational and environmental health and safety industry. ACGIH is best known for the development and publication of TLVs® and BEIs® as well as many foundation documents for the practice of industrial hygiene and occupational health.

American Industrial Hygiene Association (AIHA): Professional organization of environmental health and safety professionals practicing industrial hygiene in industry, government, labor, academic institutions, and independent organizations. AIHA develops and administers comprehensive education programs and publications that keep occupational and environmental health and safety professionals current in the field of industrial hygiene.

Ambient measurement: A measurement (usually of the concentration of a chemical or pollutant) taken in an ambient medium, normally with the intent of relating the measured value to the exposure of an organism that contacts that medium.

Amine precursor uptake and decarboxylation (APUD) cells: A group of apparently unrelated endocrine cells found throughout the body that have a number of similar characteristics and that make a number of hormones with similar structures (including serotonin, epinephrine, dopamine, neurotensin, and norepinephrine). Over 60 types of endocrine cells have been identified in the APUD system that can be found in the numerous organs throughout the body.

Ammonium molybdate: A metallic negative stain that is used to negative stain the Formvar backing on coated electron microscopic grids for transmission electron microscopy.

Amphibia: A class of anamniote tetrapods comprised of 2600 known species of toads, frogs, salamanders, and newts, many of which are poisonous but not necessary dangerous to humans; any life form adapted to or able to live in both aquatic and terrestrial environments.

Amyotrophic lateral sclerosis: Fatal neurologic disease characterized by progressive degeneration of upper and lower motor neurons in the brain and spinal cord.

Analytic study: A study designed to examine a priori hypothesized causal associations.

Anaphylaxis: An extreme, immediate immunologic reaction characterized by contraction of smooth muscle and dilation of capillaries due to the release of pharmacologically active substances (e.g., histamine) in response to administration of a foreign material; a local or systematic immediate hypersensitivity reaction resulting from the release of mediators following exposure to antigen. A life-threatening, often fatal response.

Androgens: Male sex steroids (e.g., testosterone).

Anemia: Reduction below normal of hemoglobin concentration; usually accompanied by a similar reduction of red blood cell count and hematocrit. Functionally, anemia is characterized by a decrease in red cell mass sufficient to cause reduced oxygen delivery to peripheral tissues.

Anemone: Any herb of the genus *Anemone*.

Anencephalus: Congenital absence of the upper part of the brain and the flat bones of the skull.

Anergy (tolerance): Unresponsiveness to antigenic stimulation; also referred to as *tolerance*.

Animal Care Program: "All activities conducted by and at an institution that have a direct impact on the well-being of animals, including animal and veterinary care, policies and procedures, personnel and program management and oversight, occupational health and safety, institutional animal care and use committee (IACUC) functions, and animal facility design and management."

Animal extremism: The practice of or belief in taking extreme action to promote beliefs regarding animal rights, including illegal activities that result in property damage, theft, and human injury.

Animal model: A living, nonhuman test system that models human biology or a disease state, due to similarities in taxonomy, physiology, etiology, or pathogenesis of disease. The model may be defined as an animal in its unaltered state, or by a method of preparation involving surgical, pharmacologic, genetic, or behavioral manipulation.

Animal rights: The belief that nonhuman animals have moral rights that are equal in consideration to humans. These beliefs may be practiced personally through lifestyle decisions, promoted publically, politically, or through extremism activity, or enacted through the legal system or legislation.

Animal welfare: The belief that animals should be provided with living conditions that provide for their physical and behavioral needs and that pain or distress should be avoided unless there is social or scientific justification for performing activities that are not in the animals' interest.

Animal Welfare Regulations: Laws that define requirements for the care and use of animals in research, teaching, testing, exhibition, breeding, commercial use, and transportation.

Animal Welfare Standards: Legal requirements or published guidelines that are not legally binding, but represent industry best practices to promote and ensure animal welfare.

Annexin V-FITC staining: Method used to assay apoptosis or necrosis. Annexin V labeled with fluorescein isothiocyanate (FITC) binds to phosphatidylserine residues of cellular membranes; cell populations with differential binding and, hence, fluorescence are detected by flow cytometry.

Anogenital distance (AGD): A primary landmark of sexual development, typically measured at birth, reflecting the linear distance between the genital tubercle and the anus. AGD is sexually dimorphic, being greater for males than females, and androgen dependent. The AGD in male rodents is decreased by development exposure to antiandrogens.

ANOVA: Analysis of variance.

Antibody: Complex molecules produced by plasma cells that recognize specific antigens. Antibodies, also called *immunoglobulins* (Ig), consist of two basic units. The antigen-binding section (Fab) contains variable regions with coding for antigen recognition. In mammals, the constant region of the molecule (Fc) may be grouped into several classes, designated IgA, IgD, IgE, IgG, and IgM, depending on the function of the molecule. Cross-linking of antibody molecules on the surface of a target leads to activation of complement, usually resulting in the destruction of the target.

Antibody-forming cell (AFC)/plaque-forming cell (PFC) assay: An assay that measures the ability of animals to produce specific antibodies against a T-dependent or T-independent antigen following in vivo sensitization. Due to the involvement of multiple cell populations in mounting an antibody response, the AFC assay actually evaluates several immune parameters simultaneously. It is considered to be one of the most sensitive indicator systems for immunotoxicology studies.

Antigen: A molecule that is the subject of a specific immune reaction. Antigens are recognized in a cognate fashion by the T-cell antigen receptor, the B-cell antigen receptor, or immunoglobulins (antibodies). Antigens generally are proteinaceous in nature.

Antigen-presenting cell (APC): Cells that are responsible for making antigens accessible to immune effector and regulatory cells. Following internalization and degradation of the antigen (generally by phagocytosis), a fragment of the antigen molecules is presented on the APC cell surface in association with a major histocompatibility complex (MHC) molecule. This complex is recognized by either B cells via surface-bound immunoglobulin molecule or by T cells via the T-cell antigen receptor. Induction of a specific immune response then proceeds. APCs include macrophages, dendritic cells, and certain B cells.

Antigenicity (immunogenicity): The property of eliciting an immune response, characterized by an interaction of a foreign material with endogenous antibodies or immune cells, in a subject that has been previously exposed (sensitized) to that foreign material.

Anti-Mullerian hormone (also called *Mullerian Inhibiting Substance*): A protein produced by fetal Sertoli cells that prevents formation of a female reproductive tract in male fetuses.

Aortic perfusion: Aorta is cannulated for inflow of the perfusate through the heart and the perfusate exits through the left atrium.

Aplastic anemia: Failure of blood cell production resulting from direct injury to pluripotent hematopoietic stem cells or their stromal microenvironment and characterized by varying degrees of pancytopenia (i.e., decreased erythrocytes, leukocytes [primarily neutrophils], and platelets) and hypocellular bone marrow.

Apoptosis: A series of biochemical events characterized by activation of a series of caspase enzymes that lead to the digestion of cellular DNA with the appearance of DNA laddering on agarose gels and morphological formation of intranuclear clumps in affected cells. This process is also known as *programmed cell death*, whereby cells die in a controlled, progressive manner that is regulated in part by the release of mitochondrial-initiating factors and cytochrome c. Programmed cell death is a normal cellular process for the removal of unneeded cells during organogenesis (e.g., cell death and replacement) that is accelerated by a number of toxic agents, including chemotherapeutic drugs. Thus, apoptosis is a single-cell phenomenon that is energy dependent and tightly regulated and generally occurs at lower doses of many toxicants and is important in morphogenesis and development. It is a genetically programmed form of cell death, distinct from necrosis, which is accidental cell death. Apoptosis helps regulate animal cell populations by eliminating cells that have been overproduced or mutated. The process is complementary to mitosis but opposite in function.

Apoptosis-inducing factor (AIF): A pro-apoptotic protein that when released from the mitochondrion stimulates activation of caspase 3.

Apparent volume of distribution: The volume of plasma (or blood) into which the body load appears to have been dissolved or distributed; equivalent to the body load, at any time, divided by the corresponding plasma (or blood) concentration. It is not a physiological volume but is important, as it indicates the volume of plasma that has to be cleared of chemical; independent of concentration and dose under first-order conditions.

Applied dose: The amount of a substance in contact with the primary absorption boundaries of an organism (e.g., skin, lung, gastrointestinal tract) and available for absorption.

Aquatic toxicology: The study of adverse effects on freshwater and saltwater biota and on the ecosystems that contain them.

Arachnida: A large class of invertebrates comprised chiefly of predaceous terrestrial forms such as scorpions, spiders, harvestmen, mites, ticks, and related forms; they are characterized by a cephalothorax that bears four pairs of walking appendages.

Arithmetic mean: The sum of all the measurements in a dataset divided by the number of measurements in the dataset.

Aspect ratio: Usually applied to fibers; the ratio of length to width.

Atherosclerosis: Nodular sclerosis characterized by irregularly distributed lipid deposits in the intima of the large and medium-sized arteries; such deposits are associated with fibrosis and calcification.

Atopy: General systemic or local hypersensitivity (i.e., allergy), often related to genetic predisposition; may be thought of as *unwanted reactivity.*

ATP-binding cassette (ABC) transporters*:* ABC transporters are transmembrane proteins that utilize the energy of ATP hydrolysis to translocate various substrates across cell membranes. This process can occur against a considerable concentration gradient. ABC transporters include P-glycoprotein (P-gp), the breast cancer resistance protein (BCRP), and the family of multidrug resistance–associated proteins (MRPs).

Atrial perfusion: Right atrium is cannulated for inflow of the perfusate, which exits through the right ventricle via pulmonary arterial cannula or an open slit.

AUC: Area under the plasma concentration time course.

Autoimmunity: Immune reactivity toward self.

Autologous blood perfusate: Blood used as perfusate comes from the same animal from which the organ was removed for perfusion.

Autolysis: Enzymatic self-digestion of cells or tissues that occurs after death. Autolysis complicates detection of pathologic changes in tissues or organs during necropsy and subsequent microscopic examination and can make valid observations during these activities impossible.

Autoradiography: The production of an image by the emission of radioactive decay energy from a radionuclide. It provides a qualitative visual image of the tissue distribution of a xenobiotic and can provide quantitative distribution data as well. Quantitative whole-body autoradiography (QWBA) is becoming a standard tool for absorption, distribution, metabolism, and excretion (ADME) studies.

Autosome: A chromosome that is not a sex-determining chromosome.

Azotemia: Accumulation of nitrogenous wastes such as urea or creatinine in the blood.

Background exposure: Exposures that are not related to the site—for example, exposure to chemicals at a different time or from locations other than the exposure unit of concern. Background sources may be either naturally occurring or anthropogenic (man-made).

Background level (environmental): The concentration of substance in a defined control area during a fixed period of time before, during, or after a data-gathering operation.

Base pair substitution: A gene mutation characterized by the replacement of one nucleotide pair for another in a codon.

B-Cell antigen receptor: A membrane-bound molecular complex responsible for antigen recognition by B cells. It comprises membrane immunoglobulin (mIg) and several accessory molecules. Functionally analogous, but structurally dissimilar, to the T-cell antigen receptor.

B Cell/B lymphocyte: Lymphocytes that recognize antigen via surface-bound immunoglobulins. B cells that have been exposed to specific antigen differentiate into plasma cells that are responsible for producing specific antibodies. B cells differentiate in the bone marrow in mammals and in an organ known as the bursa in birds.

Becquerel (bq): SI unit of radioactivity equaling one disintegration/s, approximately 2.7×10^{-11} curies (Ci).

Beer–Lambert law: This law relates solute concentration and cell path length to ultraviolet absorbance. The law can be expressed by the following equation:

$$A = \log \frac{I_o}{I_t} = abc$$

where

A is the absorbance
I_o is the original intensity incident on the cell
I_t is the reduced intensity transmitted from the cell
a is a proportionality constant (the absorptivity)
b is the path length of the solution
c is the concentration of the analyte

When c is in moles per liter, the constant is the molar absorptivity or molar extinction coefficient. When c is a 1% solution and b is a 1 cm path length, the term *specific absorptivity* is used (1%/1 cm is the most common form used).

Behavioral teratology: The functional deficits arising from exposure to neurotoxic agents during early development.

Benchmark dose (BD): The lower confidence limit on a dose associated with a specified level of response; a dose corresponding to a specified level of risk, generally in the range of 1%–10%.

Beta cells: Insulin-secreting cells of the endocrine pancreas.

Bias: Systemic error as opposed to a sampling error; for example, selection bias may occur when each member of the population does not have an equal chance of being selected for the sample.

Bifurcation: Usually related to airway anatomy, describing a branching of the parent airway into two or more smaller airways, often at acute angles.

Bile salt export pump (BSEP): An ATP-dependent efflux transporter expressed on the canalicular membrane of hepatocytes. BSEP drives and maintains the enterohepatic circulation of bile salts, and is the rate-limiting step of hepatocellular bile salt secretion. Mutations in BSEP or inhibition of its function by xenobiotics have been associated with liver disease or injury.

Bioaccumulation: The net uptake of chemicals from the environment from all sources.

Bioactivation: The enzymatic conversion of a chemical to a more toxic form (in the body or in vitro by an enzyme as a model of a process in the body).

Bioassay: A functional assay that depends on the use of living cells or cell components as an indicator system.

Bioaccumulation: General term that describes the net accumulation of a substance within the tissue of an organism, assuming steady-state conditions.

Bioaccumulation factor (BAF): The ratio of chemical concentration in an organism of interest to the concentration in its exposure medium.

Bioactivation: Enzymatic conversion of a less toxic or reactive compound to a more toxic or reactive compound. Xenobiotics may react covalently with endogenous molecules following bioactivation, sometimes resulting in toxicity to the organism.

Bioavailability: The fraction (or sometimes percentage) of the administered dose that enters the general circulation as the parent compound. A low bioavailability may be due to poor absorption or first-order conditions; the state of being capable of being absorbed and available to interact with the metabolic processes of an organism. Bioavailability is typically a function of chemical properties, physical state of the material to which an organism is exposed, and the ability of the individual organism to physiologically take up the chemical.

Bioconcentration: (1) The accumulation of a substance that partitions from water to an organism, assuming steady-state conditions. Often used to describe body burdens in aquatic organisms, can also be used for terrestrial organisms that experience exposure through soil pore water transfer. (2) The uptake of chemicals from water alone.

Bioconcentration factor (BCF): The tendency of a chemical to be more concentrated in an aquatic organism than the concentration in its environment, calculated by dividing the concentration of the chemical in the organism (wet weight) by the concentration of the chemical in the water.

Biocontainment: The process and equipment used for the purpose of preventing the unwanted release of hazardous material or organisms.

Biodegradation: The process of microbial transformation of a substance in the environment.

Biodegradation: The breakdown of chemicals in organisms or the environment, primarily by microorganisms.

Biodiversity: The variety of organisms considered at all levels, from genetic variants belonging to the same species through arrays of species to arrays of genera, families, and higher taxa; includes the variety of ecosystems that comprise both the communities of organisms within particular habitats and the physical conditions under which they live.

Bioexclusion: The process of preventing the introduction of unwanted microorganisms into animals or their immediate environment.

Biologically based dose–response model: A mathematical expression of the relationship between the incidence of severity of a biological effect and a dose that is based on the biological mechanism or mode of action.

Biologically effective dose: The amount of a deposited or absorbed chemical that reaches the cells or target site where an adverse effect occurs or where that chemical interacts with a membrane surface.

Biomagnification: (1) The increase in tissue contaminant concentration in higher trophic levels as a result of dietary accumulation. (2) The increasing concentration of a substance in organism tissues at successively higher trophic levels that occurs through trophic transfer, that is, ingestion of food items.

Biomarker: Observable change (not necessarily pathological) in the function of an organism, related to a specific exposure or event; a biochemical, genetic, or molecular indicator that can be used to screen disease or toxicity; parameters that can be used as an indicator of exposure, effect, or susceptibility and may be a metabolite, enzyme, or cell surface marker, among others.

Biotransformation: The biochemical modification of a xenobiotic once it enters an organism. Chemical modification can be enzymatic or nonenzymatic and may result in either reduced or increased toxicity. This process generally gives rise to compounds that are more readily excreted in the urine and feces and thus serves as a detoxification process; however, some xenobiotics are activated to more toxic metabolites by these enzymatic conversions.

Birth defect/congenital malformation: An abnormality identified in utero or within the first 2 years postnatal (i.e., death, growth or functional retardation, or alteration or dysmorphogenesis).

Blackfoot disease: A condition caused by long-term exposure to arsenic. The condition was first noted in Taiwan in regions containing high levels of arsenic in drinking water. The condition is characterized by poor circulation, leading to distal gangrene of the foot and other extremities.

Blood: A complex tissue composed of plasma and cellular elements with many different functions; the circulating tissue of the body; the fluid and its suspended formed elements that are circulated through the heart, arteries, capillaries, and veins; the means by which oxygen and nutrient materials are transported to the tissues and carbon dioxide and various metabolic products are removed for excretion.

Body burden: The amount of a particular chemical stored in a body at a particular time, especially a potentially toxic chemical in the body as a result of exposure. Body burdens can be the result of long-term or short-term storage—for example, the amount of a metal in bone, the amount of a lipophilic substance such as polychlorinated biphenyl (PCB) in adipose tissue, or the amount of carbon monoxide (as carboxyhemoglobin) in the blood; amount of radioactive material present in a human or animal.

Bolus dose: A quantity of test material administered all at once. This term is commonly applied to the single daily administration of a test material by oral gavage in toxicity studies.

Bond stretching frequency: This frequency is related to the masses of the two atoms that form the bond (M_a and M_b, in grams), the velocity of light (c), and the force constant of the bond (k, in dyn/cm). The frequency can be expressed approximately as

$$v\,(\text{in cm}^{-1}) = \frac{1}{2\pi c}\sqrt{\frac{k}{M_a M_b/\left(M_a + M_b\right)}}$$

The value of k is unique for a specific bond type (e.g., sp^3, sp^2, and sp bond have values of 5, 10, and 15×10^5 dyn/cm, respectively).

Bootstrap: A method of sampling actual data at random, with replacement, to derive an estimate of a population parameter such as the arithmetic mean or the standard error of the mean. The sample size of each bootstrap sample is equal to the sample size of the original dataset. Both parametric and nonparametric bootstrap methods have been developed.

Botulism: Fatal paralytic disease resulting from the consumption of food containing preformed toxin from *Clostridium botulinum*.

Bounding estimate: An estimate of exposure, dose, or risk that is higher than that incurred by the person in the population with the highest exposure, dose, or risk. Bounding estimates are useful in developing statements that exposures, doses, or risks are *not greater than* the estimated value.

Bowman's membrane: An acellular layer of collagen and ground substance that provides a functional interface between the stroma and epithelium of the cornea.

Bufotenin: A very toxic, water-insoluble genin and serotonin derivative that is synthetically produced and is found in certain toads and in very small amounts in the usual edible mushroom.

Bursa of fabricius: A structure located in the cloaca of avians, where bone marrow–derived lymphocytes mature into immunocompetent B cells prior to moving to the peripheral lymphoid organs.

***Caenorhabditis elegans*:** *C. elegans,* a nematode or roundworm, was the first animal to have its genome completely sequenced and all genes fully characterized.

Capture velocity: Air velocity at any point in front of the hood or at the hood opening necessary to overcome opposing air currents and to capture the contaminated air at that point by causing it to flow into the hood.

Cascade impactor: Instrument used to collect and sort aerosols onto discrete stages by separating the particles according to their aerodynamic size.

Case-control study: A study in which the past histories of those with a specific disease (the case) are compared with those who do not have the disease (the controls). The measure of association is the odds ratio (i.e., the odds of the cases having had some type of exposure compared to the odds of the controls having had that same exposure). In the context of exploratory data analysis, the case-control approach has sometimes been called *a disease in search of an exposure.*

Case reports and case series: A description of a single individual or group of individuals with the same or similar disease. This type of work lacks controls; therefore, any conclusions derived from such anecdotal information must be viewed with caution. Nonetheless, case reports and case series sometimes are useful for generating hypotheses.

Cause of death: The disease or injury that initiated the train of events leading directly to death, or the circumstances of an accident or violence that produced the fatal injury.

CBG: Corticosteroid-binding globulin.

CBI: Chemical-binding index.

cDNA: Complementary DNA enzymatically synthesized as a copy of mRNA.

Cell-mediated immunity (CMI): Antigen-specific immune reactivity mediated primarily by T lymphocytes. CMI may be expressed as immune regulatory activity (primarily mediated by CD4+ T-helper cells) or immune effector activity (mediated largely by CD8+ T-cytotoxic cells). Other forms of direct cellular activity (e.g., natural killer [NK] cells, macrophages) are generally not antigen specific (i.e., nonimmune) and are more accurately described as natural immunity.

Central tendency exposure (CTE): A risk representing the average or typical individual in the population, usually considered to be the arithmetic mean or median of the risk distribution.

Centrilobular cells: Collection of hepatic cells situated around the terminal hepatic venule (or central vein).

Centromere: Constriction along the length of a chromosome.

Chelating agent: An organic compound that forms multiple coordinate covalent bonds with metal ions yielding stable compounds that can be excreted. Chelating agents are used in the treatment of metal toxicity. An example is dimercaptopropanol (British Anti-Lewisite) to treat arsenic poisoning.

Chemical shift: The frequency at which a given nucleus absorbs in a nuclear magnetic resonance (NMR) spectrum. The precession frequency (v) for a given nucleus depends on its chemical environment and the shift in v from the standard value is given by the fundamental NMR equation:

$$v = \frac{\gamma_o^{btn}}{2\pi}$$

is called the *chemical shift* for that nucleus. Chemical shifts are rarely (if ever) expressed in absolute frequency units; instead, they are measured relative to ^{13}C a standard reference compound. For ^{1}H and NMR, the universally accepted reference is tetramethylsilane (TMS). The protons and carbons of TMS absorb at a lower frequency (are more shielded) than those of almost all other organic compounds, so their chemical shifts are arbitrarily set to 0 Hz, and most other chemical shifts measured relative to them are positive. Chemical shifts are expressed in dimensionless units designated δ. The chemical shift in δ units is defined according to the following relationship:

$$\delta = \frac{v_s v_{std}}{\text{Operating frequency}} \times 10^6 \text{ ppm}$$

Chemokine: Small peptide molecules related to cytokines and associated with a variety of physiological states, such as inflammation and immunoregulation.

Chemotaxis: Directed movement of cells through a concentration gradient of an attractant molecule, such as a chemokine.

Chilopoda: A class of about 2000 species of nocturnal, predatory, terrestrial arthropods that includes centipedes; they superficially resemble millipedes.

Cholestasis: Diminution or cessation of bile flow, accompanied by decreased excretion and enhanced retention of normal constituents found in bile.

Chondrichthyes: The vertebrae class comprised of cartilaginous fishes, including sharks, rays, skates, and chimaeras; there are about 300 species, some of which are venomous.

Chromatography: A process for separation of molecules on the basis of their affinities for a stationary phase and a mobile phase.

Chromophore: A structural moiety in an organic molecule that absorbs light in the useful part of the ultraviolet spectrum. Common chromophores are aromatic moieties and conjugated double-bond moieties.

Chromophoric resonance Raman label: These labels provide detailed vibrational and electronic spectral data when they are incorporated into the vicinity of a biologically important site. They usually are designed to mimic natural biochemical components and are themselves biologically active compounds. These labels are useful for obtaining information on protein–ligand interactions and have been utilized for studying enzyme–substrate complexes, where vibrational spectra of the substrate during enzyme catalysis can be obtained.

Chromosome: Microscopically visible organelle composed of DNA and proteins.

Chronic: Characterized by a time period of long duration; commonly used to describe long-term (6–12 months) exposure in toxicity studies.

Chronic toxicity study: A multiple-dose study in which animals are treated for ≥6 months to comprehensively assess the potential toxicological effects of a compound following long-term exposure; normally, these studies are required prior to phase II testing in humans.

Chylomicrons: A class of large lipoprotein structures that are created by the cells of the lumen of the small intestine and transport dietary fat by exocytosis from the small intestine to the lymphatic system, where they are eventually removed by lipoprotein lipase.

Ciguatoxins: A group of colorless and heat-stable lipophilic polyether neurotoxins produced by 300–400 tropical reef and semipelagic marine animals.

Cilia: Flexible, microscopic projections from cell surfaces that participate in the active movement of overlying mucous; effective rhythmic ciliary motion is essential for normal particle clearance of deposited particles from airway surfaces.

Clastogen: An agent that causes chromosomal breakage.

Clastogenicity: Chromosome breakage and/or rearrangements.

Clearance: The volume of plasma (or blood) that is cleared of chemical per unit time; equivalent to the rate of elimination, at any time, divided by the corresponding plasma (or blood) concentration. Clearance may be dependent on the blood flow and/or the metabolic activity or extraction ratio (at steady state) of the organ(s) elimination; independent of concentration and dose under first-order conditions. The volume of plasma from which a compound is completely removed by the kidneys per unit time.

Cleft phallus: See *Hypospadia*.

Clinical assays of renal function: Assays that can be performed in humans; typically involve measurement of parameters in blood (serum and plasma) or urine; usually noninvasive.

Clinical pathology: A subspecialty of pathology that uses laboratory methods such as hematology and clinical chemistry to detect, define, or corroborate a disease state.

Cluster of differentiation (CD): The CD series is used to denote cell surface markers (e.g., CD4, CD8). These markers, used experimentally as a means of identifying cell types, serve various physiological roles.

C_{max}: Maximum achieved concentration.

Cnidaria: A large family of venomous marine invertebrates of the Indo-Pacific region often found in coral reefs.

COD: Caloric optimization diet.

Coded chemicals: Chemicals labeled by code rather than names so they can be tested and evaluated without knowledge of their identity or anticipation of test results. Coded chemicals are used to avoid intentional or unintentional bias when evaluating laboratory or test method performance.

Coded microscopic evaluation: The practice of conducting the initial histopathological evaluation with the pathologist having no knowledge of treatment status of individual animals.

Coding regions: Those parts of the DNA that contain the information required to form proteins. Other parts of the DNA may have noncoding functions (e.g., start–stop, pointing, or timer functions) or as yet unresolved functions or perhaps even *noise*.

Codon: A DNA base pair triplet coding for an amino acid or stop signal.

Coefficient of inbreeding: Refers to a mathematical relationship used to express the relatedness and is expressed in mathematical values ranging between 0 and 1.

Cohort study: This type of epidemiology study is conceptually quite similar to the approach used in most toxicology experiments. The health experience (incidence of disease or mortality) of those exposed to some agent is compared with that of a group not so exposed. In epidemiology, however, the results usually are presented in terms of a relative risk or standardized morbidity (or mortality) ratio. In the context of exploratory data analysis, the cohort approach also has been called *an exposure in search of a disease*.

Collision-induced dissociation: A method in which ions are collided with neutral molecules to produce fragmentation. This process is also referred to as *MS/MS*, as a mass spectrum is produced on an ion selected from a mass spectrum.

Colubridae: The largest and most cosmopolitan family of snakes, comprised of more than 1,700 of the known species of snakes, most of which are nonvenomous; both jaws hold solid or grooved teeth, but no enlarged or hollow fangs, and in most cases, the head is wider than the neck.

Commensal: An organism commonly found in association with animals or their environment that under normal circumstances does not produce disease.

Complement: A group of approximately 20 proteinase precursors that interact in a cascading fashion. Following activation, the various precursors interact to form a complex that eventually leads to osmotic lysis of a target cell.

Computational toxicology: The application of mathematical and computer models to predict adverse effects and to better understand the mechanisms through which a given chemical induces harm (U.S. Environmental Protection Agency definition).

Conceptus: An embryo or fetus.

Conditioned response: Response to an originally neutral stimulus, such as a sound or light, that has acquired the ability to evoke the response because it was paired with an eliciting stimulus.

Confidence interval: A range of values (above, below, or above and below) about the midpoint of the sample (e.g., the mean, median, mode) that contains (with a specified level of probability, such as 95%, or a standard deviation or error, such as 67%) the true value of the population midpoint. The 95% confidence interval (also called the *fiducial limit*) is equivalent to the $p = 0.05$ region boundary.

Confidence limit: A statistical estimate that considers the influence of experimental variation of a parameter.

Confocal microscope: An instrument capable of producing high-resolution microscopic images that can be used to study ocular tissues. A confocal microscope with scanning capability can be used to determine area and depth of corneal injury.

Confounding variable, confounder: A confounder is an alternative cause for the disease in question that is unequally distributed among those exposed and unexposed to the putative agent of interest. As a consequence, it can confound or confuse the measure of association and any resulting interpretations of cause and effect.

Congenital: Present at birth.

Coniine: A highly toxic liquid alkaloid and derivative of pyridine; it is the chief toxic agent of poison hemlock.

Conjugation: A common mechanism during phase II metabolism where an endogenous compound is added to specific functional groups of a xenobiotic; generally, this increases the excretion of the xenobiotic and decreases its potential to interact at critical sites to produce toxicity.

Conjugation reaction: A metabolic process that combines a xenobiotic having an appropriate functional group with an endogenous substrate (e.g., glucuronic acid, glutathione, sulfonic acid) to yield a product having increased water solubility compared to the parent compound facilitating urinary or biliary elimination. Often the added chemical group is recognized by specific carrier proteins.

Conjunctiva: The delicate membrane that lines the eyelid and covers the exposed surface of the eyeball. Histologically, the conjunctiva is an aqueous nonkeratinized epithelium with numerous mucous-secreting cells. In the Draize eye test, effects to the conjunctiva represent a maximum of 20 out of 110 total points.

Coprophagy: The process of eating one's own feces.

Cornea: The transparent outermost covering of the anterior portion of the eye consisting of the epithelium, the stroma, and the endothelium. In the Draize eye test, effects to the cornea represent a maximum of 80 out of 110 total points.

Correlation: The relationship or interdependence between measurable varieties or ranks—that is, the extent to which, as one set of values changes, another set also changes in the same (positive correlation) or an opposite (negative correlation) direction.

Cortical collecting duct: Originates at convergence of two initial collecting tubules and extends to the corticomedullary border; nephron segment responsible for regulating the final composition of urine.

Cosmic rays: Radiation of many sorts, mostly nuclei (protons) with very high energies, originating outside the Earth's atmosphere.

Coupling constant: The separation between nuclear magnetic resonance (NMR) spectral lines due to coupling is the *coupling constant* (J). The magnitude of coupling constants depends on the number of intervening bonds and the bond geometry. Coupling constants are expressed in units of hertz (Hz).

Critical temperature: Maximum temperature at which a gas may be liquefied by application of pressure alone. Above this temperature, the substance may only exist as a gas. The critical temperature for CO_2 is 31°C.

Cross-sectional study: A prevalence study (i.e., an epidemiology study) that examines the association between health status and other variables of interest as they exist in a defined population at one particular time. This type of research can also be useful for generating etiologic hypotheses, but these hypotheses then need to be tested in analytic studies. Conceptually, it is also the first step of the more rigorous cohort method.

Cumulative distribution function (CDF): A representation, generally a function or graph, of the cumulative probability of occurrence for a random independent variable. The CDF is obtained from the probability density function (PDF) by integration in the case of a continuous random variable and by summation for discrete random variable. Each value c of the function is the probability that a random observation x will be less than or equal to c.

Curie: Standard measure of rate of radioactive decay; based on the disintegration of 1 g of radium, or 3.7×10^{10} disintegrations/s.

Cyclone separator: A process in which particle-laden air is introduced radially into the upper portion of a cylinder so it makes several revolutions inside the cylinder. The particles in the air are accelerated outward to the cylinder walls, where they either stick and are retained (low particle loading) or are swirled down to a collection port at the bottom of the cylinder (high particle loading).

Cyclopeptides: Group of toxins, produced by the *Amanita* and *Galerina* species of mushrooms, that inhibit mammalian nuclear RNA polymerase.

Cytochrome c: A major component of the mitochondrial electron transport chain that is localized in the intermitochondrial membrane space. Increased release of this protein into the cytosol is one of the major factors activating the caspase system resulting in apoptosis.

Cytochrome P450: Heme thiolate proteins associated with the endoplasmic reticulum originally named for their absorption maximum at 450 nm. These proteins, which serve as catalysts in a variety of oxidative reactions involving both endogenous and xenobiotic lipophilic compounds, are unique in their multiplicity of isozymes, substrates, reactions, and regulatory mechanisms. This family of heme-containing enzymes is involved in the so-called phase I metabolism of xenobiotics and endogenous compounds and catalyzes myriad reactions, including oxidation, reduction, dealkylation, and hydrolysis.

Cytokine: Small peptide molecules that subserve a wide range of regulatory and effector mechanisms. These include interleukins, tumor necrosis factors, interferons, colony-stimulating factors, and other growth and regulatory factors. Often referred to as *lymphokines* in the older literature.

Cytotoxic T lymphocyte (CTL): A subset of T lymphocytes bearing the CD3/CD8 surface markers; CTLs are able to kill target cells following induction of a specific immune response. The mechanism of this lysis appears to be a combination of direct lysis resulting from the extrusion of lytic granules by CTLs, as well as the induction of apoptosis in the target cell. The target cells most frequently used for assessment of CTL activity are virally infected cells and tumor cells. Measurement of CTL activity provides an indication of cell-mediated immunity.

Datum: A single point of measurement; more than one point is referred to as *data*.

Default value: A conservative value used for a model parameter or uncertainty (safety) factor when data are inadequate to justify a different value.

Definitive test: A toxicity determination designed to provide an accurate quantitative result with low variability; a second-level test conducted following pretests or range-finding tests.

Delayed skeletal ossification: Developmental delay in bone formation.

Delayed-type hypersensitivity (DTH): A form of cell-mediated immunity in which recalled exposure to an antigen results in an inflammatory reaction mediated by T lymphocytes; usually referred to as *contact hypersensitivity.*

Deposition: The process whereby the amount and location of matter (vapor, gas, or solid) is absorbed into or onto a surface (usually described as the amount per unit area during a specified time).

Descemet's membrane: An acellular layer that lies beneath the stroma and forms the basement membrane of the corneal endothelium.

Descriptive study: A study designed to describe the distribution of certain variables (e.g., a health survey of a community that gathers data on disease status and presents the resulting information in the form of what disease was present when, where, and among whom). Although not useful for etiologic research, it can be used to generate hypotheses.

Detoxication: The enzymatic conversion of a chemical to a less toxic form or, for these purposes, the conversion of a chemical to a form that can no longer be bioactivated (distinguished from detoxification, which is the process of removing the chemical from the body by physical means).

Developmental toxicology: The study of the causes, mechanisms, and sequelae of perturbed developmental events in species of animals that undergo ontogenesis; affected endpoints include death, delayed or retarded development, dysmorphology, and functional impairment.

Diagnostic drift: The gradual changes in nomenclature or application of severity grading scales that may occur in a single study group, across several groups in a single study, or when several studies are compared.

Diastole: The dilation of the heart cavities during which they fill with blood.

Dietary reference intake (DRI): Dietary Reference Intakes is the general term for a set of reference values used to plan and assess nutrient intakes of healthy people. These values, which vary by age and gender, include:

- Recommended Dietary Allowance (RDA): Average daily level of intake sufficient to meet the nutrient requirements of nearly all (97%–98%) healthy people.
- Adequate Intake (AI): Established when evidence is insufficient to develop an RDA and is set at a level assumed to ensure nutritional adequacy.
- Tolerable Upper Intake Level (UL): Maximum daily intake unlikely to cause adverse health effects. http://ods.od.nih.gov/health_information/dietary_reference_intakes.aspx.

Diffuse neuroendocrine system (DNES): Neurones and amine precursor uptake and decarboxylation (APUD) cells produce some identical biogenic amines and peptides in cells that in some ways are

similar to nerve cells and in other ways are similar to endocrine cells. These cells are located in different organs and have a common regulatory mission. Although they do not form a specific organ, they do function to control many aspects of homeostasis and are considered to be a regulatory entity.

Diffuse neuroimmunoendocrine system (DNIES): Recently, it has been shown that there are common chemical regulatory systems present among the nervous, endocrine, and immune systems. The close relationship among the amine precursor uptake and decarboxylation (APUD) cells that produce peptidergic/aminergic neurons, the DNIES system that unties many different locations, and the peptide-producing immunocompetent cells suggests that the entire system should be combined into a single regulatory homeostatic functional system and termed the *diffuse neuroimmunoendocrine system*.

Diffusion-limited uptake: Tissue uptake is limited by the diffusion through the membranes rather than by the blood flow to the tissue.

Dinoflagellata: An order of predominately marine, free-swimming protists that have two flagella; they are a staple food of shellfish and some crabs and are thus in the human food chain. Certain species may become extremely abundant in warm coastal waters, causing certain of the toxic *red tides*, although few are actually toxic.

Diploid: Two set of chromosomes, one maternal and one paternal.

Dirty bomb: A bomb that uses conventional explosives, such as dynamite, to spread radioactive material. The best known of the radiological dispersal devices; the dirty bomb does not produce a nuclear explosion but is sometimes referred to as a *Weapon of Mass Disruption*.

Discrimination behavior: Behavior based on the ability of subjects to discriminate stimulus qualities. In the typical situation, because different stimulus properties require different behaviors, discrimination abilities can be measured by the type or location of responses.

Distal tubule: Generic term describing epithelial cell types in the cortex comprising distal convoluted tubule, connecting segment, and initial collecting tubule.

Distress: A state in which an animal, unable to adapt to one or more stressors, fails to return to physiological and/or psychological homeostasis. May be manifested as clinical signs of disease, abnormal or maladaptive behaviors, or subclinically as a pathologic condition as measured by abnormal physiological parameters or organ function.

Distribution: The reversible transfer of chemical from the general circulation into the body tissues.

DNA: Deoxyribonucleic acid, the chemical substance containing the genetic code; see also *Nucleotide*.

DNA adduct: A molecule that is covalently linked to a portion of the DNA helix.

DOCA: Deoxycorticosterone.

Donor animal: An animal from which the organ or the blood was removed.

Dosage: A general term encompassing the dose, its frequency, and the duration of dosing.

Dose: The amount of a substance available for interaction with metabolic processes or biologically significant receptors after crossing the outer boundary of an organism. The *Potential Dose* is the amount ingested, inhaled, or applied to the skin. The *Applied Dose* is the amount of a substance presented to an absorption barrier and available for absorption (although not necessarily having yet crossed the outer boundary of the organism). The *Absorbed Dose* is the amount crossing a specific absorption barrier (e.g., the exchange boundaries of skin, lung, and digestive tract) through uptake processes. *Internal Dose* is a more general term denoting the amount absorbed without respect to specific absorption barriers or exchange boundaries. The amount of the chemical available for interaction by any particular organ or cell is termed the *delivered dose* for that organ or cell.

Dose equivalent (H): Unit of biologically effective dose, defined as the absorbed dose in rads multiplied by the quality factor (Q). For all x-rays, gamma rays, beta particles, and positrons likely to be used in nuclear medicine, $Q = 1$.

Dose rate: Dose per unit time (e.g., in mg/day), sometimes also called *Dosage*. Dose rates are often expressed on a per-unit-body-weight basis, yielding units such as mg/kg/day (mg/kg per day). They are also often expressed as averages over some time period (e.g., a lifetime).

Dose reconstruction: An approach to quantifying exposure from internal dose, which is in turn reconstructed after exposure has occurred, from evidence within an organism, such as chemical levels in tissues or fluids or from evidence of other biomarkers of exposure.

Dose–response: "What is there that is not poison? All things are poison and nothing [is] without poison. Solely, the dose determines that which is not a poison" (Paracelsus). The biological response to an agent is a function of the condition of exposure, including dose, duration, and route.

Dose–response assessment: The part of risk assessment associated with evaluating the relationship between the dose of an agent administered or received and the incidence or severity of an adverse health or ecological effect.

Dose–response model: A mathematical expression that relates the incidence or magnitude of a biological effect to the dose of a chemical.

Dosimetry: Estimating or measuring the quantity of material (mainly refers to particulate) at specific target sites at a particular point in time. The quantity can be measured in terms of mass number, surface area, or volume; process of measuring or estimating dose.

DPA: Decision point approach.

DR: Diet restricted.

Draculin: Anticoagulant factor present in the saliva of vampire bats.

Duct velocity: Air velocity through the duct cross section. When solid material is present in the air stream, the duct velocity must be equal to or greater than the minimum air velocity required to move the particles in the air stream.

Dust: Solid particles that are capable of temporary suspension in air or other gases. Usually produced from larger particles or masses through grinding, crushing, or other handling. Particles may be up to 300–400 μm, but those above 20–30 μm usually do not remain airborne.

Drug: Any substance or product that is used or intended to be used to modify or explore physiological systems or pathological states for the benefit of the recipient World Health Organization (WHO).

Dysplasia: Abnormal tissue development.

Early-phase regeneration (EPR): Tissue repair response, where arrested G_2 hepatocytes are activated to proceed through mitosis (see *SPR*).

Early transient incapacitation (ETI): Transient performances deficits observed in animals and humans after a large, rapidly delivered dose of ionizing radiation. Five to ten minutes after radiation exposure, behavioral performance rapidly falls to near zero, followed by partial or total recovery 10–15 min later.

EC_{50} (effective concentration–50%): A statistically or graphically determined concentration of a chemical that reduces a sublethal response parameter of interest by 50%.

Echinodermata: Phylum of marine invertebrate coelomate animals that includes starfish and sea urchins. Approximately, 85 of the nearly 6000 species are known to be venomous or poisonous.

Ecotoxicology: The study of adverse effects to organisms other than humans from exposure to natural or synthetic toxic substances in the environment.

Ecological risk assessment: The process used to establish the likelihood that adverse ecological effects are occurring or may occur as a result of exposure to one or more stressors.

Ectopic or cryptorchid testis: Malposition or displacement of the testis outside the scrotum. Typically, such testes are found within the peritoneum or inguinal canal; however, abdominal sites outside the peritoneum have been observed.

Effect concentration (EC): Concentration of a substance in media predicted to cause a measurable change in a defined proportion (e.g., 50%) of a population of organisms.

Effective dose-50% (ED_{50}): The dose of radiation or a chemical agent that would result in a given response in 50% of the population; see also *Median effective dose*.

Elapidae: A family of front-fanged, highly venomous, aquatic, burrowing, terrestrial, and arboreal snakes that includes cobras, kraits, mambas, coral snakes, death adder, the Australian copperhead, and the African garter snake; they are characterized by a pair of comparatively short, stout, permanently erect, deeply grooved fangs at the front of the mouth, and the tail is cylindrical and tapered.

Electrocardiogram: A bioelectric potential originating in the myocardium and recorded on the surface of the body; represents the sum of the electrical depolarizations of the myocardium syncytium as the wave of depolarization sweeps across the heart.

Electron paramagnetic resonance (EPR): An analytical technique that is used to study the structure and properties of free radicals. EPR spectroscopy is similar in principle and practice to nuclear magnetic resonance (NMR) spectroscopy. Whereas NMR uses energy in the radiofrequency region of the electromagnetic spectrum, EPR uses energy in the microwave region.

Elimination: The irreversible transfer of the chemical from the circulation to the organs of elimination and its subsequent removal from the body by metabolism or excretion.

Emergency response planning guidelines (ERPGs): ERPGs are values intended to provide estimates of concentration ranges for 1 h exposures above which one could reasonably anticipate observing adverse health effects. Compare to acute exposure guideline levels (AEGLs).

EMIT: Enzyme-multiplied immunoassay technique.

Empirical distribution: A distribution obtained from actual data possibly smoothed with interpolation technique. Data are not fit to a particular parametric distribution (e.g., normal, lognormal) but are described by the percentile values.

Empirical dose–response model: A mathematical model that is selected from plausible models based on agreement with experimental data.

Enantiomer: A type of stereoisomer, an enantiomer is one of a pair of isomers that are nonsuperimposable mirror images of each other. Although chemically identical (except for optical rotation), enantiomers may demonstrate widely different pharmacological and toxicological properties.

Endobiotics: Chemicals that are normally present in the biochemistry of a cell; these include chemicals that are normally found with the biochemistry of anabolic and catabolic metabolisms.

Endocrine disruptor: Chemicals or naturally occurring substances that can alter the endocrine system. Reference to chemically induce alternations in any of a wide range of endocrine functions.

Endometrium: Inner lining of the uterus that is shed during menstruation.

Endoscopy: In vivo inspection of the internal surface of a hollow organ with an instrument.

Endpoint: The biological or chemical process, response, or effect assessed by a test method.

Engineered nanoparticle: A form of nanoparticles that are designed and fabricated by material scientists for specific physical, chemical, optical, magnetic, catalytic, or morphological properties not evident in the macroscale.

Engineering standards: Specifications that do not provide for interpretation and modification of prescribed methods or procedures, even if acceptable alternative methods are available or unusual circumstances occur. This term is most commonly used in conjunction with prescribing conditions for the care and use of laboratory animals.

Enteric endocrine system (EES): An endocrine system that helps control digestive function. Over 25 hormones have been identified as affecting the gastrointestinal tract, with single-hormone-secreting cells located throughout the lumen of the stomach and small intestine.

Enteric nervous system (ENS): A nervous system that helps control digestive function; it is located primarily in the wall of the gastrointestinal tract and is an interdependent part of the autonomic nervous system that can be affected by sympathetic and parasympathetic nervous impulses. It is also referred to as the *second brain* because it can operate the gastrointestinal tract in the absence of external stimuli.

Enterohepatic circulation: Bile salts are necessary for the absorption of lipids when ingested and present in the small intestine. The bile salt forms micelles that are integral in the absorption of longer chained lipids. When the micelles are spent, they are eventually reabsorbed in the ileum of the small intestine to be returned throughout the circulatory system to the liver where the bile salts are resecreted through the bile ducts into the small intestine to again form micelles.

Environmental fate: The destiny of a chemical or biological pollutant after release into the environment. Environment fate involves temporal and spatial considerations of transport, transfer, storage, and transformation.

Environmental enrichment: Providing animals with sensory and motor stimulations, through structures and resources that facilitate the expression of species-typical behaviors and promote psychological well-being.

Enzyme: A biological catalyst (for these purposes, proteins).

Enzyme-linked immunosorbent assay (ELISA): A type of immunoassay in which specific antibodies are used to both capture and detect antigens of interest. The most popular type is the *sandwich* ELISA, in which antibodies are bound to be a substrate such as a plastic culture plates. These antibodies bind antigenic determinates on molecules (or alternatively on whole cells). Unrelated material is washed away and the plates are exposed to an antibody of a different specificity; this antibody is coupled to a detector molecule.

Epididymis: The duct that conveys sperm from the testis to the vas deferens. It consists of caput (head), corpus (body), and cauda (tail) regions that support sperm transit, maturation, and storage prior to ejaculation.

Epitope: The portion of an antigen that is recognized by an antibody or T-cell antigen receptor; also known as the *antigenic determinant*.

Epigenetics: Refers to any change in gene expression that is stable between cell divisions but do not involve changes in the DNA sequence of the organism. Mechanisms involved with epigenetic changes include methylation of cytosine residues in the DNA, remodeling of chromatin structure, regulatory processes mediated by small RNA molecules, and other gene silencing pathways. However, one of the most significant challenges with this area of research is understanding the *normal* epigenome in regard to the distinction between adaptive and adverse epigenetic changes. Without detailed characterization of the epigenome, including stable and variable methylation sites, it is not possible to evaluate whether an epigenetic alteration is an adverse effect from exposure to an exogenous agent or is part of the normal epigenetic variability. As described in a workshop by the National Academy of Sciences' Standing Committee on Use of Emerging Science for Environmental Health Decisions "Use of Emerging Science and Technologies to Explore Epigenetic Mechanisms Underlying the Developmental Basis for Disease," epigenetic testing is not yet sufficiently validated for the regulatory process because (1) no single test is ideal for epigenetic effects, (2) normal methylation patterns and long-term effects are not well understood, and (3) standardized tiered screening scheme to prioritize chemicals is lacking.

Epizootic: Refers to the initial period following introduction of a microorganism into a naïve population, during which time it undergoes rapid spread within the population and is often associated with a high incidence of clinical signs or disease.

Essential test method components: Structural, functional, and procedural elements of validated test methods that should be included in the protocol of a proposed mechanistically and functionally similar test method. These components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to essential test method components is necessary when the acceptability of a proposed test method is being evaluated based on performance standards derived from a mechanistically and functionally similar validated test method. (*Note*: Essential test method components were previously referred to as *minimum procedural standards*.)

Estrogen: Female sex steroid (e.g., estradiol).

Exencephaly: An open brain resulting from failure of the neural tube to close.

Exon: Actively transcribed DNA in a eukaryotic gene.

Exposure: Contact of a chemical, physical, or biological substance with the outer boundary of an organism. Exposure is quantified as the concentration of the agent in the medium in contact integrated over the time duration of contact.

Exposure assessment: The determination or estimation (qualitative or quantitative) of the magnitude, frequency, duration, and route of exposure; the qualitative or quantitative estimate (or measurement) of the magnitude, frequency, duration, and rote of exposure. A process that integrates information on chemical fate and transport environmental measurements, human behavior, and human physiology to estimate the average doses of chemicals received by individual receptors. For simplicity in this guidance, exposure encompasses concepts of absorbed dose (i.e., uptake and bioavailability).

Exposure concentration: The concentration of a chemical in its transport or carrier medium at the point of contact.

Exposure pathway: The physical course a chemical or pollutant takes from the source to the organism exposed.

Exposure point concentration (EPC): The contaminant concentration within an exposure unit to which receptors are exposed. Estimates of the EPC represent the concentration term used in exposure assessment.

Exposure route: The way in which a chemical enters an organism after contact (i.e., by inhalation, ingestion, or dermal absorption).

Exposure scenario: A set of facts, assumptions, and inferences about how exposure takes place; this information aids the exposure assessor in evaluating, estimating, or quantifying exposures.

Extra risk: $(P - P_o)(1 - P_o)$

where

P is the total risk

P_o is the background (spontaneous) risk in control animals or unexposed humans

Extrapolation: Using data or results from one set of conditions (e.g., animal experimental results) to predict results for a different set of conditions (e.g., humans).

Extravascular hemolysis: Destruction of red blood cells by cells of the mononuclear phagocyte system (e.g., splenic macrophages); may be a normal process for removal of senescent red blood cells or part of a pathological process for removal of abnormal red blood cells or red blood cells coated by immunoglobulin (i.e., immune-mediated hemolysis).

Eye corrosion: Irreversible ocular tissue damage following exposure to a material. Eye corrosion represents gross tissue destruction, which generally occurs rapidly after exposure.

Eye irritation: Reversible inflammatory changes in the eye and its surrounding mucous membranes following direct exposure to a material on the surface of the anterior portion of the eye.

Face velocity: Air velocity at the hood opening.

FACS analysis: Flow-assisted cell sorting; analytical method applied to data from flow cytometry experiments.

False negative: A substance incorrectly identified as negative by a test method.

False negative rate: The proportion of all positive substances falsely identified by a test method as negative (see *Two-by-two table*). It is one indicator of test method accuracy.

False positive: A substance incorrectly identified as positive by a test method.

False positive rate: The proportion of all negative substance that are falsely identified by a test method as positive (see *Two-by-two table*). It is one indicator of test method accuracy.

Favism: A hemolytic disease in individuals deficient in glucose-6-phosphate dehydrogenase resulting from consumption of fava beans.

Fertility: The ability to conceive and produce a live offspring. The fertility index should be calculated separately for male and female animals and is generally calculated as the ratio of the number of animals pregnant divided by the number of animals inseminated.

Fetal alterations (malformations, variations, and developmental delays): Any morphological change identified in a term conceptus, including frank malformation, minor deviations from normal development, and reversible delays or accelerations in development, regardless of the potential effect on subsequent viability or quality of life.

Fiber: A particle that has a length-to-width ratio of 3:1 and a length greater than 5 μm. These can be naturally occurring, man-made mineral, or synthetic organic fibers.

Fibrosis: An abnormal accumulation or proliferation of fibrous connective tissue, usually in the lung.

First-order process: A process for which the rate of reaction is proportional to the available concentration—for example, diffusion, metabolism (at low concentrations), and filtration.

Fixation: Preparation of a histologic or pathologic specimen for the purpose of maintaining the existing form and structure of its constituent elements. Maintenance of the normal form and structure of tissues and organs is critical to the evaluation of pathologic effects during microscopic examination. Common fixatives, such as formaldehyde, result in the precipitation of proteins. This fixes them in place and preserves the morphology of the specimen.

Face velocity: The measured air speed at an inlet or outlet of a ducted air system.

Flame ionization detector (FID): An instrument that measures real-time concentrations of organic vapors at

low levels from parts-per-billion (ppb) up to 50,000 parts-per-million (ppm). FIDs use the energy of a hydrogen flame to ionize gases and produce ions that can be measured as a weak current through an imposed electrical field. The resulting electrical signal is proportional to the amount of gas in the sample.

Flammability: The ability of a material to ignite and burn readily.

Flash point: The lowest temperature at which vapor is given off in sufficient quantity so the air–vapor mixture above the surface of the solvent will ignite momentarily in a flame.

Flavin-containing monooxygenases (FMOs): Enzymes containing flavin adenine dinucleotide that belong to a family of proteins that are important in the NADPH-dependent metabolism of exogenous compounds. FMOs catalyze the addition of a single oxygen atom to nucleophilic nitrogen, sulfur, and phosphorus centers of a variety of xenobiotics.

Flavonoids: Phenolic plant pigments belonging to flavone, flavanone, isoflavone, anthocyanidin, chalcone, or aurone groups.

Flow cytometry: Method to separate populations of fluorescent-labeled cells according to differences in a specific property.

Fluid mosaic membrane model: The accepted models for the description of the biological membrane. This model is a noncontinuous phospholipid bilayer that forms planar bimolecular films that separate two aqueous compartments with hydrophobic cores. Proteins are an integral part of the membrane and span the entire thickness of the lipid layer.

Folliculogenesis: Maturation of the ovarian follicle, a densely packed shell of somatic cells that contains an immature oocyte.

Food poisoning: A predominantly gastrointestinal disease resulting from the consumption of food containing any of the ever-increasing number of toxigenic microorganisms or the preformed toxins produced by them.

Formication: The unpleasant sensation of tiny insects (ant) crawling on the skin.

Fourier transformation (FT): A mathematical operation that converts information from the time domain to the frequency domain. FT can be applied to a variety of spectral techniques, including mass spectrometry, nuclear magnetic resonance, electron paramagnetic resonance, and infrared spectroscopy.

Fractional excretion: The excretion of a substance in urine relative to the rate it is filtered into the urine; an index of reabsorptive function.

Frameshift: A gene mutation characterized by the addition or deletion of one or more base pairs in a gene.

Free radical: A molecule that is inherently unstable and highly reactive with other components of living systems and produced by sufficient exposure to ionizing radiation.

Frequency distribution: A graph or plot that shows the number of observations that occur within a given interval. Usually presented as a histogram showing the relative probabilities for each value, it conveys the range of values and the count (or proportion of the sample) that was observed across that range.

FSH: Follicle-stimulating hormone.

Fugu: A puffer fish that can lead to severe tetrodotoxin poisoning if improperly prepared.

Fume: Minute solid particles arising from high-temperature or combustion processes with subsequent condensation of vapors and often accompanied by a chemical reaction, such as oxidation. Metal vapors commonly condense to create fume; exposure to fresh metal fumes can cause metal fume fever. Fumes exist initially as very small (<100 nanometers [nm]; 10^{-9} m) particles but form larger (~1 μm) particle clusters through agglomerative growth.

Functional observation battery: A set of standardized observations, typically performed with rodents, designed to assess neurobehavioral functions such as reflexes.

Functionalization: The addition to or uncovering of functional groups that are required for subsequent phase II metabolism; for example, hydroxylation of a hydrocarbon provides a functional group from which a conjugate can be formed during phase II metabolism.

Functionalization reactions: Enzymatic reactions that introduce a new functional group into a xenobiotic molecule, either by addition of a new functional group (e.g., hydroxylation) or by removal of a masking group (e.g., *N*-demethylation). Functionalization reactions are also commonly referred to as phase 1 reactions.

Gametogenesis: Production of sperm or ova.

Gamma rays: High-energy, short-wavelength electromagnetic radiation emitted from the nucleus of an atom.

Gas: A substance that exists in the gaseous state at standard temperature and pressure.

Gas chromatography–mass spectrometry: A method for introducing analytes volatized into the gas phase into the mass spectrometer through a chromatographic system where a carrier gas passes through a column packed with a solid-phase material.

Gastrointestinal transit: The rate of passage of the luminal contents of the gastrointestinal tract in the oral to anal direction, ordinarily measured with a nonabsorbable marker.

Gavage: Method of oral administration of a solution or suspension using a suitable stomach tube or feeding needle attached to a syringe. In toxicity studies, gavage is a common method of test material administration by the oral route.

Gene: Generally described as the smallest functional unit of an organism's genome.

Gene pool: The total genetic information contained in the reproductive cells of a species.

Genome: The total number of genes contained in the hereditary material of an organism; the chromosomal DNA information.

Genomics: The techniques available to identify the DNA sequence of the genome; field of study involving analysis of genetic material (i.e., DNA, RNA). Genomics can be studied at the cellular level in a population; automated methods, such as DNA microarray, have been developed for high-throughput analysis.

Genotoxic: Property of an agent making it capable of damaging or altering an organism's genetic composition.

Genotoxicity: Alteration of nucleic acids and associated components at subtoxic exposure levels, resulting in modified hereditary characteristics or DNA inactivation.

Genotype: The nucleotide composition of an organism's hereditary material; the full set of genes carried by an individual organism. Note that this term is more limited than the genome, as the genome also contains noncoding DNA and genes.

Geometric mean: The nth root of the product of n values.

Gestation/pregnancy: The interval in a pregnant animal from conception (fertilization) to the beginning of parturition.

GH: Growth hormone or somatotropin.

Globally Harmonized System (GHS): A UN initiative aiming to harmonize the classification, labeling, and SDS format of chemicals so as to promote international trade and the protection of health and environment.

Glomerular filtration rate: The volume of plasma separated from the vascular space across the renal glomerulus, expressed per unit time. The filtrate is further modified by the nephron tubule to become the final urine that is eliminated from the body. The rate at which plasma is filtered through the glomerular capillary bed into the tubular lumen is defined as the difference between the hydraulic and oncotic pressures across the glomerular capillary wall multiplied by an ultrafiltration coefficient and the surface area available for filtration.

Glycone: The activated form of glucose used in glucuronidation. The term is sometimes used for other activated forms of the endogenous compound used in xenobiotic conjugation reactions.

Glycosides: Chemicals of varied structure linked to a mono- or disaccharide by a β-glycosidic linkage; an acetal that yields sugar and a nonsugar on hydrolysis and is found more commonly in plants than in alkaloids. Toxicity varies from nontoxic to extremely toxic.

Glucuronidation: A biosynthetic conjugation reaction involving membrane-bound uridine diphosphoglucuronosyl transferases that catalyze the transfer of glucuronic acid, in the form of endogenous uridine diphosphoglucuronic acid, to a xenobiotic functional group (e.g., hydroxyl, carboxylic acid) to yield a product that has increased water solubility and properties that enhance its renal and biliary elimination.

Glutathione S-transferase (GST): A family of phase 2 (biosynthetic) enzymes that catalyze conjugation of the endogenous tripeptide glutathione with electrophilic atoms in xenobiotic substrates. The GSTs involved in xenobiotic metabolism are found in the cytosolic compartment of cells, whereas a membrane-bound form is involved in biosynthesis of endogenous compounds.

Gonadotropin-releasing hormone (GnRH): A neuropeptide that regulates the release of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

Gonadotropins: Usually considered to be follicle-stimulating hormone (FSH) and luteinizing hormone (LH); collectively includes FSH and LH.

Good laboratory practices (GLPs): Regulations promulgated by the U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA), as well as principles and procedures adopted by the Organization for Economic Cooperation and Development (OECD) and Japanese authorities, that describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submission to national regulatory agencies.

Graded response: A response to a stimulus or a treatment that can be determined quantitatively on a continuous scale. In acute toxicity studies, body weight and feed consumption are examples of measurements of graded responses.

Gray (Gy): SI standard unit of absorbed dose; 1 Gy equals 1 joule of energy per kilogram of absorber or 100 rads.

GTT: Glucose tolerance test.

Haber's rule: The Haber relationship expresses the constancy of the product of exposure concentration and duration ($CT = k$). This relationship does not hold over more than small differences in exposure time. The hypothesis states that equal values of ($C \times T$) produce equal biological effects, where C is the concentration of a chemical and T is the duration of exposure.

Half-life: The time taken for the plasma (or tissue) concentration to change by 50%; half-life is a characteristic of first-order reactions and is independent of concentration and dose.

Half-life, biologic: Time it takes an organism to eliminate half of the radionuclide by biologic processes.

Half-life, effective: Time required for the activity of a radionuclide in a biologic system to be reduced to half its initial value as a consequence of both radioactive decay and biologic elimination.

Half-life, physical: The time necessary for a radionuclide to decay to half of its initial activity.

Haploid: A single set of eukaryotic chromosomes.

Hapten: Low molecular weight molecules that are not antigenic by themselves but are recognized as antigens when bound to larger molecules such as proteins.

Hazard: The inherent property of a single chemical or mixture to cause adverse effects if an organism is exposed to it; the potential for an adverse health or ecological effect. A hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

Hazard analysis and critical control point (HACCP): A system designed to assess various stages of food processing critical in controlling microbial contamination and to propose and implement procedures aimed at minimizing the microbial burden in foods.

Hazard identification: A qualitative assessment of the types of adverse effects caused by a particular chemical, including (but not limited to) an evaluation of quality of the studies, identification of susceptible subpopulations, and assessing the relevance of humans of the effects observed in animals.

Hazardous substance: Any substance or mixture of substances that is toxic, corrosive to an irritant; has the potential to cause substantial personal injury as a result of handling or use.

Heinz body: Irreversibly denatured hemoglobin attached to the inner cell membrane of the red blood cell; caused by oxidizing agents.

***Heloderma*:** A genus of lizards comprised of two species of heavy-bodied lizards with short stout legs; the body is covered by tubercular scales.

Henry's law constant: The relationship between the solubility of a gas in a liquid to the pressure of the gas above the liquid at equilibrium, which describes the tendency of a chemical to escape from solution.

Hepatic first-pass effect: Virtually, all of the substances ingested into the circulatory system are transported directly to the liver where the substances can be metabolized via phase I and phase II reactions prior to their entry into the general circulatory system.

Hepatocytes: Liver cells.

Hepatotoxicity: Toxicity to the liver.

Heterologous blood perfusate: Blood used as perfusate that comes from another animal or a different species than the source of the organ being perfused.

Heterologous expression: Production of a recombinant protein in an artificial host system.

Heterologous expression systems: Systems that allow expression of a gene in a different organism.

Heterozygous: Two different alleles on a chromosome pair.

Hexagonal lobule: Classical morphological configuration of the functional unit of the liver as described by Kiernan in 1833.

High-end exposure (dose) estimate: A plausible estimate of individual exposure or dose for those persons at the upper end of an exposure or dose distribution, conceptually above the 90th percentile but not higher than the individual in the population who has the highest exposure or dose.

High-end risk descriptor: A plausible estimate of individual risk for those persons at the upper end of the risk distribution, conceptually above the 90th percentile but not higher than the individual in the population with the highest risks. Note that persons in the high end of the risk distribution have high risk due to high exposure, high susceptibility, or other reasons; therefore, persons in the high end of the exposure or dose distribution are not necessarily the same individuals as those in the high end of the risk distribution.

Hippocampus: Radiosensitive area of the brain involved in learning and memory functions.

Histopathology: The study of morphological changes in tissues at the light-microscopic levels.

Holozoic nutrition: Obtaining nutrients or nourishment through the ingestion of large complex organic materials or whole organisms.

Homozygous: Two similar alleles on a chromosome pair.

Hormesis: A U-shaped dose–response relationship in which adverse effects do not increase monotonically with dose but decrease initially as dose increases and then rise with higher doses. (An inverted U-shaped dose–response is observed with beneficial effects.) A dose–response relationship characterized by a low-dose stimulation and a high-dose inhibition. This type of biphasic dose–response has specific quantitative features of the magnitude and width of the stimulation and the relationship of the stimulation to the response threshold.

Host defense: The ability of an animal to protect itself against disease associated with exposure to infectious organisms, foreign tissues and chemicals, and neoplasia. Host defense may be either nonspecific or specific (immune) in nature.

Host resistance: The ability of an organism to defend immunologically against infection. Host resistance may be decreased in response to an immunosuppressive insult.

HTE: Human time equivalents.

Human health risk assessment: Process by which data on the metabolism and toxicity of a chemical are used to estimate the potential hazard to humans of exposure; often involves the extrapolation of data from laboratory animals (e.g., rats or mice) to humans; used by regulatory agencies to define safe levels of exposure of human populations to potentially toxic chemicals.

Humane endpoint: The earliest point in an animal experiment when reliable data can be collected to meet experimental goals before the animal experiences.

Humoral-mediated immunity (HMI): Specific immune responses that are mediated primarily by humoral factors (i.e., antibodies and complement). The induction of humoral immune responses generally requires the cooperation of cellular immune mechanisms.

Hybridization: The formation of a double strand from two different, more or less complementary, single nucleic acid strands.

Hydroponic: Referring to a nutrient solution capable of supporting plant growth without the support of inert material.

Hydrozoa: One of three classes of the phylum Cnidaria (referred to as Coelenterata in the past) in which the stomodaeum is absent and the mesoglea has few or no cellular elements and is usually metagenetic. Most species are marine.

Hyperplasia: A histopathologic finding characterized by an abnormal increase in the number of cells in a tissue or organ. Hyperplasia generally results from increased cell division but can result from decreased cell death.

Hypertrophy: A histopathologic finding characterized by an abnormal increase in the size of cells in a tissue or organ; for example, accumulation of fat vacuoles within a cell can increase its size.

Hypospadia: In males, a congenital malformation in which the urethra remains open on the undersurface of the penis. An extreme expression of this malformation results in cleft phallus, with a cleft running the entire length of the penis.

Ibotenic acid: A neurotoxic isoxazole that, when paired with muscimol, is largely responsible for the toxicities of *Amanita* species and certain other poisonous mushrooms.

IACUC: Institutional Animal Care and Use Committee.

ICH: International Conference on Harmonization.

IDDM: Insulin-dependent diabetes mellitus, type 1.

Idiosyncrasy: A specific (and usually unexplained) reaction of an individual to, for example, a chemical exposure to which most other individuals do not react at all (e.g., some people react to their very first aspirin with a potentially fatal shock). General allergic reactions do not fall into this category.

Immune reserve: The concept that the immune response exhibits multiple redundancies capable of modulating acute reductions in certain immune functions. This reserve would theoretically prevent a severe reduction in host resistance following temporary immunosuppression of selected parameters (e.g., NK cell function).

Immunoassay: An assay that utilizes specific antibodies as reagents. Examples include enzyme-linked immunosorbent assays (ELISAs) and radioimmunoassays (RIAs).

Immunochemistry: The use of antibodies in analytical or preparative procedures.

Immunologic contact urticaria (ICU): Contact urticaria is an immediate-type reaction, on an immunologic basis, such as in latex contact.

Immunostimulation: Enhancement of immune function above an established baseline (control) response. Immunostimulation may be beneficial (e.g., therapeutics designed to restore a depressed immune response) or detrimental (e.g., the induction of allergy/hypersensitivity or autoimmunity).

Immunosuppression: Depression of immune function below an established baseline (control) response. Immunosuppression may result from inadvertent exposure to immunosuppressive agents, deliberate (therapeutic) immunosuppression, or exposure to certain infectious agents. An important consideration in immunotoxicology is determining the degree or nature of immunosuppression necessary to alter host defense. Immunosuppression can be said to result in a state of immunodeficiency.

Immunotoxicity: The condition in which a drug, chemical, or physical agent alters the structure or function of the immune system.

Immunotoxicology: The discipline of synergistically applying cardinal principles of both immunology and toxicology to study the ability of certain materials to alter the normal immune response.

Impaction: A particle deposition process related to particle inertia whereby particles contact airway surfaces by their inability to follow the air stream around directional changes from airway bends or bifurcations.

Implantation: The embedding of the early embryo in the lining of the uterus.

Imposex: A condition caused by low concentrations of organotin compounds (e.g., tributyltins and triphenyltins) characterized by the superimposing of male sex organs on otherwise normal dioecious female gastropods. The resulting pseudohermaphrodites are incapable of reproduction.

Intravascular hemolysis: Lysis of red blood cells within circulation; if severe enough, release of hemoglobin into the plasma may be recognized as hemoglobinemia and free hemoglobin filtered into urine may be recognized as hemoglobinuria.

IO: Institutional Official for the animal care and use program.

***In silico*:** An expression used to mean performed on computer or via computer simulation.

In situ perfusion: Vascularly isolated and perfused organs are left in the animal body with the neighboring tissues.

In vitro hemolysis: Rupture or lysis of red blood cells during blood collection or handling causing the release of intracellular substances (e.g., hemoglobin, enzymes, electrolytes) into serum or plasma. If severe enough, it may be responsible for artifactual test results.

In vivo nuclear magnetic resonance (NMR) spectroscopy: A technique of value for measuring alterations in specific P-31 species within cells in real time using a surface coil and large-bore, high-resolution NMR magnets.

Incidence: The number of new cases of a particular disease in a defined population during a specified period of time. The term *incidence* is sometimes used

synonymously with *incidence rate*. Note that, in the medical literature, *prevalence* and *incidence* are often used incorrectly as equivalent terms.

Induction: As used with respect to xenobiotic metabolisms, induction refers to the process where exposure of an organism to a xenobiotic results in increased activity of specific biotransformation enzymes. Generally, but not always, induction results in more rapid metabolism of the inducing agent. Induction, as used in this context, does not require de novo protein synthesis but may result from other mechanisms.

Inflammation: A nonspecific host defense mechanism. It is characterized primarily by the infiltrating of leukocytes into the peripheral tissue, followed by release of various molecules that elicit nonspecific physiological defense mechanisms.

Inhalable: A particle size characteristic denoting that the substance can enter the respiratory tract; however, particles may not be sufficiently small to enter beyond the nose and into the airways and pulmonary regions. Contrast with *respirable*.

Inhalation: The breathing in of a substance in the air (gas, vapor, particulate, dust, fume, or mist).

Inhibin: A glycoprotein produced by Sertoli cells and by the pituitary that acts as a negative regulator of follicle-stimulating hormone (FSH) secretion.

Initiated Cell: A normal body (stem?) cell that has undergone the first transformation step in the process of cancer development to an intermediate state but that is not malignant.

Innate immunity: Host defense mechanism that does not require prior exposure to an antigen; various effector mechanisms have been described, including cellular cytotoxicity mediated by macrophages or NK cells, complement, and activity gamma delta T cells.

Insecta: A very large class of invertebrate animals representing more than 75% of known animal species. They have three main body parts: head, thorax, and abdomen.

Instillation: Slow introduction of a fluid (for a fluid-containing particulate) directly into the trachea of an experimental animal; this is a surrogate method for the introduction of fluid or particulate into the lungs following inhalation.

Insufflation: Introduction of particulate (without fluid) directly into the trachea (or bronchioles) of an experimental animal. This is a surrogate method for the introduction of material into the lungs following inhalation.

Intake: The process by which a substance crosses the outer boundary of an organism without passing an absorption barrier (e.g., through ingestion or inhalation; see *Potential dose*).

Interception: A particle deposition process related to a particle trajectory and the physical proximity to airway surfaces, resulting in particle contact and deposition. Interception is a particularly important deposition process for inhaled fibers.

Internal dose: The amount of a substance penetrating across the absorption barriers (the exchange boundaries of an organism) via either physical or biological processes. This term is synonymous with *Absorbed Dose*.

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use: An initiative intended to establish similar criteria to support the worldwide registration of drugs. Representatives from regulatory agencies and pharmaceutical companies from the United States, Europe, and Japan have been the primary participants.

International Union of Toxicology (IUTOX): An international organization representing toxicology societies worldwide.

Intestinal first-pass effect: Evidence suggests that not only do the small intestines provide for absorption of various substances, but it can also play a significant role in phase I and phase II metabolism reactions of certain substances prior to their absorption into the circulatory system.

Intralaboratory repeatability: The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period.

Intralaboratory reproducibility: The first stage of validation; a determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times.

Intravascular hemolysis: Rupture or lysis of red blood cells within the vascular system causing release of intracellular substances (e.g., hemoglobin) into the plasma. If severe enough, it may be recognized by hemoglobinema and/or hemoglobinuria.

Intron: Noncoding spacer DNA in a eukaryotic gene.

Intubation: Placement of a tube into a hollow organ. Intubation is used to place various materials into animals for toxicology studies. If the organ is in the stomach, the process is generally referred to as *gavage*.

Inulin: A polysaccharide (molecular weight, ~5000) that is not metabolized by mammalian cells and is too large to enter cells. The clearance of inulin is used to measure glomerular filtration rate. Inulin is used in vitro as an indicator of extracellular space.

IOCA: *In ovo* carcinogenicity assay.

Ionization: The formation of ions from neutral molecules. This is accomplished in mass spectrometry by a variety of methods, including bombardment with electrons, gas-phase chemical reactions, and ion desorption methods.

Infrared (IR) fingerprint region: This frequency region of the IR spectrum ranges from 910 to 1430 cm^{-1} and contains bending and stretching absorptions of characteristic groupings in an organic molecule. The region is abundant in absorption bands, and their frequencies and intensities are unique for a specific compound; hence, the absorption pattern in this region of the IR spectrum is utilized as a fingerprint for the recognition of an unknown molecule by comparison with the fingerprint frequencies of an authentic standard. It is important to note that polymorphic forms of the same compound may show significant differences in the fingerprint region; therefore, comparisons should be made in the solution in the same solvent. If solid-state spectra are utilized, the unknown and authentic standard should be crystallized from the same solvent under similar conditions.

Iris: The structure of the eye that is anatomically located posterior to the cornea. The iris forms the pupil of the eye and functions in regulating the amount of light that reaches the retina. In the Draize eye test, effects on the iris represent a maximum of 10 out of 110 total points.

IRMA: Immunoradiometric assay.

Irradiation: Exposure to radiation.

Irritant dermatitis: Chemically induced, nonimmunologic contact dermatitis; a complex syndrome of many types.

Ischemia: Local anemia due to mechanical obstruction (mainly arterial narrowing) of the blood supply.

Islet of langerhans: Specialized cells (beta cells) in the pancreas that secrete insulin.

Isoenzymes: Enzymatically active proteins that catalyze the same reactions and occur in the same species but differ in their physicochemical properties (also, *isozymes* or *isoforms*).

Isolated epithelial cells: In vitro preparation of single tubular epithelial cells, typically isolated by enzymatic (e.g., collagenase) digestion. Cells from specific nephron regions can be enriched by various separation methods; useful for short-term (up to 4 h) metabolic or cellular function studies.

Isolated perfused kidney: In vitro preparation involving the whole kidney. A cannula is inserted into the renal artery, and fluid is pumped into the kidney. The technique can be used to assess renal physiology and determine drug clearance. A chief advantage is that the model maintains an intact organ structure but does not include extrarenal tissues or factors; viability is maintained for up to 2–4 h.

Isolated perfused organ: Vascularly perfused organ is physically removed from the donor animal and maintained in an artificial chamber usually kept at physiological temperature.

Isolated perfused tubules: Intact tubules from specific nephron cell types are usually isolated by mechanical means and are perfused with micropipettes; useful for short-term (up to 2 h) metabolic, transport, and acute toxicity studies.

Isolated perfused ventilated lung: Vascularly perfused isolated lungs are also ventilated using mechanical devices.

Isolated tubular fragments: In vitro preparation of fragments of nephron segments, typically isolated by enzymatic (e.g., collagenase) digestion. The segments from specific cell types can be enriched by various separation methods; useful for short-term (up to 4 h) metabolic or cellular function studies.

ITO cells: Fat-storing cells found in the liver; also called *lipocytes* or *stellate cells*.

Juxtaglomerular apparatus: A specialized area of the glomerulus where the distal tubule from the nephron has contact with the arterioles entering and leaving the glomerulus; one factor in the control of renal blood flow.

Knockout animals: Genetically engineered animals in which one or more genes, usually present and active in the normal animal, are absent or inactive.

Kriging: A statistical interpolation method that selects the best linear unbiased estimate of the parameter in question. Often used as geostatistical method of spatial statistics for predicting values at unobserved locations based on data from the surrounding area. Information on the fate and transport of chemicals within the area lacking data can be incorporated into Kriged estimates.

Lactate dehydrogenase leakage: Assay for cellular necrosis that measures the fraction of cytosolic enzyme lactate dehydrogenase (LDH) released from cells due to membrane premeabilization; assays are performed by measuring NADH oxidation spectrophotometrically.

Lactation: The secretion of milk from the mammary glands and the period of time that a mother lactates to feed her young.

Large intestine: The region of the gastrointestinal tract from the ileum to the anus that consists of the cecum, the colon, and the rectum.

Laser capture microdissection: A potentially useful technique for excision of tissue area of interest (e.g., a tumor) via laser dissection from surrounding normal tissue so differences in gene or protein expression patterns may be compared by 2D gel electrophoresis patterns.

Latin hypercube sampling (LHS): A variant of the Monte Carlo sampling method that ensures selection of equal numbers of values from all segments of the distribution. LHS divides the distribution into regions of equal sampling coverage; hence, the values obtained will be forced to cover the entire distribution. It is more efficient than simple random sampling, as it requires fewer iterations to generate the distribution sufficiently.

LCB: Limited carcinogenicity bioassay.

LC_{50}: See *Median lethal* dose.

LD_{50}: Lethal dose of radiation or chemical agent that has been determined to cause death in 50% of a defined population; expressed in terms of weight of test substance per unit of test animal (mg/kg). See *Median lethal dose*.

$LD_{50/30}$: Median lethal dose (MLD or LD_{50}) required to kill 50% of the population of organisms within 30 days.

Leukopenia: Any situation in which the total number of leukocytes in the circulation is less than normal.

Leydig cells: Cells located in the interstitial compartment of the testes between the seminiferous tubules that synthesize testosterone; androgen-secreting cells present in the interstitium of the testes.

LH: Luteinizing hormone.

Limit of quantification (LOQ): The concentration of analyte in a specific matrix for which the probability of producing analytical values above the method detection limit is 99%.

Limit study: A study in which a single, maximal dose level of test material is administered to the test animals. Limit studies are conducted when administration of test material at higher dose levels is not required either because exposure at higher levels is not physically possible or because the test material has been shown to be of extremely low toxicity.

Linear energy transfer (LET): The amount of energy transferred by a unit dose of radiation per unit pathway traveled through matter (keV/micron of path); varies with the type of radiation. Alpha particles are high LET radiation with 10–100 s of keV/micron of path, whereas x-rays and gamma rays are low LET radiations (tenths to 10 keV/micron).

Linear extrapolation: The process of estimating a value at conditions not directly measurable using a linear relationship between the biological effect and the dose or duration of exposure. (Technically, when a measure of the background effect is available, an *interpolation* is being performed.)

Linear kinetics: With linear kinetics, there is a linear relationship between dose and plasma concentrations and body loads. Linear kinetics are characteristic of first-order reactions because the rates of reactions increase as the concentration or body load increases; therefore, parameters such as bioavailability, clearance, apparent volume of distribution, and half-life are independent of dose (see definitions in text).

Linear model: A model in which the change in a biological effect is proportional to the change in dose or duration of exposure.

Lipid peroxidation: The chain reaction formation of the mediators of lipid degradation by ionizing radiation.

Lipophilicity: The tendency of a chemical to partition into a lipid medium or a fat solvent, such as hexane, from an aqueous medium.

Liquid chromatography–mass spectrometry: A method for introducing analytes dissolved in solution into the mass spectrometer through a chromatographic system in which a liquid mobile phase passes through a column packed with a solid-phase material.

Liver acinus: Functional unit of hepatocytes as defined by Rappaport; consists of a small parenchymal mass arranged around an axis consisting of a terminal portal venule, a hepatic arteriole, a bile ductile, lymph vessels, and nerves.

LOAEL: Lowest observed-adverse-effect level.

Low-dose extrapolation model: A model that uses information on observed dose–response relationships combined with mechanistic understanding to predict the dose–response relationship below the range of observable data.

Lymph: A clear, transparent, sometimes faintly yellow and slightly opalescent fluid that is collected from the tissues throughout the body. It flows into the lymphatic vessels and is eventually added to the venous blood circulation.

Lymphoproliferation: Proliferation of lymphocytes in response to stimulation with cellular activators, including antigens or mitogens. Because the proliferation of lymphocytes is one of the initial consequences of cellular activation, lymphoproliferation is often used as a nonspecific in vitro measure of immunoresponsiveness. This assay is sometimes referred to as the *blastogenesis assay.*

Mab: Monoclonal antibody.

Macrocirculation: Comprised of the heart, great vessels (both arterial and venous), and larger arteries and veins.

Malformation: Structural defect due to abnormal development.

Magic angle spinning: A nuclear magnetic resonance (NMR)–based technique enabling the analysis of intact tissue. The term *magic angle* is derived from the fact that, when samples are spun rapidly at 54.7° relative to the applied magnetic field (the so-called magic angle), line-broadening effects that would ordinarily obfuscate a proton spectra of a solid sample can be reduced.

Major histocompatibility complex (MHC): A complex of genes coding for tissue compatibility markers. Two major classes are recognized: class I (present on all nucleated cells) and class II (present on B cell, T cells, and macrophages). MHC molecules appear to direct the course of immune reactivity and are presented in association with antigen by antigen-presenting cells. The human equivalent is termed *human leukocyte antigen* (HLA).

Malabsorption: Impairment in the uptake of ingested substances from the gastrointestinal lumen into the bloodstream because of defects in luminal digestion, mucosal transport, or gastrointestinal transit.

Margin of safety (MOS): The difference, normally expressed as fold difference, between the dose (or exposure level) that results in toxicity and the dose that results in the desired pharmacological activity.

Mass cell: A polymorphonuclear, granule-containing cell with a major role in hypersensitivity reactions.

Mass median aerodynamic diameter (MMAD): Standard method of characterizing the size distribution of a particulate atmosphere. It represents the size when 50% of the particles are larger (or smaller) than the stated size; aerodynamic diameter that divides the particles of a sample in half, based on their weight.

Mass spectrometry: An analytical technique that determines the mass of ionized molecules and their fragments and adducts.

Mass spectroscopy (MS): An analytical technique where charged particles (ions) are created from sample molecules that are then analyzed to provide information about the molecular weight of an ion and its chemical structure.

Mating performance: The ratio of mated (inseminated) animals to the number cohabited, generally expressed mathematically as the ratio of the number of female animals inseminated divided by the number cohabited with a cohort male.

Maximally exposed individual (MEI): The single individual with the highest exposure in a given population (also, most exposed individual). This term has historically been defined in various ways, including as defined here and also synonymously with worst-case or bounding estimate. Assessors are cautioned to look for contextual definitions when encountering this term in the literature.

Maximum tolerated dose (MTD): The dose that causes no more than a 10% reduction in body weight and does not produce mortality, clinical signs of toxicity, or pathologic lesions that would be predicted to shorten the natural life span of an experimental animal for any reason other than the induction of neoplasms.

MCL: Mononuclear cell leukemia.

Mean: The average value. In a normally distributed sample, this has the same value as the median (the middle value) and the mode (the most frequent value).

Measure of association: A term that represents the strength of association between variables. The relative risk, odds ratio, and standardized mortality ratio (SMR) are measures of association commonly used in epidemiology.

Median effective dose: A statistically derived single dose of a substance that can be expected to produce a particular effect in 50% of the study population (ED_{50}). The ED_{50} is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

Median lethal dose: A statistically derived single dose of a substance that can be expected to cause death in 50% of the study population (LD_{50}; LC_{50}). The LD_{50} is expressed in terms of weight of test substance per unit weight of test animal (mg/kg). The LC_{50} is expressed in terms of weight of test substance per unit volume (mg/L).

Median value: The value in a measurement dataset such that half of the measured values are greater and half are less.

Mesocosm: The replication of a subpart of a functioning ecosystem to study food web effects of toxic substances. A mesocosm may be replicate small field enclosures or ponds, or may be developed in artificial streams, tanks, or terraria in the laboratory; they typically include vertebrates as part of the food web.

Mee's lines: Horizontal white lines on the fingernails that occur after exposure to arsenic. The lines appear after the exposed nail bed grows to the exterior.

Mercapturic acid: *N*-acetylated cysteine *S*-conjugates of a xenobiotic derived from further metabolism of glutathione–xenobiotic conjugates. Most mercapturic acids are relatively inert and get excreted in the urine, though some may undergo bioactivation in the kidney, resulting in toxicity.

Messenger ribonucleic acid (mRNA): The substance carrying genetic information from the DNA to the protein production site.

Metabolic activation: The process by which relatively stable substrates are converted to highly reactive, generally electrophilic products with the capability of producing damage to critical cellular macromolecules. The term is occasionally used to refer to the metabolism of therapeutically inactive prodrugs to the active form of the drug.

Metabolome: The set of all low molecular weight compounds synthesized by an organism; the total quantitative collection of small molecular weight biochemical metabolites present in a cell, tissue, or organism that are involved in growth, energetics, maintenance, pathology, and other biochemical functions.

Metabolomics: The study of the dynamic expression, production, relative concentration, and variation of small molecular weight, endogenous biochemicals and metabolites in living systems. Characteristic changes in these discrete biomarkers before and after exposure to xenobiotics and in pathological processes may be useful for detecting and understanding toxicological mechanisms, histopathology, and environmental stressors.

Metabonome: The constituent metabolites in a biological sample.

Metabonomics: The techniques available to identify the presence (and concentrations) of metabolites in a biological sample.

Metal fume fever: An acute condition of short duration caused by exposure to fresh fumes of zinc and other metals. The condition is characterized by fever and chills, occurring 4–12 h after exposure. Recovery usually is complete within 1 day.

Metalloid: Any element with both metal and nonmetal characteristics (e.g., arsenic, boron, tellurium).

Metallothionein: Any inducible low molecular weight, cytosolic protein with a high cysteine content (approximately 30%) and characteristically deficient in aromatic amino acids and histidine. The presence of numerous cysteinyl thiol groups permits high-affinity binding of several metals (e.g., cadmium, lead, mercury, or zinc).

Metals: A grouping of elements generally characterized by opacity, ductility, luster, being electropositive with a tendency to lose electrons, and having the property of conducting heat and electricity. Heavy metals may be further defined as any element having a density greater than 5 g/cm^3, and those of toxicological significance preferentially bind to ligands containing sulfur or nitrogen (arsenic, cadmium, chromium, lead, and mercury).

Methemoglobin: A form of hemoglobin in which the ferrous ion (Fe^{2+}) of hemoglobin has been oxidized to the ferric state (Fe^{3+}); methemoglobin is unable to carry oxygen.

Methylation: A biosynthetic conjugation reaction involving methyltransferases that catalyze the transfer of a methyl group primarily from *S*-adenosyl methionine to oxygen, nitrogen, or sulfur atoms of xenobiotics. Unlike other conjugation reactions, methylation reactions do not enhance water solubility.

Micelles: These are discrete aggregates of 20–50 molecules of bile salts that mediate the absorption of fatty acids longer than 12 carbon molecules across the lumen of the small intestine. The water-soluble part of the molecules faces outward while the nonpolar nuclei face inward. Micelles are able to dissolve the aqueous component of the chyme allowing fatty acids to accumulate in the center of the micelle. The micelles are subsequently transported to the lumen, where the fatty acids are deposited and absorbed, and the micelles are then recirculated in the chyme to repeat the process.

Microarray: A series of molecular probes, immobilized on a solid substrate, such as nylon or glass, which can be used to detect the presence of specific biomolecules, such as RNA, DNA, or protein, in a biological sample.

Microcirculation: The business end of the circulation, where delivery of oxygen and nutrients and the removal of carbon dioxide and metabolites take place.

Microcosm: A small model or piece of an ecosystem contained in a test chamber and used to study the effect of contaminants on a biotic community in a controlled environment.

Microenvironment method: A method used in predictive exposure assessments to estimate exposures by sequentially assessing exposure for a series of areas (microenvironments) that can be approximated by constant or well-characterized concentrations of a chemical or other agent.

Microenvironments: Well-defined surroundings, such as a home, office, automobile, kitchen, or store, that can be treated as homogeneous (or well characterized) in the concentrations of a chemical or other agent.

Microexposure event (MEE) analysis: A method accessing risk based on the aggregate sum of a receptor's contact with a contaminated medium. MEE analysis simulates lifetime exposure as the sum of many short-term or microexposures. MEE approaches can be used to explore uncertainty associated with the model time step in probabilistic risk assessment (PRA)—for example, the use of a single value to represent a long-term average phenomenon, seasonal patterns in exposures, or intraindividual variability.

Microisolation cage: An animal cage usually constructed of plastic that completely surrounds the animals contained therein such that air entering and exiting the cage must pass through an integrated filter designed to stop the passage of unwanted microorganisms or fomites. Most commonly, such caging is maintained using sterile techniques to prevent the introduction of unwanted microorganisms.

Microperfusion: Technique to determine the function of a specific nephron segment. The segment is isolated from the glomerular filtrate between a wax and oil block, and the segment is then perfused in situ with a solution of controlled composition through a micropipette; the solution is then quantitatively collected from the same nephron segment.

Micropuncture: Specialized technique used to analyze the effects of drugs or chemicals on single nephron function in the intact kidney. It involves the insertion of a micropipette into the lumen of a specific nephron segment of interest; experiments are performed under microscopic control.

Microsomes: (1) The operational definition of microsomes is the 105,000 *g* pellet produced from a tissue homogenate after removal of nuclei, mitochrondria, and cell debris by centrifugation at 9000 *g*. Microsomes are the remnants of the endoplasmic reticulum after cellular disruption. The outer surface of microsomal vesicles represents the cytosolic side of the endoplasmic reticulum. (2) Small vesicles of endoplasmic reticulum containing numerous xenobiotic metabolizing enzymes formed during mechanical disruption of cells or tissues. Microsomes can be isolated from crude tissue homogenates by differential centrifugation and are a common in vitro model for studying xenobiotic metabolism.

Midzonal cells: Collection of hepatic cells situated between the periportal area and the centrilobular area of the lobule.

Milk sickness: Potentially fatal neurologic disease resulting from the consumption of unpasteurized milk containing the neurotoxin tremetol, derived from animals grazing on toxic plants, white snakeroot, or rayless goldenrod.

Misclassification: A type of bias in which an individual or attribute is assigned a value that is incorrect. The misclassification may be *nondifferential* (the same in all study groups) or *differential* (not equivalent across groups). Each type of misclassification may impact the results, the former usually underestimating the true measure of association and the latter often overestimating it.

Mists: Similar to fogs, a suspension of liquid particles in gas. These can be formed from condensation of vapors or from atomization of liquids (e.g., sprayers). Mists are characterized by particles larger than about 40 μm; fogs are categorized by their smaller size.

Mitochondrial membrane permeability transition (MPT): An increase in the permeability of the mitochondrial membrane due to a loss of membrane potential resulting in the release of a number of proteins such as cytochrome c and apoptosis-inducing factor (AIF).

Mitogen: Molecules capable of inducing cellular activation; may include sugars or peptides. The ability of a cell to respond to stimulation with mitogen (generally assessed by cellular proliferation) is believed to give an indication of the cell's immune responsiveness.

Mitogen-activated protein (MAP) kinase pathway: The MAP kinase pathway is a key cellular signaling pathway resulting in transcriptional activation and mitogenic proliferation. It consists of several enzymatic activation steps and can be stimulated by a number of agents, including cytokines and radiation exposure. It is believed to account for the accelerated repopulation of cells occurring after radiation exposure, which can affect the success of radiation therapy.

Mitogenesis: The induction of mitosis or cell transformation. The stimulation of cell proliferation, a natural recovery process in response to severe toxicologic insult that does not normally occur at reasonable multiples of human exposure levels, can account for the carcinogenic response toward nongenotoxic compounds that may not be meaningful in the clinical setting.

Mixed-function oxidase: A descriptive term for the cytochrome P450 enzyme system, reflecting the differing fates of the oxygen atoms derived from molecular oxygen consumed in the reaction. One oxygen atom is incorporated into the xenobiotic substrate and the other is reduced to water.

Mixed lymphocyte response/reaction (MLR): An in vitro assay that measures the ability of lymphocytes to proliferate in response to exposure to allogeneic cells. The proliferation represents the initial stage in acquisition of CTL function; the assay thus serves as a measure of cell-mediated immune function. Sometimes referred to as *mixed lymphocyte culture* (MLC).

MOE: Margin of exposure.

Mole viper: A viper of the genus *Atractaspis*.

Mollusk: Any member of the phylum Mollusca that includes cones, snails, mussels, clams, and oysters, as well as octopi and squid.

Monocyte/macrophage: Bone marrow–derived mononuclear cells that serve a wide variety of host defense needs, acting as both nonspecific phagocytic cells and as regulators of other immune and nonimmune host resistance mechanisms. A variety of forms exist, including monocytes (found in the blood), macrophages (found in peripheral tissue), Kupffer cells (liver), Langerhans cells (skin), microglia (brain), veiled cells (lymph), and others.

Monte Carlo technique: A repeated random sampling from the distribution of values for each of the parameters in a generic (exposure or dose) equation to derive an estimate of the distribution of (exposures or doses in) the population.

Moribund status: The condition of an animal as a result of the toxic properties of a test substance where death is anticipated. For toxicity determinations, animals killed for humane reasons are considered in the same way as animals that die.

Morphology: Pertaining to the form or structure of a cell, organ, or whole animal.

Motor activity: Spontaneous locomotion in a specified enclosure designed to provide quantitative indices of movement.

MOU: Memorandum of understanding.

MRA: Mutual recognition agreement.

MSH: Melanocyte-stimulating hormone.

MTD: Maximum tolerated dose.

Mucociliary transport: Mucous lining of the nasal passage extending from the respiratory epithelium to the pharynx. The purpose of mucociliary transport is to move waste solids up from the deeper lung to the pharyngeal area to be swallowed. Particle matter entrained in a mucous layer over ciliated airways is moved along to the nasopharynx for removal from the respiratory tract by expectoration or swallowing.

Mucosa-associated lymphoid tissue (MALT): Lymphoid tissue associated with the mucosal layer in various tissues, believed to act as a primary defense at secretory surfaces. It acts, to a limited extent, independently of the systemic response. Various tissues comprise this system, including gut-associated (GALT), nasal-associated (NALT), and bronchus-associated (BALT) lymphoid tissues.

Mullerian duct: Involves a protein secreted by the fetal testes that causes the potential female Mullerian tract to regress.

Multistage model of carcinogenesis: A model that describes the carcinogenesis process as a series of mutations or mutation-like events over time that result in a malignant growth.

Murine: Of the mouse.

Muscarine: Extremely toxic alkaloid that causes muscarinism following ingestion; an extremely toxic parasynthetic poison.

Muscimol: An extremely toxic central nervous system (CNS) depressant and gamma-aminobutyric acid (GABA) agonist that is isolated from the mushroom *Amanita muscaria* and causes visual damage, mental confusion, spatiotemporal dislocation, and memory loss in humans.

Mutagenesis: The production of genetic alterations by exposure to chemicals or radiation. This can result in irreparable DNA damage and subsequent tumor development.

Mutation: A stable change in the nucleotide sequence of a gene.

Mycotoxins: A group of more than a hundred toxins produced by various fungal organisms in food and feed commodities.

Myocardium: The middle layer of the heart, consisting of cardiac muscle.

NADPH-P450 reductase: A flavoprotein responsible for mediating the reduction of cytochrome P450.

Nanoparticles: Particles, either of man-made or biological origin, with at least one dimension less than 100 nm; also referred to as *Ultrafine Particles*, which have all dimensions in the nanometer range.

Nasopharyngeal: Region of the respiratory tract serving as the entry port for inspired air (including the turbinates, epiglottis, glottis, pharynx, and larynx).

National Institute for Occupational Safety and Health (NIOSH): U.S. Federal Agency created by the Occupational Safety and Health Act of 1970. NIOSH, a part of the Centers for Disease Control and Prevention, is responsible for conducting research and making recommendations for the prevention of work-related injury and illness.

Natural immunity: Host defense mechanisms that do not require prior exposure to an antigen. Various effector mechanisms have been described, including cellular cytotoxicity mediated by macrophages or NK cells, complement, and activity of gamma delta T cells.

Natural killer (NK) cells: A population of lymphocytes distinct from T and B lymphocytes; also referred to as *large granular lymphocytes* (LGL). NK cells exhibit cytotoxicity against virally infected cells and certain tumor cells. They are notable in that they do not require prior exposure to antigen to express cytotoxicity toward their targets. Assessment of NK activity provides a measurement of nonspecific host resistance.

Necropsy: Postmortem examination of a nonhuman body. A similar term, *autopsy*, generally is used to refer to postmortem examination of a human body. Necropsies are conducted in toxicology studies following spontaneous death or euthanasia of animals to detect potential pathologic effects of test material administration.

Necrosis: Form of cell death involving numerous cells; generally occurs at higher doses of toxicants. Necrosis in vivo often involves infiltration of macrophages and other inflammatory cells.

Neoantigen: A new antigen that appears on cells during malignant transformation, viral infection, or other process by which a naturally occurring antigen is modified.

Neoplasia: The pathologic process that results in the formation and growth of abnormal tissue (neoplasm). Neoplasms usually form a distinct mass of tissue (tumor) that may be benign or malignant; the growth of the tumor exceeds and is uncoordinated with that of normal tissue and persists in the same excessive manner after cessation of the stimulus that evoked the change.

Nephrotoxicity: Toxicity to the kidney observed in vivo.

Neurohypophysis: The posterior lobe of the pituitary.

Neuropsychology: A clinical discipline that specializes in the application of psychological tests to ascertain aberrations of neurobehavioral function.

New biological entity: A complex high molecular weight material that cannot be fully characterized by standard chemical analysis and which may require immunologic, biochemical, or bioassay techniques to measure the quantity present and to assess activity.

Nictitating membrane: An important ocular structure in many species of animals (but not humans or primates) that aids in protecting the cornea and conjunctiva when the eyeball is retracted; also called the *third eyelid*.

NIDDM: Non-insulin-dependent diabetes mellitus, type 2.

NME: New molecular (chemical) entity.

No-observed-adverse-effect level (NOAEL): A number applied to the highest dose that did not elicit an adverse effect in a properly designed and executed toxicological study.

Nongenotoxic carcinogen: A substance that causes cancer, not by primarily damaging the genetic material but by mechanisms that stimulate cell proliferation, thus increasing the chances for natural mutations to be reproduced, or by the selection of specific cell populations that may derange in a later stage.

Nonimmunologic contact urticaria (NICU): Contact urticaria is an immediate-type reaction, on a nonimmunologic basis.

Nonlinear kinetics: With nonlinear kinetics, a linear relationship does not exist between dose and plasma concentrations or body loads. Nonlinear kinetics is typically due to saturation of absorption, distribution, or elimination.

Nonparametric statistical methods: Methods that do not assume functional form with identifiable parameters for the statistical distribution of interest (distribution-free methods).

Nonregenerative anemia: Anemia characterized by decreased production and delivery into circulation of newly formed red blood cells (reticulocytes).

Normal equivalent deviate (NED): Units of deviation from the mean; probability of response on a transformed scale (e.g., 50% response equals a NED of 0).

Nose-only exposure: Experimental mode of inhalation exposure in which only the nose of the animal is placed in contact with the test atmosphere (variant is head-only).

No-threshold dose–response relationship: A dose–response relationship that assumes that any dose carries some probability of an effect.

NTEL: Nontumorigenic effect level.

Nuclear magnetic resonance (NMR): A technique to identify atoms in a sample by measuring the signal given off by relaxation of, for example, protons previously aligned in a strong magnetic field.

Nuclear magnetic resonance (NMR) spectroscopy: An analytical technique that is used to study the structure of molecules. NMR spectroscopy takes advantage of the behavior of atomic nuclei in the presence of a strong magnetic field. When samples are placed in a magnetic field and irradiated with energy in the radiofrequency (RF) range, they absorb RF energy. The exact frequency absorbed by an atomic nucleus in the sample molecule differs depending on the environment of the nucleus (its position within the molecule). A plot of intensity of RF absorption vs. frequency is called an *NMR spectrum* and is characteristic of the structure of the sample molecule.

Nucleotide: In this case, the basic building block of DNA and RNA: a base–sugar–phosphate complex. Three nucleotides form a codon that codes for one amino acid.

Null allele: An inactive form of a gene.

Occupational exposure limit (OEL): Broadly defined as a limit on magnitude and duration of an individual's exposure to an airborne chemical.

Octanol–water partition coefficient (K_{ow}, P): The ratio of the concentrations of a chemical in the water phase and the *n*-octanol phase after the chemical is equilibrated between equal volumes of the two solvents.

Odds ratio: The ratio of two odds. In case-control studies, it is an *exposure odds ratio* (i.e., the odds of exposure among the cases as compared to the odds of that same exposure among the controls). Although less seldom used, an odds ratio is sometimes calculated for a cohort study, but it is a *disease odds ratio* (i.e., the odds of disease among the exposed vs. the odds of that same disease among the unexposed).

Oligonucleotide: A chain (usually of less than 100 units) of individual nucleotide residues used as a molecular probe to detect or quantify the presence of specific DNA or RNA molecules.

Oocyte: Female germ cell in the ovary; precursor of ovum.

Open access publishing: Making publication available at no charge in public repositories, typically web based.

Open microscopic evaluation: The practice of conducting the initial histopathological evaluation with the pathologists having access to all available information about the animals from which the tissues were derived.

Opportunistic organism: Refers to an organism, most commonly a microorganism, that under a particular set of biological conditions can cause disease when normally it coexists with its host without producing disease.

Organelle: A subcellular structure with a specialized function within the cell.

Organic anion transport: Movement of negatively charged compounds across renal cellular plasma membrane; involves both reabsorption (lumen to plasma) or secretion (plasma to lumen) and is mediated by specific carrier proteins.

Organic cation transport: Movement of positively charged compounds across renal cellular membrane; involves both reabsorption (lumen to plasma) and secretion (plasma to lumen) mediated by specific carrier proteins.

Organogenesis: Formation and development of organs.

Orthologs: Genes that are believed to have evolved from a common ancestral gene. Orthologs may have a high degree of sequence homology, but their protein products do not necessarily have a high degree of functional homology.

OSHA: U.S. federal agency created by the Occupational Safety and Health Act (OSHA) of 1970. OSHA, a part of the Department of Labor, assures safe and healthful working conditions by setting and enforcing standards and by providing training, outreach, education, and assistance.

Osmium tetroxide (OsO_4): A toxic metallic cellular stain used in transmission electron microscopy.

Osteichthyes: The class of vertebrate animals that contains venomous fishy bones, characterized by the presence of an endoskeleton chiefly of bone. They are the dominant fishes; they invade all types of waters.

Osteomalacia: Inadequate or delayed mineralization of bone, resulting in an increased softness of the bone. It is the adult equivalent of rickets.

Osteoporosis: A loss in both the mineral and matrix phase of bone; associated with an increased tendency to fracture.

Outlier: A value that is far separated from other members of a sample. Outliers may be due to faulty sampling techniques (the values actually belong to a different population) or an error in measurement, or they may be real and very meaningful.

Overload: A term used in inhalation toxicology to describe a condition where the normal particle clearance mechanisms of the test system have been overwhelmed by the high inhaled particle concentrations, leading to particle accumulation in the lung and inflammation.

Oxalate: Any salt acid that contains the $(COO)_2^{2-}$ radical found in numerous plants and some fungi.

Oxidation: The net loss of electrons, which may also involve the addition of oxygen to a molecule.

Oxidative stress: Imbalance between prooxidants and antioxidants.

Oxygen enhancement ratio (OER): The ratio of the dose of radiation required to produce a given biological effect in the absence of oxygen (anoxia or hypoxia) compared to the same dose of radiation exposure required in the presence of oxygen. The OERs for low linear energy transfer (LET) radiation, such as x-rays or gamma rays, usually range from 2.8 to 3.0. Living systems are more radiosensitive when irradiated in the presence of oxygen.

Oxytocin: A peptide secreted by the neurohypophysis.

P-glycoprotein (P-gp): An ATP-dependent efflux transporter expressed on various cell types such as intestinal enterocytes, hepatocytes, renal proximal tubular cells, and blood–brain barrier endothelia. The activity of P-gp limits intestinal absorption and entry of molecules that are substrates into tissues such as the liver, kidney, and brain.

Palate: Roof of the mouth, consisting of the hard palate, soft palate, and uvula.

Pain: An unpleasant sensory or emotional experience associated with actual or potential tissue damage. It typically involves a noxious stimulus or event that activates nociceptors, which convey signals to the central nervous system, where they are processed and generate multiple responses.

Parturition: The action or process of giving birth to offspring.

Permissible exposure limits (PEL): Enforcible, regulatory limits on the amount or concentration of a substance in the air established by OSHA. PELs are based on an 8 h time-weighted average (TWA) exposure.

Photo ionization detector (PID): An instrument that measures real-time concentrations of organic vapors at low levels from parts-per-billion (ppb) up to 50,000 parts-per-million (ppm). PIDs use the energy of an ultraviolet lamp to ionize gases and produce ions that can be measured as a weak current through an imposed electrical field. The resulting electrical signal is proportional to the amount of gas in the sample.

Placenta: Organ within the pregnant uterus through which the fetus derives its nourishment.

Pluripotency: Refers to a stem cell that has the potential to differentiate into any of the three germ layers: endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system).

^{31}P NMR spectroscopy: A technique involving the use of a nuclear magnetic resonance spectrometer tuned to following phosphorous-31 resonance in a variety of phosphorylated molecules such as ATP.

p53: A 53,000-molecular-weight tumor suppressor/transcriptional regulating protein that is commonly mutated in cancers. It participates in cell cycle regulation, transcription, and apoptosis. Activation of p53 after radiation exposure leads to radiation-induced cell cycle delay.

Pachymetry: A means of obtaining quantitative measurements of corneal thickness using either optical or ultrasonic methods. Derived from the Greek words *pachys* (thick) and *metry* (process of measuring).

Pancytopenia: Reduction of all three formed elements of blood: red blood cells, white blood cells, and platelets.

Paracelsus: The founder of modern toxicology.

Paracrine: A type of cellular regulation in which a substance exerts a regulatory influence primarily on cells in close proximity as opposed to the endocrine system, which exerts regulatory action at locations distant from the release.

Parenteral: Introduction into the body by a route other than the alimentary canal (e.g., subcutaneous intravenous, intramuscular injection).

Particle: A small, discrete object.

Particulate: An adjective for particle-related properties.

Partition coefficient: Ratio of concentration or relative distribution of a chemical in two matrices at equilibrium.

Partitioning factors: Factors that determine which individuals and results are used to construct a reference interval (e.g., species, strain, sex, age, instrument, method).

Parturition: The interval in a pregnant animal during which delivery of an offspring occurs; it is initiated at the first signs of labor and completed at the birth of the last offspring in the litter, in multiparous animals.

Pathogenesis: The cellular events and reactions and other pathologic mechanism occurring in the development of disease.

Pathology: The medical science, and specialty practice, concerned with all aspects of disease but with special reference to the essential nature, causes, and development of abnormal conditions, as well as the structural and functional changes that result from the disease processes.

Pathology data review: A quality assurance review of pathology data to ensure the quality of the materials and procedures used to generate histopathological data.

Pathology peer review: The procedure whereby a second pathologist reviews a subset of tissues and other data from the initial pathology evaluation to verify the accuracy of toxicologically significant microscopic findings.

Pathology Working Group (PWG): A panel of expert pathologists assembled to review a specific question concerning pathology study results.

PCNA: Proliferating cell nuclear antigen.

PCR: Polymerase chain reaction.

Performance: The accuracy and reliability characteristics of a test method (see *Accuracy*, *reliability*).

Performance standard: A set of specifications that define an outcome in detail and provide criteria for assessing that outcome. The term is most commonly used in conjunction with prescribing conditions for the care and use of laboratory animals. These standards are based on validated test methods, which provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are (1) essential test method components; (2) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (3) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of reference chemicals.

Perfusion-limited uptake: Tissue uptake limited by the rate of blood flow to the tissue.

Peripheral blood leukocyte (PBL): Leukocytes derived from the peripheral circulatory system in humans. Due to the accessibility, these cells are often used in ex vivo assays of the human immune function.

Periportal cells: Collection of hepatic cells situated around the portal triad (branch of the portal vein, a hepatic arteriole, and a bile duct).

Peroxisome: Membrane-bound cell organelles present in cells of animals, plants, fungi, and protozoa that contain oxidative enzymes responsible for the formation and degradation of hydrogen peroxide. These organelles are involved in lipid, sterol, and purine metabolism, as well as peroxidative detoxification. Nongenotoxic chemicals that cause hepatic peroxisome proliferation can cause liver tumor development in rodents. This phenomenon has not been shown to be relevant to humans.

Persistent light eruption: Photoallergic contact dermatitis that flares with ultraviolet exposure continuing past cessation of photoallergen exposure.

Personal measurement: A measurement collected from an individual's immediate environment using active or passive devices to collect the samples.

Pfiesteria: An unclassified marine organism associated with human illness from a toxin not yet identifiable.

Phagocytosis: Process of ingestion by phagocytes whereby the cell membrane of the phagocyte engulfs bacteria and delivers them into the cell, where enzymatic digestion occurs; the active process of particle indigestion by cells (generally macrophages). Once within phagocytic vesicles (phagosomes), particles are subjected to hydrolytic enzymes secreted by macrophages.

Pharmacodynamics: The study of the biological and physiological effects of chemicals and their mechanisms of actions.

Pharmacogenetics: The study of inherited differences in xenobiotic metabolism. This includes genetic mechanisms of species differences and interindividual and interpopulation differences in the genotype and phenotype of cytochrome P450s and other xenobiotic metabolizing enzymes.

Pharmacokinetics: The study of the movement of a chemical (drug) in the body (absorption, distribution, metabolism, excretion). This study involves the time course of absorption, disruption, metabolism, and excretion of a foreign substance (e.g., a drug or pollutant) in an organism's body.

Phase 1 clinical trials: The first stage of clinical testing. These single- and multiple-dose studies are normally conducted in healthy male volunteers to assess the safety and systemic exposure of new drug candidates.

Phase 2 clinical trials: The second stage of clinical testing. These trials are designed to assess safety and efficacy in the target patient population; the length of these trials is determined by the time required to demonstrate clinical endpoints suggestive of efficacy.

Phase 3 clinical trials: Expanded controlled and uncontrolled trials. These trials are performed after preliminary evidence suggesting effectiveness of the drug has been obtained in phase 2 and are intended to gather additional information about the effectiveness and safety that is needed to evaluate the overall benefit–risk relationship of the drug. Phase 3 studies also provide an adequate basis for extrapolating the results to the general population and transmitting that information to physicians. Phase 3 studies usually include several hundred to several thousand people.

Phase 4 clinical trials: These are postmarketing trials that may be requested by regulatory agencies upon their review of the New Drug Application (NDA) or in response to effects that become evident as more patients become exposed to the drug. Sponsors may also choose to conduct these studies to support a line extension strategy (e.g., new formulations, expansion of the patient population).

Phase I metabolism: The first step in drug metabolism. Its purpose is to aid the elimination of foreign compounds from the body. The main reactions involved are oxidation, reduction, and hydrolysis but could also be hydration or isomerization. The aim of phase I is to add or unmask a reactive functional group to which phase II metabolic enzymes can add a highly water-soluble molecule. This is desired because the more water soluble a compound, the more readily it is excreted by the kidneys. Although some compounds can be eliminated solely as a result of phase I drug metabolism, most go on to be involved in the conjugation reactions of phase II metabolism. Also, phase I is not a necessary precursor to phase II metabolism for some foreign molecules.

Phase II metabolism: Conjugation reactions by which covalent bonds are formed between chemicals (both xenobiotics and endogenous) and small, polar, endogenous compounds (e.g., glucuronic acid, sulfate, glutathione, glycine); these reactions often, but do not always, follow phase I reactions.

Phenotype: The total of observable features of an organism, as the result of interaction between the genetic material (genotype) and the environment.

Photoallergic contact dermatitis: Allergic contact dermatitis dependent on ultraviolet (typically UVA) exposure.

Photoirritant (phototoxic): Acute irritant dermatitis dependent upon ultraviolet (typically UVA) exposure.

Physiological leukocytosis: Increased white blood cell count associated with endogenous catecholamine release, usually resulting from excitement or fear (fight-or-flight phenomenon) or pain.

Physiologically based pharmacokinetic (PBPK) model: A mechanistic model that describes quantitatively the uptake, distribution, metabolism, and excretion of a chemical; the model can also be used to quantify the dose of an active metabolite received by the target tissue.

Phytotoxin: A plant toxin composed of complex proteins that are produced by a relatively small number of plants and are structurally similar to bacterial toxins.

Pica: Compulsive ingestion of nonnutritive items, such as dirt, flaking paint, plaster, ashes, or laundry starch. Individuals with pica often have greater exposure to toxicants (e.g., ingestion of lead in paint chips).

Pinocytosis: Cellular process of actively engulfing a liquid.

Pit viper: Any snake of the family Crotalidae that had a depression or pit between the nostril and the eye.

Plasma: The liquid portion of blood in vivo. Plasma is obtained from blood collected with an anticoagulant and centrifuged to separate it from the cells.

Plenum velocity: Air velocity in the plenum. For good air distribution with slot-type hoods, the maximum plenum velocity should be one-half of the slot velocity or less.

Point estimate: A quantity calculated from values in a sample to represent an unknown population parameter. Point estimates typically represent a descriptive statistic (e.g., arithmetic mean, 95th percentile).

Point estimate risk assessment: Familiar risk assessment methodology in which a single estimate of risk is calculated from set point estimates. The results provide point estimates of risk for the central tendency exposure (CTE) and reasonable maximum exposure (RME) exposed individuals. Variability and uncertainty are discussed in a qualitative manner.

Point-of-control measurement of exposure: An approach to quantifying exposure by taking measurements of concentration over time at or near the point of contact between the chemical and an organism while the exposure is taking place.

Poison: Any substance (chemical, physical, biological) that is harmful or destructive to a biological (living) system.

Polymerase chain reaction: A method in which a region of a nucleic acid is selectively amplified by cycles of nucleotide polymerization; a technique enabling a rapid multiplication of selected parts of a DNA or RNA strand.

Polymorphisms: Multiple phenotypes of an organism determined by different alleles; in this context, the existence of an interindividual difference in DNA sequence coding for one specific gene. The effects of such a difference may vary dramatically, ranging from no effect at all to the building of inactive proteins or not even building the protein. Two structurally distinct genes for the same protein; polymorphic genes can be produced by mutations that result in nucleotide sequence differences that can lead to the production of proteins that differ functionally or that may not have altered functionality.

Polynomial dose–response model: A dose–response model in which the response is expressed mathematically as the sum of quantities containing increasing powers of dose.

Polytocous: The production of litters of offspring.

Post-implantation: Occurring after the early embryo embeds into the lining of the uterus.

Pre-implantation: Occurring before the early embryo embeds into the lining of the uterus.

Pseudopregnancy: The appearance of clinical and/or subclinical signs and symptoms associated with pregnancy when the organism is not actually pregnant.

Porifera: An animal phylum of some 5000 species of the simplest, multicellular life forms, most of which are marine.

Positron-emitting isotopes: These isotopes have relatively short half-lives (10–100 min) and decay exclusively by emission of a positron. They produce readily detectable, tissue-penetrating, γ-ray photons and are thus commonly used in tomographic techniques (position emission tomography, or PET). Examples of such isotopes are ^{11}C, ^{13}N, ^{15}O, and ^{18}F. Because of their brief existence, positron-emitting isotopes usually have to be manufactured close to the site where they are to be used.

Potential dose: The amount of a chemical contained in the material ingested, air breathed, or bulk material applied to the skin.

Power: The effect of the experimental conditions on the dependent variable relative to sampling fluctuation. When the effect is maximized, the experiment is more powerful. Power can also be defined as the probability that there will not be a type II error (1-beta). Conventionally, power should be at least 0.07.

Preanalytical variables: Factors that occur prior to analysis of samples and influence clinical pathology results.

Preanalytical variation: Variability in test results caused by factors that occur before the test is performed.

Precision: A measure of the agreement among replicate measurements of an analyte; a measure of the reproducibility of a measured value under a given set of conditions.

Prediction model: In alternative method development, the tool that is used to predict the endpoint of interest; the prediction model is an algorithm that defines how to convert results from the alternative method into a prediction of the in vivo toxicity. A formula or algorithm is used to convert the results obtained using a test method into a prediction of the toxic effect of interest. A prediction model contains four elements: (1) a definition of the specific purpose for which the test method is to be used, (2) specifications of all possible results that may be obtained, (3) an algorithm that converts each study result into a prediction of the toxic effect of interest, and (4) specifications as to the accuracy of the prediction.

Predictivity (negative): The proportion of correct negative responses among substances testing negative by a test method (see *Two-by-two table*). It is one indicator of test method accuracy. Negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the substances tested.

Predictivity (positive): The proportion of correct positive responses among materials testing positive by a test method (see *Two-by-two table*). It is one indicator of test method accuracy. Positive predictivity is a function of the sensitivity of the test method and the prevalence of positives among the substances tested.

Prevalence: The number of cases of a designated disease that exist at a particular point in time is the *point prevalence*. Various types of *period prevalence* represent the number of existing and new cases of a disease that are extant at the beginning of or at any time during a defined period of time. In the medical literature, both types of prevalence, particularly the latter, may erroneously be called *Incidence*, especially if the data are presented as a *prevalence rate*. Prevalence is also defined as the proportion of positive or negative substances in the population of substances tested (see *Two-by-two table*).

Prevalidation: In alternative methods development, the preliminary phase to validation in which the purpose of a test and its capabilities, limitations, interlaboratory transferability, and predictability are determined; the process during which a standardized test method protocol is developed and evaluated for use in validation studies. Based on the outcome of those studies, the test method protocol may be modified or optimized to increase intra- or interlaboratory reproductivity for use in further validation studies.

Primary cell culture: Isolated epithelial cells, tubules, or tubular fragments are used as seed material to grow renal cells under defined conditions on a solid matrix. It is a primary cell culture when seed material is directly derived from fresh tissue. It typically grows to confluence in 5–7 days and has limited ability to be passaged, depending on the species.

Primary enclosure: The cage, pen, or stall that forms the immediate limit of an animal's environment in a research facility.

Primary response: The immune response following initial contact with an antigen, resulting in the establishment of immunologic memory; synonymous with *immunization*.

PRL: Prolactin.

Probabilistic risk assessment (PRA): A risk assessment that uses probabilistic methods to derive a distribution of risk or hazard based on multiple sets of values sampled for random variables.

Probability density function (PDF): A representation, generally a function, graph, or histogram, of the probability of occurrence of an unknown or variable quantity. The sum of the probabilities for discrete random variables and the integral for continuous random variables (i.e., the area under the curve) is equal to 1.0. PDFs can be used to display distributions used as input to a probabilistic assessment or the distribution of risks that forms the output of that assessment.

Probit: Inverse cumulative distribution function of the normal distribution; it is the normal equivalent deviate (NED) plus 5 (e.g., 50% response equals a probit of 5).

Proliferation: Process of cell growth. Uncontrolled proliferation can result in cancer.

Proprietary test method: A test method for which manufacture and distribution are restricted by patents, copyrights, trademarks, etc.

Proteome: The entire protein complement of a biological sample.

Proteomics: The techniques available to identify the proteins in biological samples; field of study involving analysis of protein levels in cells or populations; automated methods developed for high-throughput analysis.

Protocol: The precise step-by-step description of a test method, including listing of all necessary reagents and all criteria and procedures for generating and evaluating test data.

Proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy: Nuclear magnetic resonance is a phenomenon that occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Proton NMR spectroscopy is the use of NMR phenomenon or protons (there are other magnetic nuclei) to study physical, chemical, and biological properties of samples.

Proximal tubules: Initial cell types of the tubular epithelium connected to glomerulus. They are subdivided into two overall segments—proximal convoluted (S1) and proximal straight (S2, S3) tubules. S1 cells are found in the cortex; S2 cells are found in the cortex and outer stripe of the outer medulla; and S3 cells are found in the outer stripe of the outer medulla. These are major sites in the nephron for drug metabolism, transport, reabsorption, and secretion of amino acids, glucose, organic anions, and organic cations.

Psilocybin: A hallucinogenic that is much less potent than Lysergic acid diethylamide (LSD) but much more potent than mescaline. It is moderately toxic to humans by ingestion and intraperitoneal routes and is probably toxic by all routes of exposure.

Psychophysics: A group of methods formulated to guide the testing of sensory capacities.

PTH: Parathyroid hormone.

Pulmonary: Region of the respiratory tract serving primarily as the air-exchange region composed of respiratory bronchioles, alveolar ducts, and alveoli.

Quality assurance: A management process by which adherence to laboratory testing standards, requirements, and record-keeping procedures is assessed independently by individuals other than those performing the testing.

Quality control: Procedures for establishing, monitoring, and evaluating the quality of the analytical testing process of each method to assure accuracy and reliability of test results for test subjects or patients.

Quantal response: A response to a stimulus or a treatment that can be referred to as *all or none* (it either happens or it does not happen). In acute toxicity studies, mortality is an example of a quantal response.

Racemate: Compound containing a 50:50 proportion of enantiomers.

Rad: Radiation-absorbed dose of ionizing radiation. One rad = 100 erg/g.

Radiation: Energy propagated through space or matter as waves (gamma rays, ultraviolet light) or as particles (alpha or beta rays).

Radiological dispersal device (RDD): Mechanism designed to scatter radioactive material into the environment. The amount and type of radioactive material used will determine the number of causalities, the extent of evacuations, and the area of contamination.

Radionuclide: An element, either in the environment or internal, which emits ionizing radiation.

Radioprotective agents: Chemical compounds that, when administered before irradiation, protect the organism against radiation damage.

Radiosensitizer: Substance that enhances the radiation response of biologic systems.

Radiotolerance: The eventual lack of sensitivity to radiation.

Random: Each individual member of the population has the same chance of being selected for the sample.

Randomization: Process used in toxicity studies to ensure a homogeneous population and minimize errors due to sampling bias. Randomization can be done by assigning animals to treatment groups using computer-generated random numbers or through the use of tables of random numbers.

Rate: An expression of the frequency with which an event occurs in a defined population; a measure of the *absolute risk* for the disease in that population. In a rate, the numerator is a subset of the denominator; therefore, all rates are ratios but not all ratios are rates. A rate usually has a time dimension. For a prevalence rate, it is the implied or explicit time at which the data were collected. For an incidence rate, it is the time over which the new events took place. For convenience, the denominator of either type of rate usually is presented as some power of 10 (i.e., the number of cases per 100 or per 1,000 or, for rare events, per 100,000 or even per 1,000,000).

Rate difference: The difference between two rates. In the situation where etiology has been established, the rate difference between the disease incidence in the exposed and unexposed groups is sometimes called the *excess rate* or *attributable risk*.

Raw data: Any laboratory worksheets, records, memoranda, notes, or exact copies thereof that are the result of original observations and activities from a study. This may include manually recorded information, printouts from automated instruments, computer printouts, photographs, microfilm or microfiche copies, and magnetic media (including dictated observations).

RCB: Rodent cancer bioassay.

RD_{50}: The concentration of airborne substance that produces 50% decrease in the respiratory rate in rodents, usually mice. This is the numerical output of a sensory irritation study.

REACH (Registration, Evaluation, and Authorization of Chemicals): This is a far-reaching European legislation replacing all previous patchy EU toxic substances control legislations. REACH aims to comprehensively regulate the manufacture, import, and commerce of hazardous substances and their preparations in the European Union.

Reactive or secondary thrombocytosis: Increased platelet counts observed in conjunction with generalized bone marrow stimulation as may occur with hemolysis, blood loss, and many types of acute and chronic inflammation.

Reasonable maximum exposure (RME): The highest exposure that is reasonably expected to occur at a site. The intent of the RME is to estimate a conservative exposure case (i.e., well above the average case) that is still within the range of possible exposures.

Reasonable worst case: A semiquantitative term referring to the lower portion of the high end of the exposure, dose, or risk distribution. The reasonable worst case has historically been loosely defined, including synonymously with *maximum exposure* or *worst case*, and assessors are cautioned to look for contextual

definitions when encountering this term in the literature. As a semiquantitative term, it is sometimes useful to refer to individual exposures, doses, or risks that, while in the high end of the distribution, are not in the extreme tail. For consistency, it should refer to a range that can conceptually be described as above the 90th percentile in the distribution but below about the 98th percentile.

Receptor: The sensitive site for chemical–biological interaction.

Recirculating perfusion: Perfusate flows from a reservoir through the organ being perfused and returns to the same reservoir.

Recommended exposure limit (REL): An occupational exposure limit that has been recommended by NIOSH. RELs are time-weighted average (TWA) concentrations for up to a 10 h workday during a 40 h workweek.

Red cell generative response: A response to reduced red cell mass characterized by an appropriate increase in erythropoiesis and correlative changes in related parameters.

Red cell mass parameters: Parameters that provide an estimation of whole-body red cell mass and include RBC count, hemoglobin concentration, and hematocrit.

Rederivation: Refers to a process that utilizes removal of term fetuses with subsequent cross-fostering onto mothers of the right microbiological status or the use of embryo transfer procedures to change the microbiological status of animals.

Reduction alternative: A new or modified test method that reduces the number of animals required.

Reference chemicals: Chemicals selected for use during the research, development, prevalidation, and validation of a proposed test method because their response in the in vivo reference test method or the species of interest is known. Reference chemicals should represent the classes of chemicals for which the proposed test method is expected to be used and cover the range of expected response (negative, weak to strong positive). Different sets of reference chemicals are likely to be required for the various stages of validation. After a proposed test method has been recommended or accepted as valid for its intended purposes (i.e., has been recommended as a validated test method to federal agencies), a representative subset of chemicals used during the validation process may be selected to validate a mechanistically and functionally similar test method. To the extent possible, this subset of reference chemicals should (1) be representative of the range of responses that the validated test method is capable of measuring or predicting, (2) have produced consistent results in the validated test method and in the reference test method or the species of interest, (3) reflect the accuracy of the validated test method, (4) have well-defined chemical structures, (5) be readily available, and (6) not be associated with excessive hazard or prohibitive disposals costs. This list of reference chemicals would represent the minimum number of chemicals that should be used to evaluate the performance of proposed mechanistically and functionally similar test methods with established performance standards. If any of the recommended chemicals are unavailable, other chemicals for which adequate reference data are available could be substituted. If desired, additional chemicals representing other chemical or product classes and for which adequate reference data are available can be used to more comprehensively evaluate the accuracy of the proposed test method.

Reference concentration (RfC): Air concentration of a chemical exposure (expressed in mg/m^3) that is associated with minimal or no risk of adverse effects, even in susceptible subpopulations.

Reference dose (RfD): An estimate of a daily exposure to a human population, including sensitive groups, that is likely to be without appreciable risk of deleterious health effects during a lifetime.

Reference interval: The central interval of test values (usually the central 95th percentile) obtained from a defined group of apparently healthy individuals using defined methods (see *Partitioning factors*). In contrast, a reference range is the entire range of values from those individuals, including minimum and maximum values.

Reference toxicant: Chemicals of known toxicity that are used to determine the health of a population of a test species.

Refinement alternative: A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances the well-being of animals.

Regenerative anemia: Anemia characterized by increased production and delivery of newly formed red blood cells (reticulocytes) into circulation; typically associated with hemorrhage or hemolysis.

Regional particle deposition: Particles ≤1 μm deposit by diffusion in the alveolar region of the respiratory tract; particles 1–5 μm deposit by sedimentation in the tracheal/bronchial/bronchiolar/alveolar regions; particles between 5 and 30 μm are deposited primarily by inertial impaction in the nasopharyngeal region.

Reinforcement schedule: A designated relationship between a specific behavior, such as pressing a lever in an experimental chamber, and the delivery of a reinforcer, such as a feed pellet.

Relative biological effectiveness (RBE): The ratio of a dose of a test radiation (e.g., neutron, gamma, x-ray) required to produce the same reference biological endpoint as the dose of a standard radiation of 250 kVp x-rays.

Relative risk: The ratio of the absolute risk of disease or death (the incidence rate) among the exposed population to the risk of disease among the unexposed; a measure of association that sometimes is called the *risk ratio*, *rate ratio*, or *RR*.

Relevance: The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the accuracy or concordance of a test method.

Reliability: A measure of the degrees to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and interlaboratory reducibility and intralaboratory repeatability.

Remediation action level (RAL): A concentration such that remediation of all concentrations above this level in an exposures unit will result in the 95% upper confidence limit being reduced to a level that does not pose an unacceptable risk to an individual experiencing random exposures. The RAL will depend on the mean, variance, and sample size of the concentrations within an exposure unit as well as considerations of short-term effects of the chemicals of concern.

Renal cell lines: Immortalized cultures of renal cells, derived from specific nephron cell types but often expressing properties of multiple cell types. They can be maintained indefinitely.

Renal cellular repair and regeneration: Process by which the renal epithelium synthesizes new cells to replace those damaged by chemical toxicants or in various pathological states.

Renal slices: In vitro model involving slicing renal tissue. Typically obtained with a razor and a glass microscope slide, the slices can be obtained from the cortex, outer or inner stripe of outer medulla, or the inner medulla. They are useful over the short term (up to 60 min) in metabolic and transport studies.

Replacement alternative: A new or modified test method that replaces animals with nonanimal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate).

Reproducibility: The consistency of individual test results obtained in a single laboratory (*intralaboratory reproducibility*; see *Intralaboratory reproducibility*) or in different laboratories (*interlaboratory reproducibility*) using the same protocol and test samples.

Reproductive toxicology: The study of causes, mechanisms, and sequelae of adverse effects on the reproductive system, including alterations to the reproductive organs, related endocrine system, or pregnancy outcomes, and manifested as adverse effects on sexual maturation, gamete production and transport, cycle normality, sexual behavior, fertility, gestation, parturition, lactation, pregnancy outcomes, premature reproductive senescence, or modifications in other functions dependent on the integrity of the reproductive system.

Residual analysis: Analysis of the difference between experimental and simulated data as a function of time or other controllable variables. The residuals should be random if the model is adequate.

Residual body: A lobe of cytoplasm containing residual organelles (e.g., mitochondria, Golgi apparatus, endoplasmic reticulum, ribosomes, lipid droplets) that detaches from the elongated spermatid at spermiation. Residual bodies are eliminated from the seminiferous epithelium by Sertoli cell phagocytosis.

Resinoid: Any substance that contains or resembles a resin.

Respirable: Inhalable materials that are capable of getting to and being deposited in the gas-exchange region of the lung; a particle size characteristic whereby the substance can enter the pulmonary region of the respiratory tract. These are generally low-micrometer- to submicrometer-sized particles. Contrast with *Inhalable*.

Respiratory system: Complex arrangement of organs designed primarily for the intake of oxygen and the elimination of carbon dioxide; divided into the proximal or upper airway (nose, pharynx, larynx, trachea, nonalveolarized bronchioles) and the distal or lower airway (alveolar bronchioles, alveolar ducts, alveolar air sacs).

Respiratory tract: The entire breathing system, including mouth, nose, larynx, trachea, lungs, and associated nerves and blood supply.

Retention (of particulate material): Quantity of particles present at specific respiratory tract sites that is the net difference between deposition and clearance processes; refers to particle matter and represents the quantity of particles present at specific respiratory tract sites. Retention is the net difference between deposition and clearance processes.

RIA: Radioimmunoassay.

Ring tail: A condition seen in rodents in which annular constructions of the tail occur that may lead to necrosis of all or part of the tail. This condition is presumed to be associated with abnormal environmental conditions.

Risk: Proportion or probability expressed on a scale of 0–1, or 0%–100%, that individuals process or develop a specified biological effect by a given time for a defined set of exposure conditions; the probability or degree of concern that exposure to an agent will cause an adverse effect in the species of interest.

Risk characterization: A component of risk assessment that describes the nature and magnitude of risk, including uncertainty. In assessments of Superfund sites, it includes the summary and interpretation of information gathered from previous steps in the site risk assessment (e.g., data evaluation, exposure assessment, toxicity assessment), including the results of a probabilistic analysis.

Robust: Having inferences or conclusions little affected by departure from assumptions.

Rodent lifetime: Generally taken to be 2 years for risk estimation.

Roentgen (R): Quantity of x- or gamma radiation per cubic centimeter of air that produces one electrostatic unit of charge.

Roentgen equivalent man (REM): Unit of dose equivalent; the absorbed dose in rads multiplied by the *Q* of the type of radiation.

Rough endoplasmic reticulum (RER): The endoplasmic reticulum component that contains bound ribosomes and is largely responsible for protein synthesis.

Route of exposure: Portal of entry of a chemical into the body: oral, inhalation, dermal, or injection.

S9 mix: A 9000 *g* supernatant fraction from a tissue homogenate (e.g., liver).

Safety factor: A number or factor that considers inter- and intraspecies variability, sensitivity, and extrapolatability. It is applied to a NOAEL to establish an acceptable daily intake (ADI). See *Uncertainty factor.*

Safety pharmacology: The study of the pharmacologic effects of a drug candidate that are unrelated to the desired pharmacologic effect. These studies are generally conducted at doses similar to the anticipated therapeutic dose.

Standardized aquatic microcosm (SAM): A defined aquatic microcosm (3 L vessel) containing 15 species in a well-defined medium of trace metals and vitamins and 200 g of sand to which chitin and cellulose are added.

Sample: In statistics, the collected set of data points that (if properly collected) are representative of the population (all of the values there are).

Saponins: Steroidal or terpenoid glycosides from plants and animals capable of reducing surface tension and disruption of cell membranes.

Saturation dose: Dose that overwhelms a toxification or detoxification mechanism such that no additional changes in the effect are incurred above that dose.

Sawfly: Insects, the larvae of which are hepatotoxic when ingested by ruminant livestock.

Schedule-controlled operant behavior: An approach to the study of behavioral function that relies on manipulation of reinforcement schedules.

Scombroid poisoning: An allergic fish poisoning resulting from the consumption of inadequately processed fish containing histamine and saurine formed from bacterial action.

Screen/screening test: A rapid, simple test conducted for the purposes of a general classification of substances according to general categories of hazard. The results of a screen generally are used for preliminary decision-making and to set priorities for more definitive tests. A screening test may have a truncated response range (it may be able to reliably identify active chemicals but not inactive chemicals).

Screening tests: In acute toxicity testing, tests designed to define the range of toxicity using fewer animals per dose level or fewer dose levels.

Sea snakes: Members of the Elapidae family that spend most of their lives in oceans.

Secondary enclosure: The room or space in which a primary enclosure is located.

Secondary phase regeneration (SPR): Tissue repair response where hepatocytes are mobilized from G_0/G_1 to proceed through mitosis (see *Electron paramagnetic resonance*).

Secondary response: The immune response that occurs after initial contact with an antigen (the primary response). The secondary response is quicker, of higher affinity, and more pronounced. For humoral-mediated immune reactions, the secondary response is associated with antibody class switching.

Secondary toxicity: The toxicity of a chemical determined by feeding the test animals (especially ferrets or mink) with prey that have been fed the test chemical.

SEL: Safe exposure level.

Selection and selection bias: The process by which subjects are included in a study. If systematic differences exist between those who are selected for a study and those who are not, selection bias may occur. The bias may be introduced by the subjects themselves through self-selection (into or out of the study), as a result of the sources of the subjects, or by the study investigators.

Seminiferous epithelium: The Sertoli cells and developing male germ cells within the seminiferous tubules in the testes.

Sensitivity: The number of subjects experiencing each experimental condition divided by the variance of scores in the sample; the proportion of all positive chemicals that are classified correctly as positive in a test method. It is a measure of test method accuracy (see *Two-by-two table*).

Sensitivity analysis: Evaluation of the effect of changes in the value of a particular parameter on the estimates of a state variable provided by a mathematical model. Sensitivity is expressed as the magnitude of change in the endpoint of interest (e.g., tissue dose) as a function of change in the value of a particular model parameter.

Sertoli cell: A somatic cell present in the seminiferous epithelium of mammals. Solidly attached to the basement membrane of seminiferous tubules, it produces important regulatory glycoproteins, including anti-Mullerian hormone, inhibin, and androgen-binding protein and is essential for male germ cell development. Junctions between Sertoli cells form the blood–testis barrier.

Serum: The liquid portion of blood that remains after a clot has formed. Serum is obtained from blood collected without anticoagulant and centrifuged to separate it from the cells.

Serum responsiveness: Cell proliferative reaction to the addition of serum to tissue culture medium after prior deprivation.

Severity grading of lesion: The semiquantitative application of a defined severity score to specific microscopic lesions, usually to denote the extent of tissue involvement or degree of tissue damage.

Shuttle vector: A DNA transfer agent capable of moving genes into or out of a cell.

SI units: International system of units; SI refers to *Système International d'Unites*. Radiation units include Joule/kilogram, Gray, Sievert, and Becquerel.

Sievert (Sv): SI unit of dose equivalence; the absorbed dose in grays multiplied by the *Q* of the type of radiation. One Sv = 100 rems.

Signature sequencing: Sequencing of a short stretch of cDNA close to the end of the complementary mRNA. Sequence stretches of some 20 nucleotides are sufficiently discriminative to identify the transcript of an individual gene in a mammalian tissue.

Significance level: The probability that a difference has been erroneously declared to be significant, typically 0.05 and 0.01, corresponding to 5% and 1% chance of error.

Simulation: System behavior predicted for specific exposure conditions by solving the set of differential and algebraic equations of a model.

Single nucleotide polymorphism: Interindividual variation in the genetic code at the level of one single building block (see *Nucleotide*, *polymorphisms*).

Single-pass perfusion: Perfusate flows from a reservoir through the vasculature of a perfused organ; it is collected and not reused so the perfusate goes through only once.

Single-wall carbon nanotube (SWNT): An engineered nanoparticle consisting of carbon atoms arranged in a single tubular structure with a very high aspect ratio. The diameter of the nanotubes is less than 100 nm but the tube lengths can be several micrometers.

Skin notation: Hazard warnings used worldwide to alert workers and employers to the health risks of dermal exposures to chemicals in the workplace.

Slit-lamp biomicroscope: An instrument used to study ocular tissues that consists of a microscope and high-intensity light source. It allows the eye to be illuminated and observed from different angles and can detect lesions not observable by gross examination.

Slope: The difference in the incidence or magnitude of an effect divided by the difference in dose that created the effect.

Slot velocity: Air velocity through the openings in a slot-type hood. It is used primarily as a means of obtaining uniform air distribution across the face of the hood.

Small intestine: The region of the gastrointestinal tract from the pyloric sphincter of the stomach to the cecum that consists of the duodenum, the jejunum, and the ileum; the primary site of absorption of ingested substances.

Smokes: A complex mixture of solid or liquid particles, such as soot, liquid droplets, or mineral ash particles from incomplete combustion of organic materials. Smoke particles are generally ~0.5 μm.

Smooth endoplasmic reticulum (SER): The endoplasmic component of the cell that does not contain bound ribosomes and is strongly associated with drug-metabolizing enzyme systems such as those mediated by cytochrome P450.

Society of toxicology (SOT): The preeminent professional society for toxicologists in the United States.

Soil aging: The changes that occur in the interaction of chemicals with soil during which the chemicals are bound to soil particles and become less bioavailable.

Soil sorption constant (K_{oc}): The extent of partitioning of an organic chemical between the solid and solution phases of a water-saturated soil or sediment.

Somatotropin: Growth hormone.

Species sensitivity distributions: A cumulative frequency distribution (CFD) of toxicity responses for a given substance, describing the range of concentrations that results in a measured effect for a selected set of species. A CFD describes the frequency with which a variable assumes values less than or equal to some number. Distributions can be developed for species within or among taxonomic categories and can be limited to single endpoints (e.g., mortality) or be inclusive of all measured endpoints. A desired level of protection can be selected, most frequently the 5th percentile (HC_5).

Specific activity: The radioactivity per unit mass of material. Specific activity is used to quantify radionuclides. Specific activity can be given in Curies per mole (Ci/mol) or Becquerels per mole (Bq/mol).

Specific gravity: The ratio of the density of a substance to the density of a reference material at a specified temperature. Water is the reference standard for liquids and solids (density 1 g/mL at 4°C).

Specificity: The proportion of all negative chemicals that are classified correctly as negative in a test method. It is a measure of test method accuracy (see *Two-by-two table*).

Spermatid: The haploid germ cell, arising from meiotic divisions of spermatocytes, that differentiates within the seminiferous epithelium into a spermatozoon.

Spermatocytes: Germ cells in the seminiferous epithelium derived from spermatogonia that subsequently undergo two meiotic divisions to form round spermatids.

Spermatogenesis: The process of germ cell division and differentiation that begins with the multiplication of spermatogonia and ends with the release of elongated spermatids into the lumen of the seminiferous tubules (spermiation).

Spermatogenic cycle: A complete sequential progression of the cellular associations (or stages) of spermatogenesis. The stages follow one another through an entire cycle, returning to the original stage and repeating the cycle approximately 4.5 times until spermatogonia become elongated spermatids and undergo spermiation.

Spermatogonia: The diploid germ cells in adult males that divide by mitosis to produce additional stem cell spermatogonia and spermatocytes.

Spermiation: The process by which elongated spermatids are released from the germinal epithelium into the seminiferous tubule lumen.

Spermiogenesis: Last phase of spermatogenesis during which elongated spermatids are formed from round spermatids.

Spina bifida: A congenital defect in which part of the vertebral column is absent, allowing the spinal membranes to protrude; a result of failure of the neural tube to close properly.

Spin trapping: The most commonly used indirect method for detecting free radicals. The technique involves adding a spin trap (usually a nitrone or nitroso compound) to the sample prior to radical generation. When the radical is generated, it reacts rapidly with the spin trap, producing a secondary radical or *spin adduct*, which is more stable than the parent free radical and can be detected by electron paramagnetic resonance (EPR).

Standard deviation: The most widely used measure of dispersion of the points in a frequency distribution about the midpoint (usually the mean). It is equal to the square root of the variance. For a normally distributed population, the region within one standard deviation of the mean contains 67% of the distribution.

Statistical heterospectroscopy: A statistical technique for combining nuclear magnetic resonance (NMR)- and mass spectroscopy (MS)-based metabonomic data. It provides a focus on metabolites that change consistently across the platforms on a sample-to-sample basis.

Statistical significance: An inference that the probability is low that the observed difference in quantities being measured could be due to variability in the data rather than an actual difference in the quantities themselves. The inference that an observed difference is statistically significant is typically based on a test to reject one hypothesis and accept another.

Steady state: A situation in which the rate of change is equal to zero.

Steatosis: Accumulation of lipid within hepatocytes (fatty liver).

Stem cell: A multipotential self-renewing cell in the bone marrow that serves as the precursor for all hematopoietic cell lineages, including those of the immune system.

Stereoisomers: A general term for isomers that differ only in the orientation of the atoms in space. Enantiomers, isomers that differ in their optical rotation, are a subclass of stereoisomers.

Stomach: Saclike region of the gastrointestinal tract from the esophagus to the duodenum consisting of the cardia, fundus, corpus, antrum, and pylorus; the site of hydrogen ion secretion from parietal cells of the gastric glands that activates pepsin-mediated proteolysis.

Stress–response leukogram: Typically, an increase in neutrophil count and decreases in lymphocyte and eosinophil counts following exogenous corticosteroid administration or when stressful conditions result in increased production of endogenous corticosteroids.

Solute carrier (SLC) transporters: SLC transporters are primarily unidirectional uptake transporters, although some function in a bidirectional manner. SLC transporters include over 300 members organized into 47 families, including both facilitative and secondary active transporters. Solutes, or substrates, that are recognized and transported across membranes by SLC transporters include charged and uncharged organic molecules as well as inorganic ions.

Sulfonation: A biosynthetic conjugation reaction involving cytosolic and membrane-bound sulfotransferases that catalyze the transfer of sulfate, in the form of endogenous 3′-phosphoadenosine-5′-phosphosulfate, to a xenobiotic functional group (e.g., hydroxyl) to yield a product that has increased water solubility and properties that enhance its renal and biliary elimination.

Subchronic: Characterized by a time period of intermediate duration; commonly used to describe exposure between acute and chronic durations in toxicity studies (usually 3 months).

Subchronic toxicity study: Multiple-dose study in which animals are treated for less than 6 months. They are intended to elucidate the target organs for toxicity and demonstrate dose–response relationships. They are normally required prior to any clinical testing.

Substitute method: A new or modified test method proposed for use in lieu of a currently used test method, regardless of whether that test method is for a definitive screening or adjunct purpose.

Substrate probe: Individual isozymes may show significant differences in substrate specificity. A substrate that is metabolized by a specific isozyme and does not show significant overlaps with other isozymes can be used to probe for the presence and activity of the isozyme. Substrate probes can be used in vitro and in vivo. Use of substrate probes may not be as accurate as certain other techniques for isozyme identification but are sometimes more practical.

Syncytium: A multinucleated protoplasmic mass formed by the secondary union of originally separate cells.

Synergism: Refers to a response that is greater than a multiplicative response observed in chemical interaction studies.

Syncytiotrophoblast: Epithelial covering of the highly vascular embryonic placental villi that invades the wall of the uterus to establish nutrient circulation between the embryo and the mother.

Systems biology: Is a research approach that integrates computational sciences, applied mathematics, and experiments to allow for the analysis and interpretation of complex datasets generated by systematically perturbing defined biological systems.

Systems biology verification and industrial methodology for process verification in research (IMPROVER): IMPROVER is a methodology that aims to provide a measure of quality control of industrial research and development by verifying the soundness of the methods used.

Systole: Contraction of the heart, especially of the ventricles, by which blood is driven through the aorta and pulmonary artery to transverse the systemic and pulmonary circulation, respectively.

T_3: Triiodothyronine.

T_4: Thyroxine.

T_{max}: Time to maximum achieved concentration.

T-cell receptor (TCR): The heterodimeric surface molecule on T cells that serves to recognize antigen. It always occurs in conjunction with the CD3 surface antigen, which is responsible for transmembrane signaling following antigen recognition.

T cell/T lymphocyte: Lymphocytes primarily responsible for the induction and maintenance of cell-mediated immunity, as well as regulating humoral-mediated immunity and certain nonimmune effector mechanisms. A variety of T-cell subtypes have been described, including T-helper cells, T-cytotoxic cells, T-suppressor cells, and T-inducer cells.

Tannins: Heterogeneous polyphenols of plant origin.

Target tissue dose: Concentration of a chemical in the tissue or organ where the biological effect occurs.

TBG: Thyroid-binding globulin.

TeBG: Testosterone-binding globulin.

TEF: Toxic equivalency factor.

Telomere: The terminal portion (end) of a chromosome.

Teratology: The study of the causes, mechanisms, and sequelae of perturbed developmental events in species of animals that undergo ontogenesis; in the past, the definition was limited to malformation, but the term is now generally accepted to be synonymous with developmental toxicology.

Teratogenesis: Process by which birth defects arise.

Test article: Any food additive, color additive, drug, biological product, electronic product, medical device, pesticide, or other chemical substance to be subjected to studies.

Test material: Any chemical substance to be subjected to studies. This does not include electronic products or medical devices.

Test method: A process or procedure used to obtain information on the characteristics or a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified condition. Used interchangeably with *test* and *assay* (see also *Validated test method*).

Test system: Any animal, plant, microorganism, and subparts thereof (e.g., in vitro organ systems) or physical matrix (e.g., soil or water) to which a test or control article is administered or added for study.

Testosterone: The primary male sex hormone produced by Leydig cells in the testis that, along with the metabolite dihydrotestosterone, is responsible for male reproductive tract development, maintenance of spermatogenesis, secondary sex characteristics, and sexual behavior.

Tetrodotoxin: An extremely toxic, highly lethal, crystalline neurotoxin that acts on both the central and peripheral nervous systems, causing nerve and skeletal muscle paralysis by selectively blocking the regenerative sodium conductance channel.

TFM: Test facility manager.

Therapeutic index (TI): The ratio of LD_{50}/ED_{50}. The therapeutic index is used to establish the safety margin of biologically active materials such as drugs. The higher the index, the greater the margin of safety.

Thermal neutral zone: The temperature or range of ambient temperatures at which an animal does not expend energy to heat or cool itself.

Thin ascending limb: Cell type of epithelium found in the inner medulla only in long-looped nephrons; begins after bend of Henle's loop.

Thin descending limb: Cell type of epithelium found in the inner medulla in long-looped nephrons and in the inner stripe of the outer medulla in short-looped nephrons. The thin descending limb follows proximal straight tubules as one follows nephrons from glomerular to urinary poles.

Threshold dose: Dose below which a specified biological effect does not occur for specified exposure conditions. This refers to a condition when a dose must exceed a specific amount before the agent can induce an effect.

Threshold dose–response relationship: A dose–response relationship that assumes that adverse effects occur only when a threshold dose is exceeded.

Threshold limit values (TLV): Airborne concentration of a substance that would be anticipated to produce no adverse health effects in nearly all workers exposed 8 h per day, 5 days per week, for a working lifetime. TLVs are established by the American Conference of Governmental Industrial Hygienists.

Threshold limit value (TLV®): An occupational exposure limit established by the American Conference of Government Industrial Hygienists. TLVs® are based on an 8 h time-weighted average (TWA) exposure.

Thrombocytopenia: A condition in which there is an abnormally small number of circulating platelets.

Thymus: Central lymphoid organ located in the thorax. Its function is to generate immunocompetent T cells from lymphocytes originating in the bone marrow.

Time-weighted average (TWA): The average concentration of a chemical in air (or exposure to a physical agent) for a normal 8 h workday and 40 h workweek to which a worker is exposed. TWA is calculated using the duration of exposure to different concentrations of the chemical during a specific time period.

Tissue time constant: The product of partition coefficient and volume divided by blood flow rate.

Tolerance level: The maximum legally permissible concentration of residues of a pesticide in food.

Toxic: Substance that has the capacity to produce personal injury via ingestion, inhalation, or absorption through any body surface.

Toxic neutrophils: Neutrophils with morphologic changes indicative of greatly accelerated neutrophil production, regardless of cause.

Toxicant: An agent that can result in the occurrence of a structural or functional adverse effect in a biological system.

Toxicodynamics: The process of interaction of chemical substances with target sites and the subsequent reactions leading to adverse effects.

Toxicokinetics: The process of the uptake of potentially toxic substances by the body, the biotransformations they undergo, the distribution of the substances and their metabolites in the tissues, and the elimination of the substances and their metabolites from the body. Both the amounts and the concentration of the substances and their metabolites are studied. The term has essentially the same meaning as *Pharmacokinetics*, but the latter term is usually restricted to the study of pharmaceutical substances.

Toxicological pathology: The science that integrates the disciplines of pathology and toxicology and is concerned with the effects of potentially noxious substances.

Toxicological profiles: Prepared by the Agency for Toxic Substances and Disease Registry, these profiles include extensive toxicological information for substances found at the National Priorities List (NPL) and other sites.

Toxicology: The study of the adverse effects of biological, chemical, or physical agents on living organisms and the ecosystems, including the prevention and amelioration of such adverse effects.

Toxicology data network (TOXNET): A major group of publicly available databases from the National Library of Medicine.

Toxin: A poison derived from a biological source.

Toxinology: The study of toxins.

Tracheobronchial: A region of the respiratory tract serving to deliver inspired air to deeper portions of the lung, comprised of a series of branching ducts beginning at the trachea and ending at the terminal bronchioles.

Transcript profiling: See *Transcriptomics.*

Transcription: The formation of messenger RNA (mRNA), complementary to a string of DNA.

Transcriptome: The messenger RNA (mRNA) from actively transcribed genes.

Transcriptomics: The techniques available to identify the messenger RNA (mRNA) from actively transcribed genes.

Transgenic: Referring to an organism in which new DNA is introduced into the genome. Some transgenic animal models have been suggested to complement the assessment of the potential for compounds to cause tumor development.

Transgenic animals: Genetically engineered animals carrying genes from a different species.

Translocation: Transfer of a portion of one chromosome to another chromosome.

Translational systems toxicology: Aims to extrapolate human response based on the results from animal and in vitro studies. It emphasizes the use of cell-based assays to provide more relevant data on the effects of short- and long-term exposure to toxicants.

TRH: Thyroid-releasing hormone; a tripeptide secreted by the hypothalamus.

Trophoblasts: Cells forming the outer layer of a blastocyst, which provide nutrients to the embryo and develop into a large part of the placenta.

Trypan blue exclusion: Assay for cellular necrosis; the vital dye trypan blue is only taken up by cells whose membranes have been permeabilized. The cell viability is estimated by counting the fraction of stained (i.e., blue) cells on a light microscope.

TS: Test substance.

TSH: Thyroid-stimulating hormone.

Two-by-two table: The two-by-two table can be used to calculate accuracy (concordance) ($[a + d]/[a + b + c + d]$), negative predictivity ($d/[c + d]$), positive predictivity ($a/[a + b]$), prevalence ($[a + c]/[a + b + c + d]$), sensitivity ($a/[a + c]$), specificity ($d/[b + d]$), false-positive rate ($b/[b + d]$), and false-negative rate ($c/[a + c]$).

			New Test Outcome	
Positive			**Negative**	**Total**
Reference test	Positive	a	c	$a + c$
Classification	Negative	b	d	$b + d$
	Total	$a + b$	$c + d$	$a + b + c + d$

Two-dimensional gel electrophoresis (2D gel electrophoresis): A series of electrophoretic techniques that separates proteins (gene products) on the basis of isoelectric points in one dimension followed by separation on the basis of molecular mass in the second dimension. This technique is useful for obtaining an overall assessment of both up- and downregulation of specific gene products as the result of cellular responses to chemical exposures. This technique separates proteins (gene products) in two dimensions on the basis of isoelectric point in the first dimension and on the basis of molecular mass in the second dimension. It is a powerful technique for proteomic research, particularly when coupled with computerized image analysis systems for quantification.

Tyndall phenomena: The abnormal cloudy appearance of the anterior chamber of the eye when light passes through the pupil; also called *aqueous flair.* It is the result of protein leakage from the iris into the aqueous humor causing the scattering of light and producing cloudiness.

Type I error (false positive): Concluding that there is an effect when there really is not an effect. Its probability is the alpha level.

Type II error (false negative): Concluding that there is no effect when there really is an effect. Its probability is the beta level.

Ultrafine particles: Particles that have all dimensions in the nanometer range; contrast with *Nanoparticles.*

Ultrastructural cytochemistry: A series of in situ techniques for localizing organelle-specific enzymes, such as acid phosphatase for lysosomes and peroxidase for peroxisomes, at the ultrastructural level.

Ultrastructural morphometry: A series of techniques for quantifying changes in organelle systems from electron micrographs in situ; these techniques provide useful correlative information when used in combination with biochemical measurements of chemical-induced alterations in organelle system functionality. The technique is based on the systematic evaluation of large numbers of transmission electron micrographs followed by the statistical analysis of changes in various organelle compartments induced by exposure to toxic agents. Data generated by this technique may be expressed as volume density (*Vv*) of an organelle compartment, surface density (*Sv*), or numerical density (*Nv*).

Uncertainty: Lack of knowledge about specific variables, parameters, models, or other factors. Examples include limited data regarding the concentration of a contaminant in an environmental medium and lack of information on local fish consumption practices. Uncertainty may be reduced through further study.

Uncertainty factor: A factor applied to a no-observed-adverse-effect level (NOAEL) or the lowest-observed-adverse-effect level (LOAEL) that is used to derive a reference dose or reference concentration. The aim of the uncertainty factor is to account for a lack of information on inter- and intraspecies variability, study deficiencies, or an incomplete database. The value, generally from 1 to 10, based on scientific data or judgment, allows for the unknown potential differences in doses or durations of exposure that produce effects in different individuals, different species, or different routes of exposure or are due to an inadequate database on which to base a decision.

Upper respiratory tract: The mouth, nose, sinuses, and pharynx.

Urinalysis: Assays on chemical and biophysical parameters of urine; includes measurement of parameters such as levels of metabolites, protein, glucose and creatinine excretion, urinary enzymes, and urinary specific gravity.

Urinary enzymes: Enzymes secreted into urine used to measure renal function; can often be used as biomarkers of cell-type specific toxicity and function.

Urticaria: Hives; an eruption of itching wheals (welts).

UVR: Ultraviolet radiation.

Validated test method: An accepted test method for which validation studies have been completed to determine the accuracy and reliability of this method for a specific purpose.

Validation: The process by which the reliability and accuracy of a procedure are established for a specific purpose.

Vapor: Gaseous forms of substances that normally are in the liquid or solid state; the gas-phase components of a substance that is a solid or liquid at standard temperature and pressure.

Vapor pressure: The amount of pressure exerted by a saturated vapor above its own liquid in a closed container.

Vaporization: The transfer of a chemical from a solid surface or a solution to a gas, dependent upon the chemical's diffusivity, water solubility, and vapor pressure.

Variability: The range of values expected among individuals of a given population; a term used to describe the natural variation in human (or test animal) responses to chemical exposures. It is also used to describe variation in exposures to chemicals in the environment. Variability represents true heterogeneity, diversity, or a range that characterizes an exposure variable or response (e.g., differences in body weight). Further study (e.g., increasing sample size *n)* will not reduce variability, but it can provide greater confidence in quantitative characterizations of variability.

Vasopressin (antidiuretic hormone, ADH): An octapeptide secreted by the neurohypophysis.

Vehicle: A substance to which a test material is added to provide a consistency or form suitable for its intended

use. In toxicity studies, test materials are added to vehicles to prepare solutions (e.g., water), suspensions (e.g., methyl cellulose), ointments (e.g., petrolatum), triturates (e.g., milk, sugar), and other forms to facilitate administration to the animals.

Venom: An animal toxin.

Viperidae: True vipers; a diverse genus of venomous snakes, many of which are large and extremely dangerous, even deadly.

Vipers: Snakes of the families Viperidae and Crotalidae.

Water solubility: The maximum amount of a chemical that may be dissolved in a given quantity of pure water at a given temperature.

Weapon of mass disruption: A terrorist weapon designed to frighten and disrupt the population.

Whole animal studies: Studies in which physiological function and toxicity are determined in vivo.

Whole-body exposure: Experimental mode of inhalation exposure in which the entire animal is placed within the test atmosphere.

Wolffian duct: Embryonic duct from which the male reproductive duct system, accessory sex glands, and external genitalia are derived.

Xenobiotic: A substance that is foreign to a biological system; a substance (usually) not present in the reference organism; a chemical species, synthetic in origin, that is toxic or damaging to a biological system.

X-ray microanalysis: The in situ localization of trace elements within organelles at the ultrastructural level using the electron beam of either a transmission or scanning electron microscope to displace electrons from specific energy shells (K, L, M) with the resulting release of characteristic x-rays that may be monitored by energy-dispersive or wavelength-dispersive spectrometers.

Zero-order process: A process for which the rate is constant and independent of dose or concentration; characteristic of enzymatic processes under saturating concentrations.

Zonation, hepatic lobule: Quantitative (or qualitative) distribution of enzymes in liver lobules based on morphological configuration.

Zonation, metabolic: Differences observed between enzyme activities in periportal and perivenous regions of the liver.

Zoonotic: Refers to the ability of an organism to be transmitted between species of animals; most commonly used in reference to the ability of an organism to be exchanged between animals and humans.

Zygote: A fertilized ovum.